












# Cold water immersion in recovery following a single bout resistance exercise suppresses mechanisms of miRNA nuclear export and maturation

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## Abstract

Cold water immersion (CWI) following intense exercise is a common athletic recovery practice. However, CWI impacts muscle adaptations to exercise training, with attenuated muscle hypertrophy and increased angiogenesis. Tissue temperature modulates the abundance of specific miRNA species and thus CWI may affect muscle adaptations via modulating miRNA expression following a bout of exercise. The current study focused on the regulatory mechanisms involved in cleavage and nuclear export of mature miRNA, including *DROSHA*, *EXPORTIN-5*, and *DICER*. Muscle biopsies were obtained from the *vastus lateralis* of young males ( $n = 9$ ) at rest and at 2, 4, and 48 h of recovery from an acute bout of resistance exercise, followed by either 10 min of active recovery (ACT) at ambient temperature or CWI at 10°C. The abundance of key miRNA species in the regulation of intracellular anabolic signaling (miR-1 and miR-133a) and

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angiogenesis (miR-15a and miR-126) were measured, along with several gene targets implicated in satellite cell dynamics (NCAM and PAX7) and angiogenesis (VEGF and SPRED-1). When compared to ACT, CWI suppressed mRNA expression of *DROSHA* (24 h  $p=0.025$  and 48 h  $p=0.017$ ), *EXPORTIN-5* (24 h  $p=0.008$ ), and *DICER* (24 h  $p=0.0034$ ). Of the analyzed miRNA species, miR-133a (24 h  $p<0.001$  and 48 h  $p=0.007$ ) and miR-126 (24 h  $p<0.001$  and 48 h  $p<0.001$ ) remained elevated at 24 h post-exercise in the CWI trial only. Potential gene targets of these miRNA, however, did not differ between trials. CWI may therefore impact miRNA abundance in skeletal muscle, although the precise physiological relevance needs further investigation.

#### KEYWORDS

cold water immersion, microRNA export and maturation, resistance exercise

## 1 | INTRODUCTION

Cryotherapy, most performed using cold water immersion (CWI), is widely employed during the recovery phase post-exercise or high intensity sport events. CWI has been reported to attenuate muscle soreness and increase the speed of recovery of muscular function following exercise (Fonseca et al., 2016; Moore et al., 2022; Siqueira et al., 2018). While CWI following intense resistance exercise may be beneficial for sub-maximal aerobic function (Roberts et al., 2014), there is also evidence for reduced post-exercise rates of muscle protein synthesis rates (Fuchs et al., 2020) and blunted resistance training-induced muscle hypertrophy and muscular strength (Roberts et al., 2015). CWI use with resistance training also shifts muscle cell adaptations toward a relatively slow twitch phenotype, with increased capillary formation (D'Souza, Zeng, Markworth, et al., 2018). Therefore, CWI exerts pleiotropic effects on the adaptive cellular and molecular responses within the musculature following a bout of exercise.

Given the diversity of molecular mechanisms regulated by CWI, one unexplored potential cellular mechanism influenced by the transient muscle hypothermia is microRNAs (miRNAs). miRNAs are well-established to be important components of the molecular events necessary for muscle phenotype adaptation to physical activity and exercise training (D'Souza, Bjørnsen, et al., 2017; D'Souza, Markworth, et al., 2017; D'Souza, Zeng, Figueiredo, et al., 2018; D'Souza, Zeng, Markworth, et al., 2018; D'Souza, Zeng, Markworth, et al., 2019; D'Souza, Zeng, Poppitt, et al., 2019; Margolis et al., 2017; Margolis & Rivas, 2018; Mitchell et al., 2018; Zacharewicz et al., 2014; Zeng et al., 2018). More than 2000 human miRNA species have been reported (Kozomara & Griffiths-Jones, 2014). miRNAs function to “fine-tune” protein expression, by

either binding to and promoting mRNA degradation, or by inhibiting the translational complex (Sevignani et al., 2006; Ying & Lin, 2004). Cellular abundance of miRNA species is initially regulated by transcriptional activation. miRNA species are firstly transcribed as long primary-miRNAs (pri-miRNA) that then undergo cleavage by an enzymatic complex that includes DROSHA, a ribonuclease III endonuclease, resulting in ~70 nucleotide long precursor miRNA (pre-miRNA). The generated pre-miRNAs are subsequently exported from the nucleus by EXPORTIN-5, and then undergo final cleavage by another related ribonuclease III endonuclease, DICER, generating the mature ~19–22 nucleotide miRNA (Drummond et al., 2008). Therefore, miRNA abundance is controlled by multiple mechanisms, with miRNA cleavage and trafficking being key steps. Consistent with the role of miRNA in mediating aspects of the molecular adaptations to exercise, and the adaptive regulation of transcript maturation and traffic, previous studies have reported that the gene expression of *DROSHA*, *EXPORTIN-5*, and *DICER* are all upregulated following resistance exercise (Garner et al., 2020; Russell et al., 2013).

Transient hypothermia and hyperthermia both exert a pronounced influence on miRNA abundances and their transcriptional activity in multiple tissue and cell types (Chen et al., 2018; Fukuoka et al., 2014; Liu et al., 2017; Umehara et al., 2020). More specifically to exercise, in long-distance runners who experienced hypothermia there was differential regulation of muscle miRNA species compared to runners who did not experience hypothermia (Devasthanam & Tomasi, 2017; Umehara et al., 2020). Further, cell culture studies have identified that the DICER protein is highly sensitive to both hyperthermia and hypothermia (Pilotte et al., 2011). Collectively, these findings provide a hypothetical basis for investigation of the impact of CWI on the molecular mechanisms of

miRNA regulation (DROSHA, EXPORTIN-5, and DICER) and expression of specific miRNA species related to exercise adaptations in response to CWI during exercise recovery (Peake et al., 2020). On the basis of prior cell culture studies (Devasthanam & Tomasi, 2017), we hypothesized that CWI would elevate muscle DICER expression following a single bout of resistance exercise. Further analysis was conducted to quantify various miRNA species previously implicated in the adaptive response to muscle loading including intracellular anabolic signaling (miR-1 and miR-133a; McCarthy & Esser, 2007) and angiogenesis (miR-15a and miR-126a; Fish et al., 2008; Sun et al., 2013; Wang et al., 2016; Yin et al., 2012). Finally, analysis was made of downstream mRNA and protein targets of these miRNAs involved in myogenesis (neural cell adhesion molecule; *NCAM* and paired protein box 7; *PAX7*) (Chen et al., 2010; Gagan et al., 2012; Harding & Velleman, 2016; Ivey et al., 2008; Yin et al., 2020) and angiogenesis (vascular endothelial growth factor; *VEGF*, and Sprouty-related EVH1 domain-containing protein 1; *SPRED-1*) (Cordes & Srivastava, 2009; Dang et al., 2013; Henn et al., 2019; Wang & Olson, 2009).

