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Running head: MicroRNA expression in high and low capacity runner rats

Running Head: MicroRNA regulation in high and low running capacity rats

Expression of microRNAs and target proteins in skeletal muscle of rats selectively bred for high and low running capacity

Samuel K. Pinto[1], Séverine Lamon[2], Erin J. Stephenson[3], Ming Kalanon[2], Jasmine Mikovic[2], Lauren G. Koch[4], Steven L. Britton[4], John A. Hawley[1,5], Donny M. Camera[1]

[1]Centre for Exercise and Nutrition, Mary MacKillop Institute for Health Research, Australian Catholic University, Melbourne, Victoria, Australia; [2]Institute for Physical Activity and Nutrition, School of Exercise and Nutrition Sciences, Deakin University Geelong, Victoria, Australia; [3]Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee; Department of Pediatrics, University of Tennessee Health Science Center, Memphis, Tennessee; and Children's Foundation Research Institute, Le Bonheur Children's Hospital, Memphis, Tennessee; [4]Department of Anaesthesiology, University of Michigan, Ann Arbor, Michigan, USA; [5]Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, United Kingdom

Author for correspondence:

Donny Camera

Centre for Exercise and Nutrition; Mary MacKillop Institute for Health Research

Australian Catholic University

Level 5, 215 Spring St, Melbourne VIC 3000; Australia

Email: donny.camera@acu.edu.au

Phone: +61 9953 3527

Running head: MicroRNA expression in high and low capacity runner rats

Abstract

Impairments in mitochondrial function and substrate metabolism are implicated in the etiology of obesity and type 2 diabetes. MicroRNAs (miRNAs) can degrade mRNA or repress protein translation and have been implicated in the development of such disorders. We used a contrasting rat model system of selectively bred high- (HCR) or low- (LCR) intrinsic running capacity with established differences in metabolic health to investigate the molecular mechanisms through which miRNAs regulate target proteins mediating mitochondrial function and substrate oxidation processes. Quantification of select miRNAs using the Rat miFinder miRNA PCR array revealed differential expression of 15 skeletal muscle (*m. tibialis anterior*) miRNAs between HCR and LCR rats (14 with higher expression in LCR; $P<0.05$). Ingenuity Pathway Analysis predicted these altered miRNAs to collectively target multiple proteins implicated in mitochondrial dysfunction and energy substrate metabolism. Total protein abundance of citrate synthase (CS; miR-19 target) and voltage-dependent anion channel 1 (miR-7a target) were higher in HCR compared to LCR cohorts (~57 and ~26%, respectively; $P<0.05$). A negative correlation was observed for miR-19a-3p and CS (r =0.59, $P=0.02$) protein expression in LCR. To determine if miR-19a-3p can regulate CS *in vitro* we performed luciferase reporter and transfection assays in C2C12 myotubes. MiR-19a-3p binding to the CS untranslated region did not change luciferase reporter activity, however miR-19a-3p transfection decreased CS protein expression (~70%; $P<0.05$). The differential miRNA expression targeting proteins implicated in mitochondrial dysfunction and energy substrate metabolism may contribute to the molecular basis mediating the divergent metabolic health profiles of LCR and HCR rats.

Key words: Mitochondrial dysfunction, substrate oxidation, gene expression, citrate synthase

Introduction

Metabolic disorders such as type 2 diabetes and obesity are characterized by a loss of 'metabolic plasticity' where skeletal muscle is unable to effectively transition between lipid- and carbohydrate-based oxidation in response to the prevailing hormonal mileau (17). Development of these clinical conditions is determined by a complex interaction of environmental (lifestyle) and genetic (heritable) factors. Through two-way artificial selection breeding for treadmill running capacity, intrinsically high capacity runner (HCR) and low capacity runner (LCR) rats provide an excellent model system for studying the genetic factors mediating extremes in metabolic health. The HCR rats present with over 8-fold greater intrinsic aerobic running capacity at generation 28 compared to LCR rats and over 40% of the variance of the running capacity phenotype due to additive genetic variance (narrow-sense heritability, $h2 = 0.47 \pm 0.02$ in HCRs and 0.43 ± 0.03 in LCRs) (31). This superior aerobic capacity and metabolic health profile of HCR rats has, in part, been attributed to an increased activity of skeletal muscle proteins involved in mitochondrial function and substrate oxidation (15, 29, 33, 38) compared to the impaired mitochondrial function observed in LCR animals (34, 38). Thus, investigating the gene-regulatory mechanisms mediating these processes in a translational animal model system may provide new insight to the molecular basis controlling metabolic health.

