Pflugers Arch - Eur J Physiol (2015) 467:389–398
DOI 10.1007/s00424-014-1517-z

MUSCLE PHYSIOLOGY

MicroRNA-23a has minimal effect on endurance exercise-induced adaptation of mouse skeletal muscle

Shogo Wada · Yoshio Kato · Shuji Sawada · Katsuji Aizawa · Jong-Hoon Park · Aaron P. Russell · Takashi Ushida · Takayuki Akimoto

Received: 5 March 2014 / Revised: 1 April 2014 / Accepted: 6 April 2014 / Published online: 23 April 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract Skeletal muscles contain several subtypes of myofibers that differ in contractile and metabolic properties. Transcriptional control of fiber-type specification and adaptation has been intensively investigated over the past several decades. Recently, microRNA (miRNA)-mediated posttranscriptional gene regulation has attracted increasing attention. MiR-23a targets key molecules regulating contractile and metabolic properties of skeletal muscle, such as myosin heavy-chains and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α). In the present study, we analyzed the skeletal muscle phenotype of miR-23a transgenic (miR-23a Tg) mice to explore whether forced expression of miR-23a affects markers of mitochondrial content, muscle fiber composition, and muscle adaptations induced by 4 weeks of voluntary wheel running. When compared with wild-type mice, protein markers of mitochondrial content, including PGC-1α, and cytochrome *c* oxidase complex IV (COX IV), were significantly decreased in the slow soleus muscle, but not the fast plantaris muscle of miR-23a Tg mice. There was a decrease in type IId/x fibers only in the soleus muscle of the Tg mice. Following 4 weeks of voluntary wheel running, there was no difference in the endurance exercise capacity as well as in several muscle adaptive responses including an increase in muscle mass, capillary density, or the protein content of myosin heavy-chain IIa, PGC-1α, COX IV, and cytochrome *c*. These results show that miR-23a targets PGC-1α and regulates basal metabolic properties of slow but not fast twitch muscles. Elevated levels of miR-23a did not impact on whole body endurance capacity or exercise-induced muscle adaptations in the fast plantaris muscle.

Keywords MicroRNAs · Muscle fiber type · Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha · Endurance performance

Abbreviations

ALS	Amyotrophic lateral sclerosis
COX IV	Cytochrome *c* oxidase subunit IV
miRNA	MicroRNA
MyHC	Myosin heavy-chain
NFAT	Nuclear factor of activated T cells
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
Tg	Transgenic
UTR	Untranslated region
WT	Wild-type

S. Wada · K. Aizawa · J.-H. Park · T. Ushida · T. Akimoto (✉)
Division of Regenerative Medical Engineering, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-0033, Japan
e-mail: akimoto@m.u-tokyo.ac.jp

Y. Kato
Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8562, Japan

S. Sawada
Cooperative Major in Advanced Health Science, Tokyo University of Agriculture and Technology/Waseda University, Mikajima 3-8-1, Tokorozawa, Saitama 359-1192, Japan

J.-H. Park
Department of Physical Education, Konkuk University, Seoul 143-701, Korea

A. P. Russell
Centre for Physical Activity and Nutrition Research, School of Exercise and Nutrition Sciences, Deakin University, Burwood 3125, Australia

Introduction

Skeletal muscle comprises approximately 40 % of total body mass. It plays a key role in maintaining whole body health due to its role in fat and glucose oxidation, its sensitivity to insulin, and its capacity to store fuel in the form of lipids and glycogen. Skeletal muscle is a highly adaptable tissue that increases its metabolic capacity and its resistance to contraction-induced fatigue in response to endurance exercise. These adaptations involve coordinated increases in mitochondrial biogenesis, angiogenesis, insulin sensitivity, substrate oxidation, fuel storage, and oxidative myosin heavy-chain isoforms [8, 9, 16, 41, 44]. By contrast, physical inactivity combined with an energy-rich diet contributes to perturbed skeletal muscle metabolism and increased muscle fatigue that may lead to metabolic diseases, including type 2 diabetes and obesity [18]. Understanding the molecular factors contributing to the endurance exercise-induced improvement in skeletal muscle metabolic function may identify novel therapeutic targets to treat metabolic disorders.