## 2 | METHODS

Nine physically active men ( $22.1 \pm 2.2$  years old), with a history of at least 12 months experience in strength training completed a bout of single-leg strength exercise on two separate occasions using opposite legs, separated by at least 1 week (Roberts et al., 2015). The 8-repetition maximum (RM) strength of unilateral knee extension ( $71 \pm 12.0$  kg) and unilateral  $45^\circ$  leg press ( $299 \pm 44.8$  kg) for both legs was assessed 4–5 days prior to the first experimental exercise bout. At the same time, familiarization for the single-leg squats and walking lunges was performed. On the day of each trial, the resistance exercise bout included six sets of  $45^\circ$  leg press and knee extensions (8,8,10,12,10, and 10 RM), and three sets of single-leg squats and walking lunges (12 repetitions in each set).

For the CWI trial, 5 min after exercise, the participants sat in an inflatable bath (iCool iBody, Miami, Australia) for 10 min. The bath contained water that was maintained at  $10 \pm 0.3^\circ\text{C}$ , and the participants were immersed to the level of their waist. For the control active recovery (ACT; light recovery exercise performed at room temperature) trial, instead of undergoing CWI the participants exercised at room temperature on a stationary bicycle ergometer (Wattbike, Nottingham, UK) at a light, self-selected intensity ( $59.5 \pm 9.4$  W) for 10 min. Participants were prevented from showering or bathing for a further 2 h. The order of the CWI and ACT trials was randomized and counterbalanced.

Participants consumed their habitual diet, excluding alcohol, for the 48 h before trial 1, which included their preferred pre-exercise meal of choice 2 h before the pre-exercise biopsy. They were provided with and consumed 30 g of whey protein (Body Science, Gold Coast, Australia) at the end of exercise, before CWI and ACT, after which they fasted until the 2 h biopsy. Following this, another 30 g of the same whey protein was consumed. Participants consumed a habitual diet, excluding alcohol, between the 2 and 48-h biopsies. Subjects were asked to refrain from strenuous activity over the 48 h before trial 1, and between the 2 and 48-h biopsies. All activity and diet were recorded by participants in diaries relating to trial one, and then replicated for trial 2.

Muscle biopsies were collected from the vastus lateralis before, 2, 24, and 48 h post-exercise and immediately snap frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until further analysis. All participants were informed of the requirements and potential risks of the studies prior to providing their written informed consent. The experimental procedures adhered to the standards set by the latest version of the Declaration of Helsinki and were approved by the Human Research Ethics Committee of The University of Queensland (2013001426).

### 2.1 | Immunoblotting

Muscle tissue ( $\sim 25$  mg) was homogenized in ice-cold RIPA lysis buffer (Millipore, Temecula, CA, USA) supplemented with the Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific). After centrifugation 15,000 g for 10 min at  $4^\circ\text{C}$ , supernatants were collected, and total protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein were boiled in Laemmli buffer at  $95^\circ\text{C}$  for 5 min. Twenty micrograms of protein was separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Inc., CA) using the semidry Trans-Blot Turbo™ device (Bio-Rad). Membranes were incubated overnight with primary antibodies (Abcam) against Drosha (ab85027), Exportin-5 (ab129006), Dicer (ab13502), VEGF (ab46154), SPRED-1 (ab77079), and EGF1 (ab9695). Antibodies were used at 1:1000 dilution, except VEGF and SPRED1, which were used at 1:2000 and 1:500, respectively. The following day membranes were incubated for 1 h at room temperature with appropriate anti-rabbit or anti-mouse horseradish peroxidase (HRP) conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, PA) (1:10,000). The membranes were exposed on a ChemiDoc image device (Bio-Rad) using enhanced chemiluminescence reagent (ECL Select kit; GE Healthcare Ltd.). Bands were quantified

using ImageJ software (NIH, Bethesda, MD). Western blot data were normalized to the housekeeping protein GAPDH (Abcam, ab9485) (1: 10,000).

## 2.2 | RNA extraction and quantitative real-time PCR

Total RNA was extracted from 20 mg of muscle tissue using the AllPrep® DNA/RNA/miRNA Universal Kit (QIAGEN GmbH). 1500 ng of input RNA were then used for cDNA synthesis using High-Capacity RNA-to-cDNA™ kit (Life Technologies). Messenger RNA (mRNA) was measured by RT-PCR on a LightCycler 480 II (Roche Applied Science) using SYBR Green I Master Mix (Roche Applied Science). Target mRNAs were *DROSHA*, *DICER*, *EXPORTIN-5*, *NCAM*, *PAX7*, *VEGF*, and *SPRED-1*. Primers were designed using BLAST software (Table 1). Relative fold changes were determined using the  $2^{-\Delta\Delta CT}$  method (Schmittgen & Livak, 2008). The geometric mean of three reference genes was used for normalization (Vandesompele et al., 2002). The human reference genes (Eisenberg & Levanon, 2013) chromosome 1 open reading frame 43 (*C1orf43*), charged multivesicular body protein 2A (*CHMP2A*) and ER membrane protein complex subunit 7 (*EMC7*) were identified as the least variable, and therefore used as reference genes (Table 1).

## 2.3 | miRNA reverse transcription and PCR

Ten nanograms of RNA from muscle tissue was used for cDNA synthesis using TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific) and miRNA abundances were measured by RT-PCR on a QuantStudio 6 instrument (Thermo Fisher Scientific) using Applied Biosystems Fast Advanced Master Mix (Thermo Fisher Scientific).

Target miRNAs were chosen from a literature search of those previously implicated in the regulation of satellite cell dynamics and angiogenesis (Table 2). The geometric mean of three miRNAs were used for normalization, using miRNAs that showed the least variance in the current sample set (miR-191, -361 and -320a). Data were analyzed using the  $2^{-\Delta\Delta CT}$  method (Schmittgen & Livak, 2008).

## 2.4 | Statistical analyses

All data were tested for normality using Shapiro–Wilk and equal variance using Levene's Test. Where appropriate, data were log transformed and normality and equal

TABLE 1 mRNA sequences. Forward and reverse sequences of analyzed genes.

| Gene                        | Sequence                    |
|-----------------------------|-----------------------------|
| <i>DROSHA</i> (Forward)     | CGATCCAGGCCAGATGAATGA       |
| <i>DROSHA</i> (Reverse)     | TAGGACGACAGGGCTTGATG        |
| <i>EXPORTIN-5</i> (Forward) | ATCCTGTCTCGTCCTTAC          |
| <i>EXPORTIN-5</i> (Reverse) | TCTGGGGCCTTACTTTCTTC        |
| <i>DICER</i> (Forward)      | AAAATTGGCGAACTGGATGACC      |
| <i>DICER</i> (Reverse)      | TTTGGGTAGCACTGCCTTCG        |
| <i>NCAM 1</i> (Forward)     | GCAGCGAAGAAAAGACTCTGG       |
| <i>NCAM 1</i> (Reverse)     | GCAGATGTACTCTCCGGCAT        |
| <i>PAX7</i> (Forward)       | CCTTTGGAAGTGTCCACCCC        |
| <i>PAX7</i> (Reverse)       | TCGCCATTGATGAAGACCC         |
| <i>VEGF</i> (Forward)       | TCTTCAAGCCATCCTGTGT         |
| <i>VEGF</i> (Reverse)       | CTTTCTTTGGTCTGCATTC         |
| <i>SPRED-1</i> (Forward)    | CGTTTCAAAGTCTGCTGATG        |
| <i>SPRED-1</i> (Reverse)    | CATTTGCTTGTAAGTCA<br>TCTGCC |
| <i>C1ORF43</i> (Forward)    | CTATGGGACAGGGTCTTTGG        |
| <i>C1ORF43</i> (Reverse)    | TTTGGCTGCTGACTGGTGAT        |
| <i>CHMP2A</i> (Forward)     | CGCTATGTGCGCAAGTTTGT        |
| <i>CHMP2A</i> (Reverse)     | GGGGCAACTTCAGCTGTCTG        |
| <i>EMC7</i> (Forward)       | GGGCTGGACAGACTTTCTAATG      |
| <i>EMC7</i> (Reverse)       | CTCCATTCCCGTCTCATGTACG      |