MicroRNAs (miRNAs) are short, non-coding RNAs that regulate gene expression by binding to mRNA, subsequently instigating degradation or repressing protein translation (2, 10). Altered miRNA expression has been implicated in the pathogenesis of several metabolic conditions including obesity and type 2 diabetes through the regulation of key metabolic signaling networks involved in glucose and lipid handling, and mitochondrial metabolism (9, 13, 43). Additionally, divergent miRNA expression has recently been characterized in mice

with inherently high or low physical activity levels as well in human 'high' and 'low' responders to resistance exercise (5, 6). These findings suggest that miRNAs may contribute to the metabolic adaptation profile induced by physical/exercise activity. Whether miRNAs contribute to the signaling pathways that mediate the intrinsic skeletal muscle metabolic phenotypes divergent between HCR and LCR rats is unknown. We aimed to determine the miRNA expression profile and interactions with predicted protein targets implicated in metabolic health in skeletal muscle from HCR and LCR rats. We hypothesized that HCR and LCR rats would present divergent miRNA expression profiles in a non-exercise condition, with HCR rats displaying a miRNA profile that upregulates proteins promoting efficient substrate oxidation and enhanced mitochondrial function.

Materials and Methods

Experimental animals

HCR and LCR rats derived from genetically heterogeneous N:NIH stock rats by two-way artificial selection for maximal treadmill running capacity were used in this study. The breeding program and aerobic capacity testing procedures have been described in detail previously (20). Parent rats from generation 27 of selection were bred at the University of Michigan (Ann Arbor, MI, USA) and their female offspring, HCR (n = 12) and LCR (n = 12), transported to Royal Melbourne Institute of Technology (RMIT) University (Bundoora, Australia) at ~8 weeks (wk) of age. We have previously reported maximal respiratory capacity and fasting serum insulin concentrations from this LCR/ HCR generation (REF). HCRs from later generations (i.e.: 23-27) have shown similar increases in running capacity and Citrate Synthase activity above LCRs compared to earlier generations (7-11) (16, 34, 39, 41). Rats were allowed 2 wk to acclimate to RMIT facilities as previously described (38). Neither HCR or LCR rats underwent any form of exercise training during the study period.

Rats received ad libitum access to water and a standard chow diet whilst being housed under a 12:12 hour light-dark cycle in a temperature controlled environment (22 °C). Experimental procedures were approved by the University Committee on Use and Care of Animals at the University of Michigan and the RMIT University Animal Ethics Committee prior to the onset of the study.

Tissue collection

At 11 wk of age, rats were weighed and anesthetized using pentobarbital sodium (60 mg/kg body wt). The *m. tibialis anterior* (TA) was immediately excised, freeze clamped in liquid nitrogen and stored at -80 °C for subsequent analysis.

RNA extraction and quantification

RNA extraction from skeletal muscle tissue was performed using TRIzol in accordance with the manufacturer's instructions and described previously (3). Briefly, approximately 20 mg of tissue was homogenized in TRIzol and chloroform was added to form an aqueous upper phase which was precipitated by adding isopropanol. The remaining RNA pellet was washed and re-suspended in 35 µL's RNase-free water. RNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

Reverse Transcription (RT) and Real-Time PCR

A miScript II RT Kit (catalogue #218160; Qiagen, Melbourne, Australia) was used to synthesize cDNA from RNA samples using a BioRad thermal cycler (BioRad Laboratories, Gladesville, Australia) in accordance with the manufacturer's instructions. Changes in miRNA expression were quantified using a Rat miFinder miRNA PCR Array (catalogue #MIRN-001ZD-24; Qiagen, Melbourne, Australia) in a 96-well RT cycler CFX96 (BioRad

