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

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

2

3   Expression of microRNAs and target proteins in skeletal muscle of rats selectively bred for

4   high and low running capacity

5

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26   **Abstract**

27   Impairments in mitochondrial function and substrate metabolism are implicated in the

28   etiology of obesity and type 2 diabetes. MicroRNAs (miRNAs) can degrade mRNA or

29   repress protein translation and have been implicated in the development of such disorders.

30   We used a contrasting rat model system of selectively bred high- (HCR) or low- (LCR)

31   intrinsic running capacity with established differences in metabolic health to investigate the

32   molecular mechanisms through which miRNAs regulate target proteins mediating

33   mitochondrial function and substrate oxidation processes. Quantification of select miRNAs

34   using the Rat miFinder miRNA PCR array revealed differential expression of 15 skeletal

35   muscle (*m. tibialis anterior*) miRNAs between HCR and LCR rats (14 with higher expression

36   in LCR; $P<0.05$). Ingenuity Pathway Analysis predicted these altered miRNAs to collectively

37   target multiple proteins implicated in mitochondrial dysfunction and energy substrate

38   metabolism. Total protein abundance of citrate synthase (CS; miR-19 target) and voltage-

39   dependent anion channel 1 (miR-7a target) were higher in HCR compared to LCR cohorts

40   (~57 and ~26%, respectively; $P<0.05$). A negative correlation was observed for miR-19a-3p

41   and CS (r =0.59, $P=0.02$) protein expression in LCR. To determine if miR-19a-3p can

42   regulate CS *in vitro* we performed luciferase reporter and transfection assays in C2C12

43   myotubes. MiR-19a-3p binding to the CS untranslated region did not change luciferase

44   reporter activity, however miR-19a-3p transfection decreased CS protein expression (~70%;

45    $P<0.05$). The differential miRNA expression targeting proteins implicated in mitochondrial

46   dysfunction and energy substrate metabolism may contribute to the molecular basis

47   mediating the divergent metabolic health profiles of LCR and HCR rats.

48

49

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

51 **Introduction**

52 Metabolic disorders such as type 2 diabetes and obesity are characterized by a loss of

53 'metabolic plasticity' where skeletal muscle is unable to effectively transition between lipid-

54 and carbohydrate-based oxidation in response to the prevailing hormonal mileau (17).

55 Development of these clinical conditions is determined by a complex interaction of

56 environmental (lifestyle) and genetic (heritable) factors. Through two-way artificial selection

57 breeding for treadmill running capacity, intrinsically high capacity runner (HCR) and low

58 capacity runner (LCR) rats provide an excellent model system for studying the genetic factors

59 mediating extremes in metabolic health. The HCR rats present with over 8-fold greater

60 intrinsic aerobic running capacity at generation 28 compared to LCR rats and over 40% of the

61 variance of the running capacity phenotype due to additive genetic variance (narrow-sense

62 heritability, h2 = 0.47 ± 0.02 in HCRs and 0.43 ± 0.03 in LCRs) (31). This superior aerobic

63 capacity and metabolic health profile of HCR rats has, in part, been attributed to an increased

64 activity of skeletal muscle proteins involved in mitochondrial function and substrate

65 oxidation (15, 29, 33, 38) compared to the impaired mitochondrial function observed in LCR

66 animals (34, 38). Thus, investigating the gene-regulatory mechanisms mediating these

67 processes in a translational animal model system may provide new insight to the molecular

68 basis controlling metabolic health.

69

70 MicroRNAs (miRNAs) are short, non-coding RNAs that regulate gene expression by binding

71 to mRNA, subsequently instigating degradation or repressing protein translation (2, 10).

72 Altered miRNA expression has been implicated in the pathogenesis of several metabolic

73 conditions including obesity and type 2 diabetes through the regulation of key metabolic

74 signaling networks involved in glucose and lipid handling, and mitochondrial metabolism (9,

75 13, 43). Additionally, divergent miRNA expression has recently been characterized in mice

76   with inherently high or low physical activity levels as well in human 'high' and 'low'

77   responders to resistance exercise (5, 6). These findings suggest that miRNAs may contribute

78   to the metabolic adaptation profile induced by physical/exercise activity. Whether miRNAs

79   contribute to the signaling pathways that mediate the intrinsic skeletal muscle metabolic

80   phenotypes divergent between HCR and LCR rats is unknown. We aimed to determine the

81   miRNA expression profile and interactions with predicted protein targets implicated in

82   metabolic health in skeletal muscle from HCR and LCR rats. We hypothesized that HCR and

83   LCR rats would present divergent miRNA expression profiles in a non-exercise condition,

84   with HCR rats displaying a miRNA profile that upregulates proteins promoting efficient

85   substrate oxidation and enhanced mitochondrial function.