TABLE 2 miRNAs analyzed. Classification and identification number.

| miR             | ID number  |
|-----------------|------------|
| hsa-miR-126-3p  | 477887_mir |
| hsa-miR-133a-3p | 478511_mir |
| hsa-miR-1-3p    | 477820_mir |
| hsa-miR-15a-5p  | 477858_mir |
| hsa-miR-361-5p  | 478056_mir |
| hsa-miR-320a    | 478594_mir |
| hsa-miR-191-5p  | 477952_mir |

variance were restored. These log transformed data were used to perform statistical analysis. Raw data are presented in the figures. All data were analyzed with SigmaPlot software (Windows v. 12.1, Systat 218 Software Inc.) using two-way repeated measures ANOVA, with time as a within-subject factor and trial as a between-subject factor. Where time × trial interactions were observed, pair-wise differences from pre versus post-exercise within and between the ACT and CWI trials were assessed with Holm–Sidak post hoc tests. Statistical significance was accepted at  $p < 0.05$ .



### 3 | RESULTS

#### 3.1 | Regulators of miRNA biogenesis and maturation

*DROSHA* (trial×time interaction  $p=0.045$ ) mRNA expression was reduced after the CWI trial, compared with the ACT trial, when measured in the biopsied muscle samples 24, and 48 h following the completion of the exercise ( $p=0.025$  and  $p=0.017$ , respectively) (Figure 1a). Drosha protein abundances remained unaltered after the CWI trial yet were significantly lower than after the ACT trial at all timepoints (2, 24, and 48 h;  $p=0.039$ ,  $p=0.033$ , and  $p=0.014$ , respectively) (Figure 1b).

*EXPORTIN-5* (trial×time interaction  $p=0.047$ ) mRNA increased at 24 and 48 h post-exercise in the ACT trial only. Additionally, *EXPORTIN-5* was lower at 24 h after the CWI trial, compared with the ACT trial ( $p=0.008$ ) (Figure 1c). Protein expression of *EXPORTIN-5* was unresponsive to either exercise or recovery modality (trial×time interaction  $p=0.416$ ) (Figure 1d).

The mRNA of the *DICER* showed a trial×time interaction ( $p=0.034$ ) (Figure 1e). At 24 h following exercise, it was attenuated in the CWI trial compared to the ACT trial ( $p=0.004$ ). At 24 h *DICER* mRNA was also elevated compared to pre-exercise levels only in the ACT group ( $p=0.007$ ). The protein expression of *DICER* (Figure 1f) also showed an interaction (trial×time  $p=0.031$ ) and was attenuated in the CWI trial compared to the ACT trial at both 24 and 48 h ( $p=0.002$  and  $p=0.003$ ), respectively.

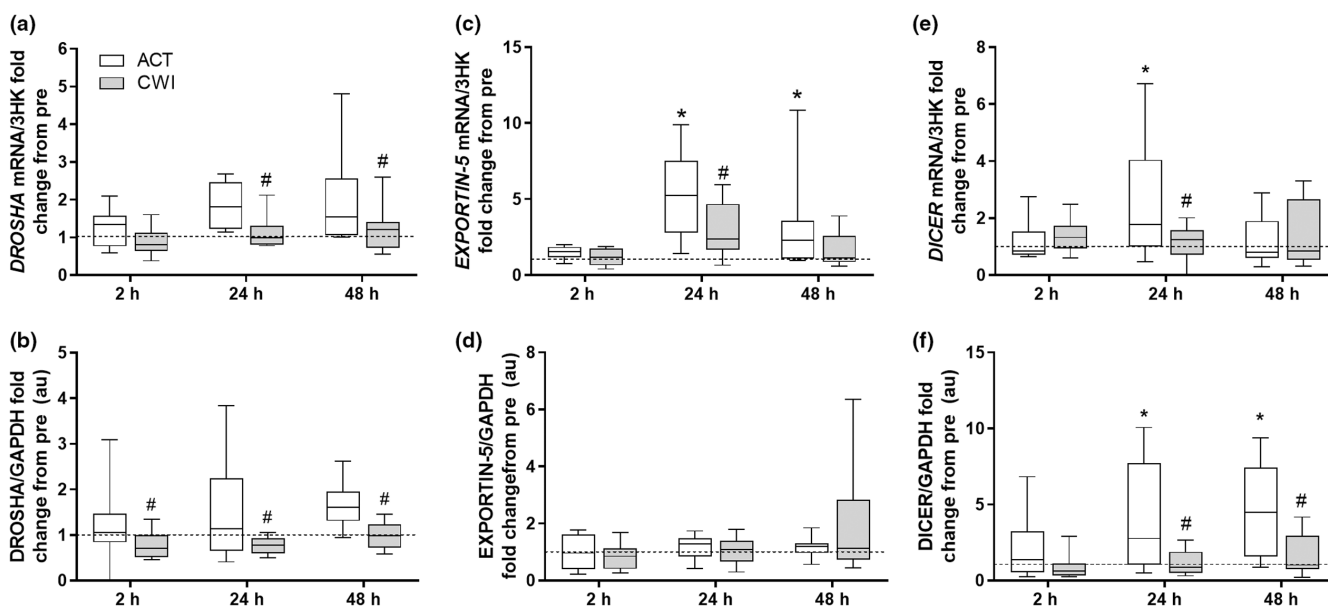
*DICER* protein in the ACT trial was also elevated at 24 and 48 h compared with rest ( $p=0.002$  and  $p<0.001$ ), respectively.