Laboratories, Gladesville, Australia) for 40 cycles (two steps: 95ºC for 15 s followed by 60ºC for 30 s). This microarray contained the 84 most abundantly expressed and best characterized miRNAs present in rats. These miRNA targets can be found via the link: http://www.sabiosciences.com/mirna_pcr_product/HTML/MIRN-001Z.html This microarray was selected as many of these miRNAs have been previously shown to regulate targets shown to have roles in substrate oxidation and mitochondrial function (4, 8, 11, 27) and is therefore relevant to the HCR and LCR experimental model. Six housekeeping control RNAs were also measured on this microarray for normalization. The relative amounts of each miRNA in PCR analysis was normalised to the average of these six (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, RNU6–2) house-keeping genes. There were no changes in the absolute CT of each individual house-keeping gene or the average between LCR and HCR cohorts (data not shown). The $2^{\Delta\Delta CT}$ method of relative quantification was used to calculate relative amounts of miRNAs (28).

miRNA target prediction

Protein/mRNA targets of miRNAs differentially expressed ($P < 0.05$) between HCR and LCR skeletal muscle were predicted using the microRNA Target Filter function of Qiagen's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). IPA's microRNA Target Filter incorporates multiple target prediction programs including TargetScan, TarBase, miRecords and the Ingenuity Knowledge Base. Predicted relationships were filtered to be either 'highly predicted' by algorithms or 'experimentally observed' by previous research. Predicted targets were then filtered to be implicated in 'Mitochondrial Dysfunction' and 'TCA Cycle II (Eukaryotic)' in skeletal muscle. These filter criteria were selected for investigation as LCR rats exhibit impaired skeletal muscle mitochondrial and TCA cycle function compared to HCR (34, 38). Predicted targets meeting these criteria were

identified for 11 of the 15 miRNAs differentially expressed between HCR and LCR rats. A minimum of one predicted protein/mRNA target was selected for further protein expression analysis (described subsequently) for each of the 11 differentially expressed miRNAs which presented protein/mRNA targets implicated in 'Mitochondrial Dysfunction' and 'TCA cycle II (Eukaryotic)'.

Western Blotting (skeletal muscle)

Approximately 30 mg of TA was homogenized in ice-cold buffer as previously described (39). Lysates were centrifuged at 12,000 *g* for 20 min at 4 °C and the supernatant was transferred to a sterile microcentrifuge tube and aliquoted to measure protein concentration using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Lysate was then re-suspended in 4X Laemmli sample buffer with 40 µg of protein loaded onto 4–20% Mini-PROTEAN TGX Stain-Free™ Gels (BioRad Laboratories, Gladesville, Australia). Post electrophoresis, gels were activated on a Chemidoc according to the manufacturer's instructions (BioRad Laboratories, Gladesville, Australia) and then transferred to polyvinylidine fluoride (PVDF) membranes. After transfer, a stain-free image of the PVDF membranes (14) for total protein normalization was obtained before membranes were rinsed briefly in distilled water and blocked with 5% non-fat milk, washed with 10 mM of Tris–HCl, 100 mM of NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1000) overnight at 4 °C. Membranes were then incubated with secondary antibody (1:2000), and proteins were detected via enhanced chemiluminescence (Thermo Fisher, Scoresby, Australia) and quantified by densitometry (ChemiDoc™ XRS+ System; BioRad Laboratories, California, USA). HCR and LCR samples were run on the same gel. Primary antibodies used were polyclonal caspase-3 (CASP3) (#9662), leucine-rich repeat kinase 2 (LRRK2) (#5559) (Cell Signaling, Beverly, MA, USA), polyclonal ATP synthase

mitochondrial F1 complex assembly factor 1 (ATPAF1) (#ab101518), beta-site APP cleaving enzyme 1 (BACE1) (#ab2077), Citrate Synthase (CS) (ab96600) and monoclonal Glycerol-3-Phosphate Dehydrogenase 2 (GPD2) (ab188585), MAP2K4 (ab33912), VDAC1 (ab14734) (Abcam, Cambridge, UK). Volume density of each target protein band was normalized to the total protein loaded into each lane using stain-free technology (14), with data expressed in arbitrary units (Figure 7).