86

87   **Materials and Methods**

88   *Experimental animals*

89   HCR and LCR rats derived from genetically heterogeneous N:NIH stock rats by two-way

90   artificial selection for maximal treadmill running capacity were used in this study. The

91   breeding program and aerobic capacity testing procedures have been described in detail

92   previously (20). Parent rats from generation 27 of selection were bred at the University of

93   Michigan (Ann Arbor, MI, USA) and their female offspring, HCR (n = 12) and LCR (n =

94   12), transported to Royal Melbourne Institute of Technology (RMIT) University (Bundoora,

95   Australia) at ~8 weeks (wk) of age. We have previously reported maximal respiratory

96   capacity and fasting serum insulin concentrations from this LCR/ HCR generation (REF).

97   HCRs from later generations (i.e.: 23-27) have shown similar increases in running capacity

98   and Citrate Synthase activity above LCRs compared to earlier generations (7-11) (16, 34, 39,

99   41). Rats were allowed 2 wk to acclimate to RMIT facilities as previously described (38).

100 Neither HCR or LCR rats underwent any form of exercise training during the study period.

101 Rats received ad libitum access to water and a standard chow diet whilst being housed under

102 a 12:12 hour light-dark cycle in a temperature controlled environment (22 °C). Experimental

103 procedures were approved by the University Committee on Use and Care of Animals at the

104 University of Michigan and the RMIT University Animal Ethics Committee prior to the onset

105 of the study.

106

107 *Tissue collection*

108 At 11 wk of age, rats were weighed and anesthetized using pentobarbital sodium (60 mg/kg

109 body wt). The *m. tibialis anterior* (TA) was immediately excised, freeze clamped in liquid

110 nitrogen and stored at -80 °C for subsequent analysis.

111

112 *RNA extraction and quantification*

113 RNA extraction from skeletal muscle tissue was performed using TRIzol in accordance with

114 the manufacturer's instructions and described previously (3). Briefly, approximately 20 mg of

115 tissue was homogenized in TRIzol and chloroform was added to form an aqueous upper

116 phase which was precipitated by adding isopropanol. The remaining RNA pellet was washed

117 and re-suspended in 35 µL's RNase-free water. RNA was quantified using a NanoDrop 2000

118 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

119

120 *Reverse Transcription (RT) and Real-Time PCR*

121 A miScript II RT Kit (catalogue #218160; Qiagen, Melbourne, Australia) was used to

122 synthesize cDNA from RNA samples using a BioRad thermal cycler (BioRad Laboratories,

123 Gladesville, Australia) in accordance with the manufacturer's instructions. Changes in

124 miRNA expression were quantified using a Rat miFinder miRNA PCR Array (catalogue

125 #MIRN-001ZD-24; Qiagen, Melbourne, Australia) in a 96-well RT cycler CFX96 (BioRad

126 Laboratories, Gladesville, Australia) for 40 cycles (two steps: 95ºC for 15 s followed by 60ºC

127 for 30 s). This microarray contained the 84 most abundantly expressed and best characterized

128 miRNAs present in rats. These miRNA targets can be found via the link:

129 http://www.sabiosciences.com/mirna_pcr_product/HTML/MIRN-001Z.html This microarray

130 was selected as many of these miRNAs have been previously shown to regulate targets

131 shown to have roles in substrate oxidation and mitochondrial function (4, 8, 11, 27) and is

132 therefore relevant to the HCR and LCR experimental model. Six housekeeping control RNAs

133 were also measured on this microarray for normalization. The relative amounts of each

134 miRNA in PCR analysis was normalised to the average of these six (SNORD61, SNORD68,

135 SNORD72, SNORD95, SNORD96A, RNU6–2) house-keeping genes. There were no

136 changes in the absolute CT of each individual house-keeping gene or the average between

137 LCR and HCR cohorts (data not shown). The $2^{\Delta\Delta CT}$ method of relative quantification was

138 used to calculate relative amounts of miRNAs (28).