#### 3.2 | miRNA regulators of myogenesis

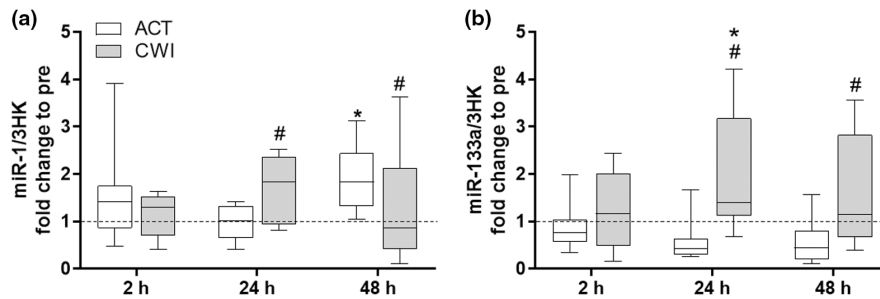
miR-1 and -133a are reported inhibitors of myogenic cell differentiation and proliferation. Both miRNAs showed trial×time interactions ( $p=0.008$  and  $0.005$ , respectively) (Figure 2a,b). At 24 h there was an increase in miR-1 after the CWI trial compared with the ACT trial ( $p=0.023$ ), while at 48 h this pattern was reversed, with a lower miR-1 expression after in the CWI trial compared to the ACT trial ( $p=0.04$ ). Expression of miR-1 was also increased at 48 h compared to pre-exercise levels in the ACT trial alone ( $p=0.012$ ). miR-133a showed an increased expression at both 24 and 48 h after the CWI trial compared with the ACT trial ( $p<0.001$  and  $p=0.007$ ), respectively. In the CWI trial, miR-133a was also elevated at 24 h following exercise when compared with pre-exercise levels ( $p=0.008$ ).

#### 3.3 | miRNA regulators of angiogenic signaling

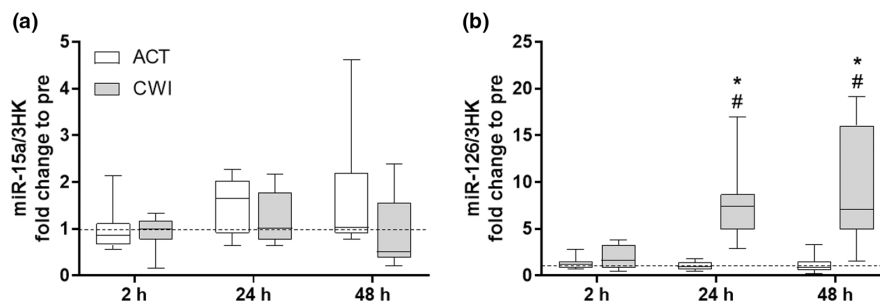
miR-15a (a negative regulator of VEGF expression) was also not altered after exercise or CWI (trial×time interaction  $p=0.215$ ) (Figure 3a). miR-126 showed a trial×time interaction  $p<0.001$  (Figure 3b). Expression of miR-126



**FIGURE 1** Effect of CWI on components regulating microRNA biogenesis. Fold change relative to respective pre-exercise (a, b) *DROSHA* mRNA and protein, (c, d) *EXPORTIN-5* mRNA and protein, and (E, F) *DICER* mRNA and protein, respectively. \* $p < 0.05$  respective pre-exercise values; #  $p < 0.05$  difference between recovery strategies at the respective time point. Data are expressed as box and whisker plots with spread plotted as 95% CI. The horizontal dotted line reflects pre-exercise values.



**FIGURE 2** Effect of CWI on miRNAs that regulate myogenesis. Fold change relative to respective pre-exercise. (a) miR-1 and (b) miR-133a, respectively. \* $p < 0.05$  respective pre-exercise values; #  $p < 0.05$  difference between recovery strategies at the respective time point. Data are expressed as box and whisker plots with spread plotted as 95% CI. The horizontal dotted line reflects pre-exercise values.



**FIGURE 3** Effect of CWI on miRNAs that regulate angiogenesis. Fold change relative to respective pre-exercise. (a) miR-15a and (b) miR-126, respectively. \* $p < 0.05$  respective pre-exercise values; #  $p < 0.05$  difference between recovery strategies at the respective time point. Data are expressed as box and whisker plots with spread plotted as 95% CI. The horizontal dotted line reflects pre-exercise values.

was elevated only in the CWI trial at both 24 and 48 h compared with after the ACT trial ( $p < 0.001$  and  $p < 0.001$ , respectively) and pre-exercise levels ( $p < 0.001$  and  $p < 0.001$ , respectively).

### 3.4 | Satellite cell gene expression

*NCAM* mRNA and *PAX7* mRNA showed time main effects following exercise ( $p < 0.001$  and  $p = 0.01$ , respectively) (Figure 4a,b). *NCAM* mRNA was significantly elevated at 24 and 48 h and was not regulated by recovery modality ( $p < 0.001$  and  $p = 0.021$ , respectively). *PAX7* mRNA was significantly elevated at 2, 24, and 48 h and was not regulated by recovery modality.

### 3.5 | Angiogenic gene and protein expression

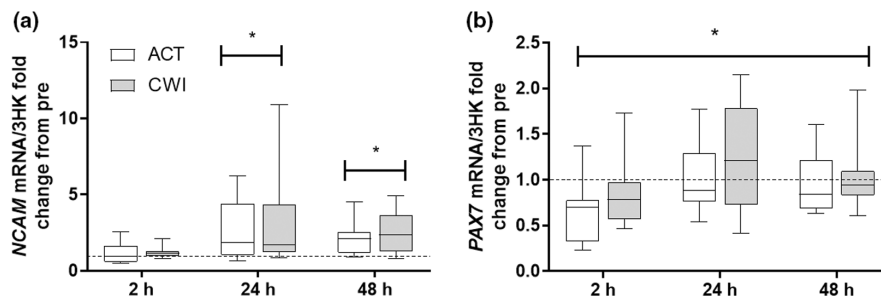
*VEGF* mRNA demonstrated a main time effect  $p < 0.001$  with a specific increase at 2 h following exercise in both trials ( $p < 0.001$ ) (Figure 5a). However, *VEGF* protein was not altered by exercise or CWI (trial  $\times$  time interaction  $p = 0.906$ ) (Figure 5b). The inhibitor of angiogenesis, *SPRED-1* mRNA (trial  $\times$  time interaction  $p = 0.027$ ) was

elevated above pre-exercise in both the CWI and ACT trials at 2 h following exercise ( $p = 0.021$  and  $p = 0.047$ , respectively) (Figure 5c). At 48 h there was an increased *SPRED-1* mRNA expression in the CWI trial compared with both the ACT trial and respective pre-exercise values ( $p = 0.003$  and  $p < 0.001$ ). Expression of *SPRED-1* protein (trial  $\times$  time interaction  $p = 0.037$ ) was increased from pre-exercise values in the ACT trial only at both 24 and 48 h ( $p = 0.002$  and  $p = 0.035$ ) (Figure 5d). Additionally, a significant attenuation in *SPRED-1* protein expression was evident at 48 h after the CWI trial compared with after the ACT trial ( $p = 0.039$ ).

Representative blots of analyzed proteins are shown in Figure 6.