Citrate synthase activity

CS activity was measured to identify whether differences in CS protein abundance were also accompanied by differences in activity. Skeletal muscle homogenates (n = 10) from freeze clamped TA muscles (10-20 mg) were prepared over ice in buffer [175 mM KCl and 2 mM EDTA (pH 7.4), 1:50 or 1:100 dilution]. Homogenates underwent three freeze-thaw cycles and CS activity was measured according to the method of Srere (37) with modifications as described previously (38).

Cell Culture

Stock C2C12 (mouse) myoblasts (ATCC, Manassas, VA, USA) were maintained at 37°C (95% O2-5% CO2) in high glucose (4.5g/L D-Glucose) culture medium with 2mM glutamine and 110 mg/L sodium pyruvate (Dulbecco's modified Eagle's medium (DMEM)), containing 10% fetal bovine serum (FBS; Life Technologies, Melbourne, Australia). For differentiation experiments, when cultures approached confluence (~90% confluent), medium was changed to differentiation medium (DMEM, supplemented with 2% horse serum; Life Technologies, Melbourne, Australia). Differentiation medium was replaced every 24 h.

Luciferase Reporter Assay

C2C12 myoblasts (1-2 x 10^5/mL) were seeded in black-walled 96-well plates. Twenty-four hours after seeding, cells were co-transfected with 150 ng pNanoglo2 vector (Promega, Alexandria, Australia) containing either: no insertion (empty control); the putative rat miR-19a-3p Citrate Synthase target site (including the predicted seed site with 10 base pairs on either side; Primer sequence- Forward: 5' CAGCAGCCTCAtttgcacagattttcaGTGACTCAGAccgcggG 3', Reverse: 5' CTAGCccgcggTCTGAGTCACtgaaaatctgtgcaaaTGAGGCTGCTGAGCT); or its mutant control, cloned between SacI and NheI downstream of the Nanoluc luciferase (Primer sequence- Forward 5' CAGCAGCCTCAcaaccaatcgagaactGTGACTCAGAccgcggG 3', Reverse: 5' CTAGCccgcggTCTGAGTCACagttctcgattggttgTGAGGCTGCTGAGCT 3'; together with 5 nM miR-19a-3p mimics (mirVana™ miRNA mimic, Life technologies, Mulgrave, Australia), or an irrelevant miRNA control (miR-99b-5p), using Lipofectamine 2000 (Thermo Fisher, Scoresby, Australia) following the manufacturer's protocol. Four hours' post-transfection, the media was removed and replaced with culture medium. Twenty-four hours later, cells were assayed for Firefly and Nanoluc luciferase expression using the Nano-Glo® Dual-luciferase® Reporter assay kit (Promega, Alexandria, Australia) following the manufacturer's protocol. The data reported are the results of three independent experiments performed in six replicates.

MiRNA transfection

C2C12 myoblasts were cultured (as above) and seeded (1.5 x 10^5 cells per well) into six-well plates 24 h before transfection. Myoblasts were transiently transfected with 1nM of miR-19a-3p mimic and a scramble negative control (mirVana™miRNA mimic, Life technologies) using Lipofectamine 2000 (Thermo Fisher; Scoresby, Australia). The myoblasts were placed in transfection medium for 4 h. Following this period, the transfection medium was switched

to culture medium until their harvest. RNA and protein were extracted for RT-PCR gene expression and Western Blot analysis, respectively.