139

140 *miRNA target prediction*

141 Protein/mRNA targets of miRNAs differentially expressed ($P < 0.05$) between HCR and LCR

142 skeletal muscle were predicted using the microRNA Target Filter function of Qiagen's

143 Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity).

144 IPA's microRNA Target Filter incorporates multiple target prediction programs including

145 TargetScan, TarBase, miRecords and the Ingenuity Knowledge Base. Predicted relationships

146 were filtered to be either 'highly predicted' by algorithms or 'experimentally observed' by

147 previous research. Predicted targets were then filtered to be implicated in 'Mitochondrial

148 Dysfunction' and 'TCA Cycle II (Eukaryotic)' in skeletal muscle. These filter criteria were

149 selected for investigation as LCR rats exhibit impaired skeletal muscle mitochondrial and

150 TCA cycle function compared to HCR (34, 38). Predicted targets meeting these criteria were

151   identified for 11 of the 15 miRNAs differentially expressed between HCR and LCR rats. A

152   minimum of one predicted protein/mRNA target was selected for further protein expression

153   analysis (described subsequently) for each of the 11 differentially expressed miRNAs which

154   presented protein/mRNA targets implicated in 'Mitochondrial Dysfunction' and 'TCA cycle

155   II (Eukaryotic)'.

156

157   *Western Blotting (skeletal muscle)*

158   Approximately 30 mg of TA was homogenized in ice-cold buffer as previously described

159   (39). Lysates were centrifuged at 12,000 g for 20 min at 4 °C and the supernatant was

160   transferred to a sterile microcentrifuge tube and aliquoted to measure protein concentration

161   using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Lysate was then re-

162   suspended in 4X Laemmli sample buffer with 40 µg of protein loaded onto 4–20% Mini-

163   PROTEAN TGX Stain-Free™ Gels (BioRad Laboratories, Gladesville, Australia). Post

164   electrophoresis, gels were activated on a Chemidoc according to the manufacturer's

165   instructions (BioRad Laboratories, Gladesville, Australia) and then transferred to

166   polyvinylidine fluoride (PVDF) membranes. After transfer, a stain-free image of the PVDF

167   membranes (14) for total protein normalization was obtained before membranes were rinsed

168   briefly in distilled water and blocked with 5% non-fat milk, washed with 10 mM of Tris–

169   HCl, 100 mM of NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1000)

170   overnight at 4 °C. Membranes were then incubated with secondary antibody (1:2000), and

171   proteins were detected via enhanced chemiluminescence (Thermo Fisher, Scoresby,

172   Australia) and quantified by densitometry (ChemiDoc™ XRS+ System; BioRad

173   Laboratories, California, USA). HCR and LCR samples were run on the same gel. Primary

174   antibodies used were polyclonal caspase-3 (CASP3) (#9662), leucine-rich repeat kinase 2

175   (LRRK2) (#5559) (Cell Signaling, Beverly, MA, USA), polyclonal ATP synthase

176 mitochondrial F1 complex assembly factor 1 (ATPAF1) (#ab101518), beta-site APP cleaving

177 enzyme 1 (BACE1) (#ab2077), Citrate Synthase (CS) (ab96600) and monoclonal Glycerol-3-

178 Phosphate Dehydrogenase 2 (GPD2) (ab188585), MAP2K4 (ab33912), VDAC1 (ab14734)

179 (Abcam, Cambridge, UK). Volume density of each target protein band was normalized to the

180 total protein loaded into each lane using stain-free technology (14), with data expressed in

181 arbitrary units (Figure 7).

182

183 *Citrate synthase activity*

184 CS activity was measured to identify whether differences in CS protein abundance were also

185 accompanied by differences in activity. Skeletal muscle homogenates ($n = 10$) from freeze

186 clamped TA muscles (10-20 mg) were prepared over ice in buffer [175 mM KCl and 2 mM

187 EDTA (pH 7.4), 1:50 or 1:100 dilution]. Homogenates underwent three freeze-thaw cycles

188 and CS activity was measured according to the method of Srere (37) with modifications as

189 described previously (38).