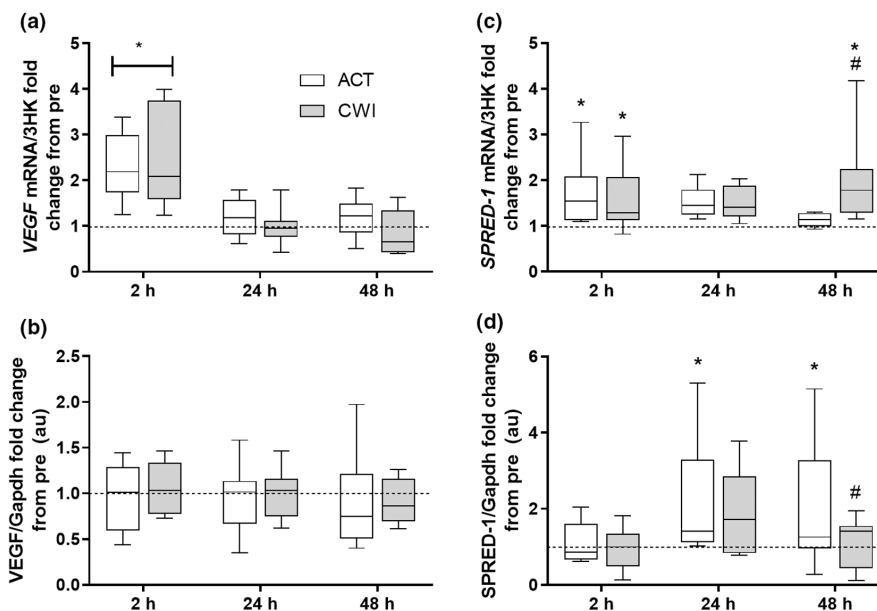
## 4 | DISCUSSION

The current study investigated the mechanisms regulating skeletal muscle miRNAs following a single bout of leg resistance exercise, followed by either CWI or ACT. It was demonstrated that brief CWI exposure has a small yet persistent impact to suppress exercise-induced increases in gene and protein abundances of *DROSHA*, *EXPORTIN-5*, and *DICER*. Therefore, our findings are contrary to our hypothesis that CWI would transiently increase muscle



**FIGURE 4** Effect of CWI on satellite cell dependent myogenesis. Fold change relative to respective pre-exercise. (a) *NCAM mRNA* and (b) *PAX7 mRNA*. \* $p < 0.05$  respective pre-exercise values; #  $p < 0.05$  difference between recovery strategies at the respective time point. Data are expressed as box and whisker plots with spread plotted as 95% CI. The horizontal dotted line reflects pre-exercise values.

**FIGURE 5** Effect of CWI on angiogenesis markers. Fold change relative to respective pre-exercise. (a, b) *VEGF mRNA* and protein, and (c, d) *SPRED-1 mRNA* and protein. \* $p < 0.05$  respective pre-exercise values; #  $p < 0.05$  difference between recovery strategies at the respective time point. Data are expressed as box and whisker plots with spread plotted as 95% CI. The horizontal dotted line reflects pre-exercise values.



levels of DICER, given the previous evidence for the temperature sensitivity of this protein (Pilotte et al., 2011; Russell et al., 2013). This study further examined whether the altered responsiveness of these major components of miRNA processing to CWI exerted an impact on the measured abundances of several key myogenic miRNA species. There was a divergence in the abundance of miRNA-1, miRNA-133-a, and miRNA-126, with a tendency for greater expression after the CWI trial. Nevertheless, further potential targets for the actions of these miRNAs, including *NCAM* and *PAX7* (targets of miRNA 1 and -133-a), as well as *VEGF* and *SPRED-1* (targets of miRNA-15a and -126), were not altered by CWI.

There has been limited analysis of the responsiveness of the pathway responsible for miRNA biogenesis, transport, and maturation to acute exercise stress and post-exercise recovery modalities. In untrained individuals, a single bout of cycle ergometry alone, or with an additional bout of knee extensor resistance exercise, was found to increase muscle mRNA expression of *DROSHA*,

*EXPORTIN-5*, and *DICER* at 1 and 3 h post-exercise (Garner et al., 2020; Russell et al., 2013). Additionally, following 10 days of endurance training, the initial increase in expression observed 3 h following the final acute exercise bout was attenuated, relative to the activation following the first (untrained) exercise bout (Russell et al., 2013). Consistent with these prior studies, we report that miRNA biosynthetic pathways are induced in human muscle at 24 and 48 h of recovery from an acute bout of resistance exercise (although we found no change at 2 h). We chose to focus on these later time-points so that we could also identify corresponding changes in protein expression. Indeed, to our knowledge the current study is the first to report on the protein level modulation of these important miRNA regulatory factors in human skeletal muscle. In this regard, our data indicate a substantial increase in intramuscular DICER protein at 24 and 48 h post-exercise. The most striking finding in the current study is that CWI almost completely abolished all exercise-induced changes in *DROSHA*, *EXPORTIN-5*, and *DICER*. Precisely how this

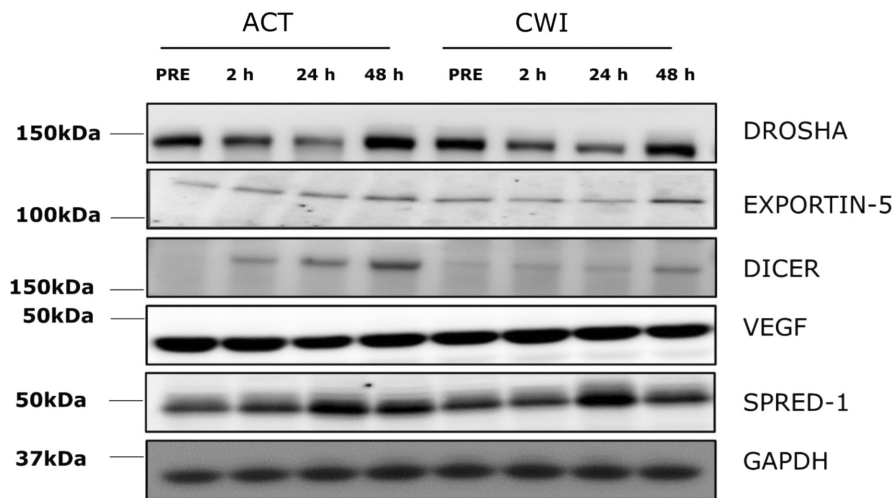


FIGURE 6 Representative immunoblot images.

may impact upon muscle adaptations to exercise is unclear and warrants further investigation.

DROSHA or DICER knockout (KO) mice were recently shown to display significant alterations in muscle miRNA abundances (Garner et al., 2020; Vechetti Jr et al., 2019). Despite this, these KO mice did not differ obviously from wild type controls with regard to muscle phenotype (Vechetti Jr et al., 2019) or endurance performance (Oikawa et al., 2019). Therefore in the current study, analysis of key myoMiRs (muscle-specific miRNAs) was also made (McCarthy & Esser, 2007). Of the analyzed miRNA species, we included miR-1 and -133a, because they have previously been identified as inhibitors of satellite cell activity and muscle protein synthesis (Drummond et al., 2008; D'Souza, Zeng, Markworth, et al., 2019). From the current study, it is apparent that miR-1 and -133a expression were elevated after the CWI compared to ACT. This new finding may help explain the deleterious effects of CWI on resistance exercise-induced muscle anabolic signaling, protein synthesis, and hypertrophy previously reported by us and others (Figueiredo et al., 2016; Roberts et al., 2015). miR-1/133 targets several pro-growth genes and has been shown to be downregulated during muscle hypertrophy in mice (McCarthy & Esser, 2007). On this basis of prior published work showing that CWI can stimulate muscle angiogenesis (D'Souza, Zeng, Markworth, et al., 2018) analysis was included for miR-15a and miR-126, which are reported to be important regulators of angiogenesis-related pathways. No change was seen in the anti-angiogenic miR-15a, but the abundance of pro-angiogenic miR-126 was markedly elevated from pre-exercise levels at 24 and 48 h following exercise in the CWI trial when compared to ACT.