Real Time Quantitative PCR and Western Blotting

C2C12 cells were homogenised in TRIZOL and RNA extracted using an RNeasy Mini Kit (Qiagen, Chadstone, Australia) according to the manufacturer's directions. First-strand cDNA synthesis was performed using either the SuperScript® VILO™ cDNA Synthesis kit (Thermo Fisher, Scoresby, Australia) or TaqMan® MicroRNA Reverse Transcription Kit in a final reaction volume of 20 µl according to the manufacturer's directions. Quantification of mRNA (in duplicate) was performed on a BioRad CFX96 thermal cycler (BioRad, Gladesville, Australia). Taqman-FAM-labelled primer/probes for citrate synthase (Cat No. Mm00466043_m1) and miR-19a-3p (Cat No. 000395) were used in a final reaction volume of 20 µl. PCR conditions were 2 min at 50 ºC for UNG activation, 10 min at 95 ºC then 40 cycles of 95 ºC for 15 s and 60 ºC for 60 s. β-actin (Cat No. Mm02619580_g1) and SnoRNA202 (Cat No. 001232) were used as a housekeeping gene to normalize threshold cycle (CT) values for mRNA and miRNA analyses, respectively. The relative amounts of mRNAs were calculated using the relative quantification (ΔΔCT) method (28).

For Western Blot analyses, proteins were lysed in a 1 × modified RIPA (Merck Millipore, North Ryde, Australia) containing 1:1000 protease inhibitor cocktail (Sigma-Aldrich, Castle Hill, Australia) and 1:100 Halt phosphatase inhibitor cocktail (Thermo Fisher, Scoresby, Australia) and left on ice for 30 min prior to centrifugation to remove insoluble material. Lysates containing twenty micrograms of protein were electrophoresed and transferred as described above with a stain-free image of the PVDF membranes obtained for total protein normalization. Transfected and scrambled samples from the same time point of collection

were run on the same gel, and the same polyclonal CS antibody as mentioned above was used to measure CS protein expression. Volume density of each target protein band was normalized to the total protein loaded into each lane using stain-free technology (10), with data expressed in arbitrary units (Figure 7).

Statistical analyses

A two-tailed unpaired t-test (GraphPad Prism Version 5.03) was used to detect differences between HCR and LCR groups in miRNA expression, protein abundance, enzyme activity and for all *in vitro* analyses of C2C12 cells. All data was subjected to the normality test using the Shapiro-Wilk test (SigmaPlot 12.0). Linear regression analysis was performed to determine associations between miRNA species and their predicted protein targets in HCR and LCR phenotypes (GraphPad Prism Version 5.03). All values are expressed as arbitrary units (AU) and presented as mean ± standard deviation (SD). Statistical significance was set at $P < 0.05$.

Results

Differential miRNA expression

There was a higher expression in LCR compared to HCR for let-7i-5p (~147% percent change), -7e-5p (~93%), miR-7a-5p (~35%), -19a-3p (~66%), -24-3p (~37%), -26a-5p (~58%), -28-5p (~54%), -30a-5p (~67%), -99a-5p (~54%), -181a-5p (~81%), -194-5p (~39%), -223-3p (~59%), -374-5p (~68%) and -376c-3p (~121%), while miR-103-3p was more highly expressed ($P < 0.05$) in HCR than LCR (~31%; Figure 1). All differentially expressed miRNAs had a mean Ct value < 32. The other 69 miRNAs analyzed were not significantly different between HCR and LCR rats (Table 1).

Bioinformatics analysis of differentially expressed miRNAs

The microRNA Target Filter function of Qiagen's IPA predicted 5672 mRNAs (2964 in skeletal muscle) to be targeted by the 15 miRNAs differentially expressed between HCR and LCR skeletal muscle samples. Eleven of the 15 differentially expressed miRNAs were predicted to target 19 mRNAs implicated in skeletal muscle mitochondrial dysfunction and TCA cycle function (Figure 2).

Protein abundance of miRNA targets

There was a greater protein abundance of CS (~57%) and VDAC1 (~26%) in HCR compared to LCR rats ($P < 0.05$; Figure 3A, B). Levels of GPD2 (~28%) were higher in LCR rats ($P < 0.05$; Figure 3C). There were no changes in the expression of CASP3, LRRK2, ATPAF1, BACE1, or MAP2K4 between HCR and LCR rats (Figure 3).

miRNA-protein correlations

A significant negative correlation was observed for miR-19a-3p and CS expression in LCR rats (r = 0.59, $P = 0.02$; Figure 4) compared to the HCR (r = XX, P = 0.76, data not shown). No other correlations between miRNAs and target proteins were found.

Citrate Synthase Activity

CS activity was significantly greater in HCR relative to LCR rats (~58%; $P < 0.05$, Figure 5).