190

191 *Cell Culture*

192 Stock C2C12 (mouse) myoblasts (ATCC, Manassas, VA, USA) were maintained at 37°C

193 (95% O2-5% CO2) in high glucose (4.5g/L D-Glucose) culture medium with 2mM glutamine

194 and 110 mg/L sodium pyruvate (Dulbecco's modified Eagle's medium (DMEM)), containing

195 10% fetal bovine serum (FBS; Life Technologies, Melbourne, Australia). For differentiation

196 experiments, when cultures approached confluence (~90% confluent), medium was changed

197 to differentiation medium (DMEM, supplemented with 2% horse serum; Life Technologies,

198 Melbourne, Australia). Differentiation medium was replaced every 24 h.

199

200 *Luciferase Reporter Assay*

201 C2C12 myoblasts (1-2 x 10^5/mL) were seeded in black-walled 96-well plates. Twenty-four

202 hours after seeding, cells were co-transfected with 150 ng pNanoglo2 vector (Promega,

203 Alexandria, Australia) containing either: no insertion (empty control); the putative rat miR-

204 19a-3p Citrate Synthase target site (including the predicted seed site with 10 base pairs on

205 either side; Primer sequence- Forward: 5'

206 CAGCAGCCTCAtttgcacagattttcaGTGACTCAGAccgcggG 3', Reverse: 5'

207 CTAGCccgcggTCTGAGTCACtgaaaatctgtgcaaaTGAGGCTGCTGAGCT); or its mutant

208 control, cloned between SacI and NheI downstream of the Nanoluc luciferase (Primer

209 sequence- Forward 5' CAGCAGCCTCAcaaccaatcgagaactGTGACTCAGAccgcggG 3',

210 Reverse: 5' CTAGCccgcggTCTGAGTCACagttctcgattggttgTGAGGCTGCTGAGCT 3';

211 together with 5 nM miR-19a-3p mimics (mirVana™ miRNA mimic, Life technologies,

212 Mulgrave, Australia), or an irrelevant miRNA control (miR-99b-5p), using Lipofectamine

213 2000 (Thermo Fisher, Scoresby, Australia) following the manufacturer's protocol. Four

214 hours' post-transfection, the media was removed and replaced with culture medium. Twenty-

215 four hours later, cells were assayed for Firefly and Nanoluc luciferase expression using the

216 Nano-Glo® Dual-luciferase® Reporter assay kit (Promega, Alexandria, Australia) following

217 the manufacturer's protocol. The data reported are the results of three independent

218 experiments performed in six replicates.

219

220 *MiRNA transfection*

221 C2C12 myoblasts were cultured (as above) and seeded (1.5 x 10^5 cells per well) into six-well

222 plates 24 h before transfection. Myoblasts were transiently transfected with 1nM of miR-19a-

223 3p mimic and a scramble negative control (mirVana™miRNA mimic, Life technologies)

224 using Lipofectamine 2000 (Thermo Fisher; Scoresby, Australia). The myoblasts were placed

225 in transfection medium for 4 h. Following this period, the transfection medium was switched

226   to culture medium until their harvest. RNA and protein were extracted for RT-PCR gene

227   expression and Western Blot analysis, respectively.

228

229   *Real Time Quantitative PCR and Western Blotting*

230   C2C12 cells were homogenised in TRIZOL and RNA extracted using an RNeasy Mini Kit

231   (Qiagen, Chadstone, Australia) according to the manufacturer's directions. First-strand cDNA

232   synthesis was performed using either the SuperScript® VILO™ cDNA Synthesis kit

233   (Thermo Fisher, Scoresby, Australia) or TaqMan® MicroRNA Reverse Transcription Kit in a

234   final reaction volume of 20 µl according to the manufacturer's directions. Quantification of

235   mRNA (in duplicate) was performed on a BioRad CFX96 thermal cycler (BioRad,

236   Gladesville, Australia). Taqman-FAM-labelled primer/probes for citrate synthase (Cat No.

237   Mm00466043_m1) and miR-19a-3p (Cat No. 000395) were used in a final reaction volume

238   of 20 µl. PCR conditions were 2 min at 50 ℃ for UNG activation, 10 min at 95 ℃ then 40

239   cycles of 95 ℃ for 15 s and 60 ℃ for 60 s. β-actin (Cat No. Mm02619580_g1) and

240   SnoRNA202 (Cat No. 001232) were used as a housekeeping gene to normalize threshold

241   cycle (CT) values for mRNA and miRNA analyses, respectively. The relative amounts of

242   mRNAs were calculated using the relative quantification (ΔΔCT) method (28).