Activation and the subsequent proliferation, differentiation, and fusion of satellite cells following resistance exercise is thought to be central in myofiber repair following resistance exercise. *NCAM* and *PAX7* mRNA is

typically upregulated in human muscle biopsies within hours of exercise, and remains upregulated for up to 24 h following exercise (Nederveen et al., 2017). In this study, we observed a small increase in *NCAM* and *PAX7* mRNA following exercise. The absence of differences between the CWI and ACT trials at 24 and 48 h suggests no effect of CWI on muscle expression of these myogenic factors. However, when paired with the observed miRNA changes, CWI and ACT may negatively influence the translation of target *NCAM* and *PAX7* mRNA species. These data are consistent with our previous observations of abolished increase in Pax7 positive cells for up to 48 h following CWI treatment, when compared to ACT after intense resistance exercise (D'Souza, Bjørnsen, et al., 2017). This in turn may contribute to the impaired muscle adaptation to training, as previously reported with CWI (D'Souza, Zeng, Markworth, et al., 2018; Roberts et al., 2015).

We have previously identified that CWI recovery across a period of resistance training results in a noticeable increase in muscle angiogenesis (D'Souza, Zeng, Markworth, et al., 2018). Although we observed that the proangiogenic *VEGF* mRNA rapidly increased following exercise, consistent with previous studies (Gavin et al., 2007; Hoier & Hellsten, 2014), we did observe corresponding changes in VEGF protein content. The anti-angiogenic factor *SPRED-1* mRNA demonstrated a modest (less than 2-fold) increase, also without marked changes in its protein abundance. The robust upregulation of miR-126 after CWI versus ACT at 24 and 48 h may contribute to the observed reduction in *SPRED-1* protein content at 48 h post-CWI (D'Souza, Zeng, Markworth, et al., 2018; Meng et al., 2012; Mitchell et al., 2018; Wang et al., 2008; Wang et al., 2016). Therefore, it is possible that a reduction in the inhibitory actions of *SPRED-1* may contribute to the greater angiogenesis following repeated exercise and CWI, as previously reported. However, this requires careful validation given that we also demonstrate a possibility for



suppressed inhibition of angiogenesis, through downregulated SPRED-1 gene expression.

The pattern of gene and protein expression for either myogenesis or angiogenesis, including the abundance of key miRNA species fail to adequately explain the molecular response to CWI, and how these might affect the resultant phenotype adaptations. It is of interest to note the measured increased abundance of miR-133a and -126 at 2h post CWI contrast with the suppressive impacts on the miRNA processing genes. Without detailed analysis inclusive of greater time points, measurements of miRNA synthesis rates, and more comprehensive analysis of a wider array of miRNA species any conclusions can only be speculative. There is indeed the possibility that there are differing processing mechanisms in the maturation of classes or groups of miRNAs (Vechetti Jr et al., 2019). Furthermore, miRNAs are thought to function as distance signaling molecules and transported via extracellular vesicles (D'Souza, Woodhead, Zeng, et al., 2018; Ji & Guo, 2019). Hence, whether these measured miRNAs were indeed solely synthesized within the sampled biopsied tissue was not elucidated. Future studies, potentially in-vitro with the use of cultured myoblasts might be a strategy to delineate the independent impact of cold stimuli more comprehensively on temperature sensitive genes and miRNA.

An important feature of the current study is that the analysis of miRNA abundance was performed using probes that cannot differentiate immature from mature miRNA forms. Critically, small RNA sequencing will need to be applied in future studies, not only to investigate the abundances of miRNA isoforms, but also to provide the opportunity to assess the global changes in all non-coded small RNA species. The current study demonstrate that CWI blocks the increase in DROSHA and DICER protein levels following resistance exercise. How this affects the cellular concentrations of mature miRNA and the resultant functions remains unclear. Our results did not provide any specific molecular signature that was indicative of the phenotype adaptations of CWI in response to training. Apart from the rapid (2h) increased in *VEGF* mRNA expression, our results show little evidence for any consistent trend that might indicate a transcriptional mechanism of our previously reported increased angiogenic response to CWI. However, our findings that CWI following resistance exercise promote expression of miR-1/133 may help explain how CWI blunts the anabolic signaling and muscle protein synthesis in response to resistance exercise.

#### AUTHOR CONTRIBUTIONS

The study was designed by Randall F. D'Souza, Llion A Roberts, James F. Markworth, Truls Raastad, Jeff S.

Coombes, Jonathan M. Peake, David Cameron-Smith, Cameron J. Mitchell. Performed experiments: Randall F. D'Souza, Vandre C. Figueiredo, James F. Markworth, Nina Zeng, and Christopher P. Hedges. Sample Collection: Llion A Roberts and Jonathan M. Peake. Analyzed data: Randall F. D'Souza, James F. Markworth, Christopher P. Hedges. Drafted manuscript: Randall F. D'Souza. All authors critically evaluated and have approved the final content of the manuscript. Randall F. D'Souza is responsible for the final content of the manuscript.


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#### REFERENCES

- Chen, H.-S., Tong, H.-S., Zhao, Y., Hong, C.-Y., Bin, J.-P., & Su, L. (2018). Differential expression pattern of exosome long non-coding RNAs (lncRNAs) and MicroRNAs (miRNAs) in vascular endothelial cells under heat stroke. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, 24, 7965–7974.
- Chen, J.-F., Tao, Y., Li, J., Deng, Z., Yan, Z., Xiao, X., & Wang, D.-Z. (2010). microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. *Journal of Cell Biology*, 190, 867–879.