Luciferase reporter assay and miR-19a-3p transfection

There were no changes in Nanoluc luciferase activity in cells co-transfected with the miR-19a-3p mimic and either the full length CS 3'UTR or the predicted miR_19a-3p target site on CS 3'UTR compared to cells transfected with an irrelevant miRNA (data not shown).

Transfection of miRNA mimics significantly increased levels of miR-19a-3p expression by ~8,165 % following 4 h transfection (Figure 6A). Citrate synthase mRNA levels were unchanged following miR-19a-3p transfection (Figure 6B) however there was a ~70% reduction in CS protein abundance compared to the scrambled negative control 4 h transfection (Figure 6C).

Discussion

MicroRNAs have emerged as key regulators of metabolic health through their ability to repress gene and protein expression (2) and may mediate underlying differences in intrinsic metabolic function between individuals. Using an animal model of inherited low- or high intrinsic running capacity that simultaneously associates with poor or good metabolic health (21), we report evidence for divergent skeletal muscle miRNA expression profiles . Specifically, 15 miRNAs with predicted mRNA targets involved in mitochondrial dysfunction and substrate oxidation were differentially expressed between HCR and LCR rats. Moreover, we show the abundance of predicted protein targets CS and VDAC1 were altered between phenotypes in accordance with miRNA expression profile. These findings suggest a regulatory role for specific skeletal muscle miRNAs of target proteins central to mitochondrial content and function.

MicroRNAs are critical regulators of skeletal muscle metabolism via the negative regulation of proteins involved in mitochondrial function and energy substrate oxidation (42).We therefore investigated the molecular events that may influence the diverse transcriptional differences in mitochondrial function and substrate handling previously reported between LCR and HCR rats (25, 32, 34, 39). Of the 84 most abundant miRNAs present in rats, there was a total of 5672 predicted protein/mRNA targets (2964 in skeletal muscle) arising from

the 15 differentially expressed miRNAs measured by IPA's microRNA Target Filter, demonstrating the potentially widespread role for miRNAs in determining the differential between HCR and LCR intrinsic phenotypes. Eleven of these differentially expressed miRNAs showed predicted protein targets implicated in mitochondrial dysfunction as identified by IPA. Numerous studies have attributed the impaired metabolic phenotype of LCR rats partly to a decrease in the abundance of skeletal muscle proteins critical to mitochondrial function (15, 34, 38). Therefore, we hypothesised that miRNAs may be a contributing regulatory mechanism to the divergent mitochondrial features and metabolic phenotypes previously characterized between HCR and LCR rats.

The first novel finding of our work was the greater miR-19a-3p expression in LCR compared to HCR rats (~63% percent change; Figure 1), which has predicted targets involved in mitochondrial dysfunction and the TCA cycle. We quantified the abundance of these predicted targets (Beta-site APP cleaving enzyme 1 and Citrate Synthase) to investigate putative interactions, finding a ~57% decrease in citrate synthase (CS) protein expression in TA from LCR rats compared to HCR rats (Figure 3). This decrease in protein expression was also supported by a reduction in citrate synthase activity (Figure 5). This is in agreement with previous reports of greater CS abundance and activity in the *m. gastrocnemius*, *m. soleus* and *m. extensor digitorum longus* of HCR rats relative to LCR rats (12, 15, 30, 33, 34, 38, 40). CS is a rate limiting enzyme of the TCA cycle located in the mitochondrial matrix and is often used as a surrogate measure for skeletal muscle mitochondrial content (22). Attenuated CS activity and abundance has been reported in the skeletal muscle of type 2 diabetic and obese individuals (18, 19, 36). Here, we report an inverse correlation between miR-19a-3p and CS expression in muscle from LCR rats, which is the first experimental evidence that miR-19a-3p may play a role in determining the mitochondrial capacity of skeletal muscle.