243

244   For Western Blot analyses, proteins were lysed in a 1 × modified RIPA (Merck Millipore,

245   North Ryde, Australia) containing 1:1000 protease inhibitor cocktail (Sigma-Aldrich, Castle

246   Hill, Australia) and 1:100 Halt phosphatase inhibitor cocktail (Thermo Fisher, Scoresby,

247   Australia) and left on ice for 30 min prior to centrifugation to remove insoluble material.

248   Lysates containing twenty micrograms of protein were electrophoresed and transferred as

249   described above with a stain-free image of the PVDF membranes obtained for total protein

250   normalization. Transfected and scrambled samples from the same time point of collection

251 were run on the same gel, and the same polyclonal CS antibody as mentioned above was used

252 to measure CS protein expression. Volume density of each target protein band was

253 normalized to the total protein loaded into each lane using stain-free technology (10), with

254 data expressed in arbitrary units (Figure 7).

255

256 *Statistical analyses*

257 A two-tailed unpaired t-test (GraphPad Prism Version 5.03) was used to detect differences

258 between HCR and LCR groups in miRNA expression, protein abundance, enzyme activity

259 and for all *in vitro* analyses of C2C12 cells. All data was subjected to the normality test using

260 the Shapiro-Wilk test (SigmaPlot 12.0). Linear regression analysis was performed to

261 determine associations between miRNA species and their predicted protein targets in HCR

262 and LCR phenotypes (GraphPad Prism Version 5.03). All values are expressed as arbitrary

263 units (AU) and presented as mean ± standard deviation (SD). Statistical significance was set

264 at $P < 0.05$.

265

266 **Results**

267 *Differential miRNA expression*

268 There was a higher expression in LCR compared to HCR for let-7i-5p (~147% percent

269 change), -7e-5p (~93%), miR-7a-5p (~35%), -19a-3p (~66%), -24-3p (~37%), -26a-5p

270 (~58%), -28-5p (~54%), -30a-5p (~67%), -99a-5p (~54%), -181a-5p (~81%), -194-5p

271 (~39%), -223-3p (~59%), -374-5p (~68%) and -376c-3p (~121%), while miR-103-3p was

272 more highly expressed ($P < 0.05$) in HCR than LCR (~31%; Figure 1). All differentially

273 expressed miRNAs had a mean Ct value < 32. The other 69 miRNAs analyzed were not

274 significantly different between HCR and LCR rats (Table 1).

275

276   *Bioinformatics analysis of differentially expressed miRNAs*

277   The microRNA Target Filter function of Qiagen's IPA predicted 5672 mRNAs (2964 in

278   skeletal muscle) to be targeted by the 15 miRNAs differentially expressed between HCR and

279   LCR skeletal muscle samples. Eleven of the 15 differentially expressed miRNAs were

280   predicted to target 19 mRNAs implicated in skeletal muscle mitochondrial dysfunction and

281   TCA cycle function (Figure 2).

282

283   *Protein abundance of miRNA targets*

284   There was a greater protein abundance of CS (~57%) and VDAC1 (~26%) in HCR compared

285   to LCR rats ($P < 0.05$; Figure 3A, B). Levels of GPD2 (~28%) were higher in LCR rats ($P <$

286   0.05; Figure 3C). There were no changes in the expression of CASP3, LRRK2, ATPAF1,

287   BACE1, or MAP2K4 between HCR and LCR rats (Figure 3).

288

289   *miRNA-protein correlations*

290   A significant negative correlation was observed for miR-19a-3p and CS expression in LCR

291   rats (r = 0.59, P = 0.02; Figure 4) compared to the HCR (r = XX, P = 0.76, data not shown).

292   No other correlations between miRNAs and target proteins were found.

293

294   *Citrate Synthase Activity*

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

296

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

298   There were no changes in Nanoluc luciferase activity in cells co-transfected with the miR-

299   19a-3p mimic and either the full length CS 3'UTR or the predicted miR_19a-3p target site on

300   CS 3'UTR compared to cells transfected with an irrelevant miRNA (data not shown).

301   Transfection of miRNA mimics significantly increased levels of miR-19a-3p expression by

302   ~8,165 % following 4 h transfection (Figure 6A). Citrate synthase mRNA levels were

303   unchanged following miR-19a-3p transfection (Figure 6B) however there was a ~70%

304   reduction in CS protein abundance compared to the scrambled negative control 4 h

305   transfection (Figure 6C).