- Cordes, K. R., & Srivastava, D. (2009). MicroRNA regulation of cardiovascular development. *Circulation Research*, *104*, 724–732.
- Dang, L. T., Lawson, N. D., & Fish, J. E. (2013). MicroRNA control of vascular endothelial growth factor signaling output during vascular development. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *33*, 193–200.
- Devasthanam, A. S., & Tomasi, T. B. (2017). Dicer protein levels elevated by mild hyperthermia promote a pro-survival phenotype. *Oncotarget*, *8*, 67001–67016.
- Drummond, M. J., McCarthy, J. J., Fry, C. S., Esser, K. A., & Rasmussen, B. B. (2008). Aging differentially affects human skeletal muscle microRNA expression at rest and after an anabolic stimulus of resistance exercise and essential amino acids. *American Journal of Physiology-Endocrinology and Metabolism*, *295*, E1333–E1340.
- D'Souza, R. F., Bjørnsen, T., Zeng, N., Aasen, K. M., Raastad, T., Cameron-Smith, D., & Mitchell, C. J. (2017). MicroRNAs in muscle: Characterizing the powerlifter phenotype. *Frontiers in Physiology*, *8*, 383.
- D'Souza, R. F., Markworth, J. F., Aasen, K. M., Zeng, N., Cameron-Smith, D., & Mitchell, C. J. (2017). Acute resistance exercise modulates microRNA expression profiles: Combined tissue and circulatory targeted analyses. *PLoS One*, *12*, e0181594.
- D'Souza, R. F., Woodhead, J. S., Zeng, N., Blenkinsop, C., Merry, T. L., Cameron-Smith, D., & Mitchell, C. J. (2018). Circulatory exosomal miRNA following intense exercise is unrelated to muscle and plasma miRNA abundances. *American Journal of Physiology-Endocrinology and Metabolism*, *315*, E723–E733.
- D'Souza, R. F., Zeng, N., Figueiredo, V. C., Markworth, J. F., Durainayagam, B. R., Mitchell, S. M., Fanning, A. C., Poppitt, S. D., Cameron-Smith, D., & Mitchell, C. J. (2018). Dairy protein supplementation modulates the human skeletal muscle microRNA response to lower limb immobilization. *Molecular Nutrition & Food Research*, *62*, 1701028.
- D'Souza, R. F., Zeng, N., Markworth, J. F., Figueiredo, V. C., Hedges, C. P., Petersen, A. C., Della Gatta, P. A., Cameron-Smith, D., & Mitchell, C. J. (2019). Whey protein supplementation post resistance exercise in elderly men induces changes in muscle miRNA's compared to resistance exercise alone. *Frontiers Nutrition*, *91*. <https://doi.org/10.3389/fnut.2019.00091>
- D'Souza, R. F., Zeng, N., Markworth, J. F., Figueiredo, V. C., Roberts, L. A., Raastad, T., Coombes, J. S., Peake, J. M., Cameron-Smith, D., & Mitchell, C. J. (2018). Divergent effects of cold water immersion versus active recovery on skeletal muscle fiber type and angiogenesis in young men. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *314*, R824–R833.
- D'Souza, R. F., Zeng, N., Poppitt, S. D., Cameron-Smith, D., & Mitchell, C. J. (2019). Circulatory microRNAs are not effective biomarkers of muscle size and function in middle-aged men. *American Journal of Physiology-Cell Physiology*, *316*, C293–C298.
- Eisenberg, E., & Levanon, E. Y. (2013). Human housekeeping genes, revisited. *Trends in Genetics*, *29*, 569–574.
- Figueiredo, V. C., Roberts, L. A., Markworth, J. F., Barnett, M. P., Coombes, J. S., Raastad, T., Peake, J. M., & Cameron-Smith, D. (2016). Impact of resistance exercise on ribosome biogenesis is acutely regulated by post-exercise recovery strategies. *Physiological Reports*, *4*, e12670.
- Fish, J. E., Santoro, M. M., Morton, S. U., Yu, S., Yeh, R.-F., Wythe, J. D., Ivey, K. N., Bruneau, B. G., Stainier, D. Y., & Srivastava, D. (2008). miR-126 regulates angiogenic signaling and vascular integrity. *Developmental Cell*, *15*, 272–284.
- Fonseca, L. B., Brito, C. J., Silva, R. J. S., Silva-Grigoletto, M. E., da Silva, W. M., & Franchini, E. (2016). Use of cold-water immersion to reduce muscle damage and delayed-onset muscle soreness and preserve muscle power in jiu-jitsu athletes. *Journal of Athletic Training*, *51*, 540–549.
- Fuchs, C. J., Kouw, I. W., Churchward-Venne, T. A., Smeets, J. S., Senden, J. M., Lichtenbelt, W. D. M., Verdijk, L. B., & van Loon, L. J. (2020). Postexercise cooling impairs muscle protein synthesis rates in recreational athletes. *The Journal of Physiology*, *598*, 755–772.
- Fukuoka, M., Yoshida, M., Eda, A., Takahashi, M., & Hohjoh, H. (2014). Gene silencing mediated by endogenous microRNAs under heat stress conditions in mammalian cells. *PLoS One*, *9*, e103130.
- Gagan, J., Dey, B. K., & Dutta, A. (2012). MicroRNAs regulate and provide robustness to the myogenic transcriptional network. *Current Opinion in Pharmacology*, *12*, 383–388.
- Garner, R. T., Solfest, J. S., Nie, Y., Kuang, S., Stout, J., & Gavin, T. P. (2020). Multivesicular body and exosome pathway responses to acute exercise. *Experimental Physiology*, *105*, 511–521.
- Gavin, T., Drew, J., Kubik, C., Pofahl, W., & Hickner, R. (2007). Acute resistance exercise increases skeletal muscle angiogenic growth factor expression. *Acta Physiologica*, *191*, 139–146.
- Harding, R. L., & Velleman, S. G. (2016). MicroRNA regulation of myogenic satellite cell proliferation and differentiation. *Molecular and Cellular Biochemistry*, *412*, 181–195.
- Henn, D., Abu-Halima, M., Wermke, D., Falkner, F., Thomas, B., Köpple, C., Ludwig, N., Schulte, M., Brockmann, M. A., & Kim, Y.-J. (2019). MicroRNA-regulated pathways of flow-stimulated angiogenesis and vascular remodeling in vivo. *Journal of Translational Medicine*, *17*, 1–19.
- Hoier, B., & Hellsten, Y. (2014). Exercise-induced capillary growth in human skeletal muscle and the dynamics of VEGF. *Microcirculation*, *21*, 301–314.
- Ivey, K. N., Muth, A., Arnold, J., King, F. W., Yeh, R.-F., Fish, J. E., Hsiao, E. C., Schwartz, R. J., Conklin, B. R., & Bernstein, H. S. (2008). MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. *Cell Stem Cell*, *2*, 219–229.
- Ji, C., & Guo, X. (2019). The clinical potential of circulating microRNAs in obesity. *Nature Reviews Endocrinology*, *15*, 731–743.
- Kozomara, A., & Griffiths-Jones, S. (2014). miRBase: Annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Research*, *42*, D68–D73.
- Liu, J., Zhu, G., Xu, S., Liu, S., Lu, Q., & Tang, Z. (2017). Analysis of miRNA expression profiling in human umbilical vein endothelial cells affected by heat stress. *International Journal of Molecular Medicine*, *40*, 1719–1730.
- Margolis, L. M., Lessard, S. J., Ezzyat, Y., Fielding, R. A., & Rivas, D. A. (2017). Circulating microRNA are predictive of aging and acute adaptive response to resistance exercise in men. *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences*, *72*, 1319–1326.
- Margolis, L. M., & Rivas, D. A. (2018). Potential role of microRNA in the anabolic capacity of skeletal muscle with aging. *Exercise and Sport Sciences Reviews*, *46*, 86–91.
- McCarthy, J. J., & Esser, K. A. (2007). MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy. *Journal of Applied Physiology*, *102*, 306–313.