To confirm whether miR-19a-3p can directly bind and regulate CS transcription, C2C12 myoblasts were co-transfected with a reporter plasmid containing a section of the putative rat miR-19a-3p Citrate Synthase target site, as well as the miR-19-3p mimic, an irrelevant miRNA that did not have a predicted binding site on the CS 3'UTR (miR-99b-5p) or no mimic at all. No reduction in luminescence levels was observed with miR-19a-3p, indicating that miR-19a-3p did not bind to the CS 3'UTR. CS gene expression data further supports this as no down-regulation of CS mRNA expression was observed following miR-19a-3p transfection. In contrast, overexpression of miR-19a-3p in C2C12 myoblasts decreased CS protein levels 4 h after the onset of transfection when compared to a scrambled control. This interaction may be direct and occur at the protein level to inhibit protein translation while allowing normal mRNA transcription. Alternatively, miR-19a-3p may interact with CS in area outside the 3'UTR to regulate its mRNA expression (23). Our findings therefore suggest that miR-19a-3p mediate signalling events controlling energy substrate metabolism and mitochondrial content, and reveal novel mechanistic information to the regulatory control of CS expression in skeletal muscle.

Another major finding from our study was the higher miR-7a expression in LCR rats (~35% percent change; Figure 1). miR-7a has been implicated in the development of insulin resistance through its down-regulation of insulin receptor substrate 1 expression and inhibition of insulin-stimulated Akt phosphorylation and glucose uptake (26). Considering LCR rats present impaired skeletal muscle insulin signalling and IRS1 phosphorylation relative to HCR (33), and miR-7a was more highly expressed in LCR rats, it is possible miR-7a may play a role in the attenuated insulin signalling response between these cohorts. Two protein targets of miR-7a identified by IPA in the 'Mitochondrial Dysfunction' filter were

VDAC1 and BACE1. VDAC1 is an outer mitochondrial membrane protein involved in the TCA cycle responsible for transporting calcium ions and metabolites including ATP across the outer mitochondrial membrane (35). VDAC1 deficient mice have been shown to display impaired glucose tolerance and exercise capacity due to impaired mitochondria-bound hexokinase activity (1). In our study, the first to compare VDAC1 protein expression between LCR and HCR rats, we observed significantly lower VDAC1 protein expression in the LCR cohort. This raises the possibility that miR-7a and VDAC1 may contribute to the divergent metabolic profiles previously established between LCR and HCR (33). Further work incorporation miR-7a over-expression analyses are required to better understand the capacity for it to regulate cellular energy production and metabolism processes.

Of the other protein targets analysed from the differentially expressed miRNAs between LCR and HCR cohorts, protein levels of Glycerol-3-Phosphate Dehydrogenase 2 (GPD2) were higher in LCR compared to HCR rats. GPD2 is a mitochondrial membrane protein centrally involved in glycolysis and was a predicted target of miR-30a. While increased GPD2 abundance in LCR skeletal muscle was unexpected based on higher miR-30a expression profile in LCR compared to HCR rats, this higher abundance of GPD2 indicates a greater reliance on glycolysis for energy production compared to HCR rats. Indeed, previous work from our laboratory has demonstrated that LCR skeletal muscle is more reliant on carbohydrate than fat metabolism at rest (33). These findings suggest other signalling mechanisms or miRNAs further to those investigated here are likely to regulate GPD2 protein expression. The miR-103-3p was another miRNA that presented higher expression in the HCR cohort of the differentially expressed miRNAs. Little is known about the role and validated targets of miR-103 with this the first study to investigate its expression in rat skeletal muscle. IPA analysis identified BACE1 and CASP3 to be targets of miR-103 within

the mitochondrial dysfunction filter; however both of these proteins presented similar expression patterns between cohorts. Previous research has suggested a role for miR-103 in myogenic differentiation with increased miR-103 expression observed in myoblasts following differentiation (7). It is possible that potential increases in myogenic differentiation regulated by miR-103 may contribute to increased skeletal muscle oxidative capacity in HCR rats previously identified by our group (32) by promoting increased muscle mass and represents an avenue for further investigation.