306

307   **Discussion**

308   MicroRNAs have emerged as key regulators of metabolic health through their ability to

309   repress gene and protein expression (2) and may mediate underlying differences in intrinsic

310   metabolic function between individuals. Using an animal model of inherited low- or high

311   intrinsic running capacity that simultaneously associates with poor or good metabolic health

312   (21), we report evidence for divergent skeletal muscle miRNA expression profiles .

313   Specifically, 15 miRNAs with predicted mRNA targets involved in mitochondrial

314   dysfunction and substrate oxidation were differentially expressed between HCR and LCR

315   rats. Moreover, we show the abundance of predicted protein targets CS and VDAC1 were

316   altered between phenotypes in accordance with miRNA expression profile. These findings

317   suggest a regulatory role for specific skeletal muscle miRNAs of target proteins central to

318   mitochondrial content and function.

319

320   MicroRNAs are critical regulators of skeletal muscle metabolism via the negative regulation

321   of proteins involved in mitochondrial function and energy substrate oxidation (42).We

322   therefore investigated the molecular events that may influence the diverse transcriptional

323   differences in mitochondrial function and substrate handling previously reported between

324   LCR and HCR rats (25, 32, 34, 39). Of the 84 most abundant miRNAs present in rats, there

325   was a total of 5672 predicted protein/mRNA targets (2964 in skeletal muscle) arising from

326 the 15 differentially expressed miRNAs measured by IPA's microRNA Target Filter,

327 demonstrating the potentially widespread role for miRNAs in determining the differential

328 between HCR and LCR intrinsic phenotypes. Eleven of these differentially expressed

329 miRNAs showed predicted protein targets implicated in mitochondrial dysfunction as

330 identified by IPA. Numerous studies have attributed the impaired metabolic phenotype of

331 LCR rats partly to a decrease in the abundance of skeletal muscle proteins critical to

332 mitochondrial function (15, 34, 38). Therefore, we hypothesised that miRNAs may be a

333 contributing regulatory mechanism to the divergent mitochondrial features and metabolic

334 phenotypes previously characterized between HCR and LCR rats.

335

336 The first novel finding of our work was the greater miR-19a-3p expression in LCR compared

337 to HCR rats (~63% percent change; Figure 1), which has predicted targets involved in

338 mitochondrial dysfunction and the TCA cycle. We quantified the abundance of these

339 predicted targets (Beta-site APP cleaving enzyme 1 and Citrate Synthase) to investigate

340 putative interactions, finding a ~57% decrease in citrate synthase (CS) protein expression in

341 TA from LCR rats compared to HCR rats (Figure 3). This decrease in protein expression was

342 also supported by a reduction in citrate synthase activity (Figure 5). This is in agreement with

343 previous reports of greater CS abundance and activity in the *m. gastrocnemius*, *m. soleus* and

344 *m. extensor digitorum longus* of HCR rats relative to LCR rats (12, 15, 30, 33, 34, 38, 40).

345 CS is a rate limiting enzyme of the TCA cycle located in the mitochondrial matrix and is

346 often used as a surrogate measure for skeletal muscle mitochondrial content (22). Attenuated

347 CS activity and abundance has been reported in the skeletal muscle of type 2 diabetic and

348 obese individuals (18, 19, 36). Here, we report an inverse correlation between miR-19a-3p

349 and CS expression in muscle from LCR rats, which is the first experimental evidence that

350 miR-19a-3p may play a role in determining the mitochondrial capacity of skeletal muscle.

351

352 To confirm whether miR-19a-3p can directly bind and regulate CS transcription, C2C12

353 myoblasts were co-transfected with a reporter plasmid containing a section of the putative rat

354 miR-19a-3p Citrate Synthase target site, as well as the miR-19-3p mimic, an irrelevant

355 miRNA that did not have a predicted binding site on the CS 3'UTR (miR-99b-5p) or no

356 mimic at all. No reduction in luminescence levels was observed with miR-19a-3p, indicating

357 that miR-19a-3p did not bind to the CS 3'UTR. CS gene expression data further supports this

358 as no down-regulation of CS mRNA expression was observed following miR-19a-3p