- Meng, S., Cao, J.-T., Zhang, B., Zhou, Q., Shen, C.-X., & Wang, C.-Q. (2012). Downregulation of microRNA-126 in endothelial progenitor cells from diabetes patients, impairs their functional properties, via target gene Spred-1. *Journal of Molecular and Cellular Cardiology*, *53*, 64–72.
- Mitchell, C. J., D'Souza, R. F., Schierding, W., Zeng, N., Ramzan, F., O'Sullivan, J. M., Poppitt, S. D., & Cameron-Smith, D. (2018). Identification of human skeletal muscle miRNA related to strength by high-throughput sequencing. *Physiological Genomics*, *50*, 416–424.
- Moore, E., Fuller, J. T., Buckley, J. D., Saunders, S., Halson, S. L., Broatch, J. R., & Bellenger, C. R. (2022). Impact of cold-water immersion compared with passive recovery following a single bout of strenuous exercise on athletic performance in physically active participants: A systematic review with meta-analysis and meta-regression. *Sports Medicine*, *52*, 1667–1688.
- Nederveen, J. P., Snijders, T., Joanisse, S., Wavell, C. G., Mitchell, C. J., Johnston, L. M., Baker, S. K., Phillips, S. M., & Parise, G. (2017). Altered muscle satellite cell activation following 16 wk of resistance training in young men. *American Journal Of Physiology-Regulatory, Integrative And Comparative Physiology*, *312*, R85–R92.
- Oikawa, S., Lee, M., Motohashi, N., Maeda, S., & Akimoto, T. (2019). An inducible knockout of Dicer in adult mice does not affect endurance exercise-induced muscle adaptation. *American Journal of Physiology-Cell Physiology*, *316*, C285–C292.
- Peake, J. M., Markworth, J. F., Cumming, K. T., Aas, S. N., Roberts, L. A., Raastad, T., Cameron-Smith, D., & Figueiredo, V. C. (2020). The effects of cold water immersion and active recovery on molecular factors that regulate growth and remodeling of skeletal muscle after resistance exercise. *Frontiers in Physiology*, *11*, 737.
- Pilotte, J., Dupont-Versteegden, E. E., & Vanderklish, P. W. (2011). Widespread regulation of miRNA biogenesis at the Dicer step by the cold-inducible RNA-binding protein, RBM3. *PLoS One*, *6*, e28446.
- Roberts, L. A., Nosaka, K., Coombes, J. S., & Peake, J. M. (2014). Cold water immersion enhances recovery of submaximal muscle function after resistance exercise. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *307*, R998–R1008.
- Roberts, L. A., Raastad, T., Markworth, J. F., Figueiredo, V. C., Egner, I. M., Shield, A., Cameron-Smith, D., Coombes, J. S., & Peake, J. M. (2015). Post-exercise cold water immersion attenuates acute anabolic signalling and long-term adaptations in muscle to strength training. *The Journal of Physiology*, *593*, 4285–4301.
- Russell, A. P., Lamon, S., Boon, H., Wada, S., Güller, I., Brown, E. L., Chibalin, A. V., Zierath, J. R., Snow, R. J., & Stepto, N. (2013). Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training. *The Journal of Physiology*, *591*, 4637–4653.
- Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, *3*, 1101–1108.
- Sevignani, C., Calin, G. A., Siracusa, L. D., & Croce, C. M. (2006). Mammalian microRNAs: A small world for fine-tuning gene expression. *Mammalian Genome*, *17*, 189–202.
- Siqueira, A. F., Vieira, A., Bottaro, M., Ferreira-Júnior, J. B., Nobrega, O. T., de Souza, V. C., Marqueti, R. C., Babault, N., & Durigan, J. L. Q. (2018). Multiple cold-water immersions attenuate muscle damage but not alter systemic inflammation and muscle function recovery: A parallel randomized controlled trial. *Scientific Reports*, *8*, 10961.
- Sun, C.-Y., She, X.-M., Qin, Y., Chu, Z.-B., Chen, L., Ai, L.-S., Zhang, L., & Hu, Y. (2013). miR-15a and miR-16 affect the angiogenesis of multiple myeloma by targeting VEGF. *Carcinogenesis*, *34*, 426–435.
- Umehara, T., Kagawa, S., Tomida, A., Murase, T., Abe, Y., Shingu, K., & Ikematsu, K. (2020). Body temperature-dependent microRNA expression analysis in rats: Rno-miR-374-5p regulates apoptosis in skeletal muscle cells via Mex3B under hypothermia. *Scientific Reports*, *10*, 15432.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*, 1–12.
- Vechetti, I. J., Jr., Wen, Y., Chaillou, T., Murach, K. A., Alimov, A. P., Figueiredo, V. C., Dal-Pai-Silva, M., & McCarthy, J. J. (2019). Life-long reduction in myomiR expression does not adversely affect skeletal muscle morphology. *Scientific Reports*, *9*, 5483.
- Wang, L., Lee, A. Y. W., Wigg, J. P., Peshavariya, H., Liu, P., & Zhang, H. (2016). miR-126 regulation of angiogenesis in age-related macular degeneration in CNV mouse model. *International Journal of Molecular Sciences*, *17*, 895.
- Wang, S., Aurora, A. B., Johnson, B. A., Qi, X., McAnally, J., Hill, J. A., Richardson, J. A., Bassel-Duby, R., & Olson, E. N. (2008). The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Developmental Cell*, *15*, 261–271.
- Wang, S., & Olson, E. N. (2009). AngiomiRs—Key regulators of angiogenesis. *Current Opinion in Genetics & Development*, *19*, 205–211.
- Yin, J., Qian, Z., Chen, Y., Li, Y., & Zhou, X. (2020). MicroRNA regulatory networks in the pathogenesis of sarcopenia. *Journal of Cellular and Molecular Medicine*, *24*, 4900–4912.
- Yin, K.-J., Olsen, K., Hamblin, M., Zhang, J., Schwendeman, S. P., & Chen, Y. E. (2012). Vascular endothelial cell-specific MicroRNA-15a inhibits angiogenesis in hindlimb ischemia. *Journal of Biological Chemistry*, *287*, 27055–27064.
- Ying, S.-Y., & Lin, S.-L. (2004). Intron-derived microRNAs—Fine tuning of gene functions. *Gene*, *342*, 25–28.
- Zacharewicz, E., Della Gatta, P., Reynolds, J., Garnham, A., Crowley, T., Russell, A. P., & Lamon, S. (2014). Identification of microRNAs linked to regulators of muscle protein synthesis and regeneration in young and old skeletal muscle. *PLoS One*, *9*, e114009.
- Zeng, N., D'Souza, R. F., Mitchell, C. J., & Cameron-Smith, D. (2018). Sestrins are differentially expressed with age in the skeletal muscle of men: A cross-sectional analysis. *Experimental Gerontology*, *110*, 23–34.

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