While there were no other differences in the expression levels of target proteins from other miRNAs differentially expressed between LCR and HCR rats, many of these miRNAs have been shown to be implicated in metabolic disorders and the regulation of mitochondrial function and protein expression. For instance, global and skeletal muscle specific overexpression of the let-7 family (including the differentially expressed let-7i and -7e miRNAs investigated in our work) has been reported to impair glucose tolerance and induce insulin resistance (9, 44). As transgenic mouse experiments have shown that let-7 targets the insulin receptor in skeletal muscle (44), it is possible the increased expression of let-7i and -7e in LCR rats may contribute to the previously reported impaired insulin signaling responses in LCR rats (24, 25, 32). An important limitation of our results is that analysis was only confined to the tibialis anterior. Differences in type IIb and type IIx fibre types exist between LCR and HCR cohorts within the Tibialis Anterior (Seifort), thus we cannot rule out that differences in miRNA expression or citrate synthase activity may be influenced by these discrepancies in fibre type. Moreover, it is also plausible that other tissues (i.e.: heart) may impact miRNA expression differently between LCR and HCR compared to our observed results in the tibialis anterior.

In conclusion, we demonstrate highly divergent skeletal muscle miRNA expression profiles between LCR and HCR rats, targeting multiple predicted protein/mRNA targets involved in mitochondrial function and substrate metabolism. These findings suggest that altered miRNA expression may mediate some of the metabolic features intrinsic to HCR and LCR rats and demonstrate the potential for miRNAs to regulate metabolic function and provide insight into the gene-regulatory mechanisms modulating intrinsic running capacity and its link to metabolic health. Further work investigating the effect of exercise in the LCR/HCR model would provide additional information regarding the regulation of miRNA expression in skeletal muscle. Future research is also warranted to identify and validate specific gene targets of miRNAs differentially expressed between HCR and LCR phenotypes and elucidate their potential regulatory role in metabolic health. Such interactions need to be confirmed in human skeletal muscle in order to become potential novel targets for mitochondrial-based therapies for the treatment of metabolic-related conditions aimed at increasing energy expenditure or enhancing substrate oxidation.

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Table 1. Relative expression of the 65 miRNAs which were not significantly different ($P < 0.05$) between TA of HCR and LCR rats as determined by qRT-PCR (n = 9). Values are means ± SD.

Figure 1. Relative expression of miRNAs differentially expressed (*$P < 0.05$) in the TA of generation 27 HCR and LCR rats as determined by qRT-PCR ($n = 9$). Values are means ± SD.

Figure 2. Pathway analysis of the 11 differentially expressed miRNAs between HCR and LCR rats and their 19 protein/mRNA targets within the 'Mitochondrial Dysfunction' and 'TCA Cycle II (Eukaryotic)' pathways in skeletal muscle as predicted by the microRNA Target Filter of Qiagen's Ingenuity Pathway Analysis. Relationships are either 'highly predicted' by algorithms or 'experimentally observed' in previous literature.

Figure 3. A) ATPAF1 (target of miR-26a, miR-28-5p, let-7i-5p and let-7e-5p), B) BACE1 (target of miR-103-3p, miR-374-5p, miR-7a-5p and miR-19a-3p-3p), C) CASP3 (target of miR-103-rp, let-7e-5p and let-7i-5p), D) CS (target of miR-19a-3p-3p), E) GPD2 (target of miR-30a-5p), F) LRRK2 (target of miR-19a-3p-3p and miR-181a-5p), G) MAP2K4 (target of miR-24-3p and miR-374-5p) and H) VDAC1 (target of miR-7a-5p) total protein content in the TA of HCR and LCR rats (n = 9). Values are arbitrary units expressed relative to Stain-Free total protein loading. (*) Significantly different ($P < 0.05$) between LCR and HCR cohorts. Values are means ± SD.

Figure 4. Correlation analysis between miR-19a-3p and its predicted protein target CS in the TA of LCR rats (n=9).

Figure 5. CS activity in the TA of HCR and LCR rats (n = 10). Values are means ± SD (*P < 0.05).

Figure 6. A) MicroRNA expression levels of miR-19a-3p normalized to SnoRNA202 after transfection in C2C12 cells; B) mRNA and C) protein expression of the miR-19a-3p predicted target CS following transfection (*P < 0.05).

Figure 7. Representative stain-free image of total protein loading for A) TA of HCR and LCR rats; and B) C2C12 cells following miR-19-3p transfection.

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