359 transfection. In contrast, overexpression of miR-19a-3p in C2C12 myoblasts decreased CS

360 protein levels 4 h after the onset of transfection when compared to a scrambled control. This

361 interaction may be direct and occur at the protein level to inhibit protein translation while

362 allowing normal mRNA transcription. Alternatively, miR-19a-3p may interact with CS in

363 area outside the 3'UTR to regulate its mRNA expression (23). Our findings therefore suggest

364 that miR-19a-3p mediate signalling events controlling energy substrate metabolism and

365 mitochondrial content, and reveal novel mechanistic information to the regulatory control of

366 CS expression in skeletal muscle.

367

368 Another major finding from our study was the higher miR-7a expression in LCR rats (~35%

369 percent change; Figure 1). miR-7a has been implicated in the development of insulin

370 resistance through its down-regulation of insulin receptor substrate 1 expression and

371 inhibition of insulin-stimulated Akt phosphorylation and glucose uptake (26). Considering

372 LCR rats present impaired skeletal muscle insulin signalling and IRS1 phosphorylation

373 relative to HCR (33), and miR-7a was more highly expressed in LCR rats, it is possible miR-

374 7a may play a role in the attenuated insulin signalling response between these cohorts. Two

375 protein targets of miR-7a identified by IPA in the 'Mitochondrial Dysfunction' filter were

376   VDAC1 and BACE1. VDAC1 is an outer mitochondrial membrane protein involved in the

377   TCA cycle responsible for transporting calcium ions and metabolites including ATP across

378   the outer mitochondrial membrane (35). VDAC1 deficient mice have been shown to display

379   impaired glucose tolerance and exercise capacity due to impaired mitochondria-bound

380   hexokinase activity (1). In our study, the first to compare VDAC1 protein expression between

381   LCR and HCR rats, we observed significantly lower VDAC1 protein expression in the LCR

382   cohort. This raises the possibility that miR-7a and VDAC1 may contribute to the divergent

383   metabolic profiles previously established between LCR and HCR (33). Further work

384   incorporation miR-7a over-expression analyses are required to better understand the capacity

385   for it to regulate cellular energy production and metabolism processes.

386

387   Of the other protein targets analysed from the differentially expressed miRNAs between LCR

388   and HCR cohorts, protein levels of Glycerol-3-Phosphate Dehydrogenase 2 (GPD2) were

389   higher in LCR compared to HCR rats. GPD2 is a mitochondrial membrane protein centrally

390   involved in glycolysis and was a predicted target of miR-30a. While increased GPD2

391   abundance in LCR skeletal muscle was unexpected based on higher miR-30a expression

392   profile in LCR compared to HCR rats, this higher abundance of GPD2 indicates a greater

393   reliance on glycolysis for energy production compared to HCR rats. Indeed, previous work

394   from our laboratory has demonstrated that LCR skeletal muscle is more reliant on

395   carbohydrate than fat metabolism at rest (33). These findings suggest other signalling

396   mechanisms or miRNAs further to those investigated here are likely to regulate GPD2 protein

397   expression. The miR-103-3p was another miRNA that presented higher expression in the

398   HCR cohort of the differentially expressed miRNAs. Little is known about the role and

399   validated targets of miR-103 with this the first study to investigate its expression in rat

400   skeletal muscle. IPA analysis identified BACE1 and CASP3 to be targets of miR-103 within

401   the mitochondrial dysfunction filter; however both of these proteins presented similar

402   expression patterns between cohorts. Previous research has suggested a role for miR-103 in

403   myogenic differentiation with increased miR-103 expression observed in myoblasts

404   following differentiation (7). It is possible that potential increases in myogenic differentiation

405   regulated by miR-103 may contribute to increased skeletal muscle oxidative capacity in HCR

406   rats previously identified by our group (32) by promoting increased muscle mass and

407   represents an avenue for further investigation.

408

409   While there were no other differences in the expression levels of target proteins from other

410   miRNAs differentially expressed between LCR and HCR rats, many of these miRNAs have

411   been shown to be implicated in metabolic disorders and the regulation of mitochondrial

412   function and protein expression. For instance, global and skeletal muscle specific

413   overexpression of the let-7 family (including the differentially expressed let-7i and -7e

414   miRNAs investigated in our work) has been reported to impair glucose tolerance and induce

415   insulin resistance (9, 44). As transgenic mouse experiments have shown that let-7 targets the

416   insulin receptor in skeletal muscle (44), it is possible the increased expression of let-7i and -

417   7e in LCR rats may contribute to the previously reported impaired insulin signaling responses

418   in LCR rats (24, 25, 32). An important limitation of our results is that analysis was only

419   confined to the tibialis anterior. Differences in type IIb and type IIx fibre types exist between

420   LCR and HCR cohorts within the Tibialis Anterior (Seifort), thus we cannot rule out that

421   differences in miRNA expression or citrate synthase activity may be influenced by these

422   discrepancies in fibre type. Moreover, it is also plausible that other tissues (i.e.: heart) may

423   impact miRNA expression differently between LCR and HCR compared to our observed

424   results in the tibialis anterior.

425

426   In conclusion, we demonstrate highly divergent skeletal muscle miRNA expression profiles

427   between LCR and HCR rats, targeting multiple predicted protein/mRNA targets involved in

428   mitochondrial function and substrate metabolism. These findings suggest that altered miRNA

429   expression may mediate some of the metabolic features intrinsic to HCR and LCR rats and

430   demonstrate the potential for miRNAs to regulate metabolic function and provide insight into

431   the gene-regulatory mechanisms modulating intrinsic running capacity and its link to

432   metabolic health. Further work investigating the effect of exercise in the LCR/HCR model

433   would provide additional information regarding the regulation of miRNA expression in

434   skeletal muscle. Future research is also warranted to identify and validate specific gene

435   targets of miRNAs differentially expressed between HCR and LCR phenotypes and elucidate

436   their potential regulatory role in metabolic health. Such interactions need to be confirmed in

437   human skeletal muscle in order to become potential novel targets for mitochondrial-based

438   therapies for the treatment of metabolic-related conditions aimed at increasing energy

439   expenditure or enhancing substrate oxidation.

440

451   **Table 1.** Relative expression of the 65 miRNAs which were not significantly different (P <

452   0.05) between TA of HCR and LCR rats as determined by qRT-PCR (n = 9). Values are

453   means ± SD.

454   **Figure 1.** Relative expression of miRNAs differentially expressed (*$P < 0.05$) in the TA of

455   generation 27 HCR and LCR rats as determined by qRT-PCR (*n* = 9). Values are means ±

456   SD.

457   **Figure 2.** Pathway analysis of the 11 differentially expressed miRNAs between HCR and

458   LCR rats and their 19 protein/mRNA targets within the 'Mitochondrial Dysfunction' and

459   'TCA Cycle II (Eukaryotic)' pathways in skeletal muscle as predicted by the microRNA

460   Target Filter of Qiagen's Ingenuity Pathway Analysis. Relationships are either 'highly

461   predicted' by algorithms or 'experimentally observed' in previous literature.

462

463   **Figure 3.** A) ATPAF1 (target of miR-26a, miR-28-5p, let-7i-5p and let-7e-5p), B) BACE1

464   (target of miR-103-3p, miR-374-5p, miR-7a-5p and miR-19a-3p-3p), C) CASP3 (target of

465   miR-103-rp, let-7e-5p and let-7i-5p), D) CS (target of miR-19a-3p-3p), E) GPD2 (target of

466   miR-30a-5p), F) LRRK2 (target of miR-19a-3p-3p and miR-181a-5p), G) MAP2K4 (target of

467   miR-24-3p and miR-374-5p) and H) VDAC1 (target of miR-7a-5p) total protein content in

468   the TA of HCR and LCR rats (n = 9). Values are arbitrary units expressed relative to Stain-

469   Free total protein loading. (*) Significantly different (P < 0.05) between LCR and HCR

470   cohorts. Values are means ± SD.

471

472   **Figure 4.** Correlation analysis between miR-19a-3p and its predicted protein target CS in the

473   TA of LCR rats (n=9).

474

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

476   0.05).

477   **Figure 6.** A) MicroRNA expression levels of miR-19a-3p normalized to SnoRNA202 after

478   transfection in C2C12 cells; B) mRNA and C) protein expression of the miR-19a-3p

479   predicted target CS following transfection (*P < 0.05).

480

481   **Figure 7.** Representative stain-free image of total protein loading for A) TA of HCR and

482   LCR rats; and B) C2C12 cells following miR-19-3p transfection.

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