MicroRNAs (miRNAs) are a class of small (~23 nucleotides) nonconding RNA that negatively regulates gene expression by inducing translational suppression or degradation of target mRNAs [14, 22, 31]. The ability of miRNAs to tightly regulate cellular networks and physiological functions makes them exciting candidates for potential therapeutic targets. MiRNAs play important roles in skeletal muscle health. For example, several muscle-enriched miRNAs including miR-1 and miR-133 are essential for skeletal muscle development [7, 19, 23, 50, 51]. MiR-208a is implicated in muscle metabolic homeostasis [13], miR-378 suppresses mitochondrial fatty acid metabolism [6], whereas miR-499, miR-208b, and miR-23a regulate myosin heavy-chain expression [24, 45, 47]. Skeletal muscle miRNA expression levels are sensitive to endurance exercise [27, 38]. However, the miRNAs influencing exercise-induced skeletal muscle adaptations, such as increases in mitochondrial biogenesis, angiogenesis, and oxidative myosin heavy-chain isoforms, have not been identified.

The regulation of miR-23a may play a role in maintaining skeletal muscle health. We have demonstrated that miR-23a binds to and downregulates peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) in a 3′UTR-dependent manner in vitro [37]. Additionally, transgenic (Tg) mice overexpressing miR-23a have a reduction in skeletal muscle PGC-1α mRNA and protein and several PGC-1α downstream targets required for efficient mitochondrial function [37]. PGC-1α is a transcriptional coactivator that is highly expressed in tissues with high-energy demands such as skeletal muscle, cardiac muscle, brain, and brown adipose tissue [10]. It transcribes gene-encoding proteins that regulate numerous muscle-adaptive responses including mitochondrial biogenesis, angiogenesis, substrate oxidation, and the development of oxidative myosin heavy-chain isoforms [9, 11, 30, 48, 49]. Muscle specific overexpression of PGC-1α increases mitochondrial biogenesis and the amount of oxidative muscle fibers [23], whereas PGC-1α deficiency in skeletal muscle reduces mitochondrial metabolism and oxidative muscle fiber content [15]. Endurance exercise in mice and humans induces skeletal muscle PGC-1α expression [3, 12, 36, 42, 43] and also reduces miR-23a expression [38, 39]. By contrast, skeletal muscle of patients with amyotrophic lateral sclerosis (ALS) presents an elevation in miR-23a and an associated decrease in PGC-1α mRNA and protein and impaired oxidative enzyme activity [37].

Several studies [2, 11, 29], but not all [34], suggest that PGC-1α plays an important role in exercise-induced adaptive mitochondrial biogenesis. Whether the regulation of miR-23a is necessary for exercise-induced skeletal muscle adaptations, particularly those regulated by PGC-1α, is unknown. We therefore hypothesized that forced expression of miR-23a in skeletal muscle would attenuate the endurance exercise-induced increase in PGC-1α and several well-established skeletal muscle adaptations, including increased mitochondrial biogenesis and capillary density as well as transition of myosin heavy-chain IIb to IIa. To test this hypothesis, we investigated changes in skeletal muscle mitochondrial content, angiogenesis, and myosin heavy-chain isoforms at rest and following 4 weeks of voluntary wheel running in wild-type (WT) mice and Tg mice overexpressing miR-23a.

Materials and methods

Ethics statement

All animal experiment protocols were approved by the Animal Care and Use Committees of the University of Tokyo.

Animal experiments

Eight-week-old female miR-23a Tg mice and their WT littermates were used for all experiments. Generation of miR-23a Tg mice were described elsewhere [46]. Briefly, the pCXbG-miR-23a plasmid vector was linearized using *Sal*I–*Eco*RI sites and injected into C57BL6/J oocytes. Genotyping was carried out by PCR, and fluorescence microscopy was used to confirm transgene expression in adult skeletal muscles. All mice were housed in temperature-controlled quarters (21 °C) with a 12-h light/12-h dark cycle and provided with water and food *ad libitum*. For voluntary running, animals were subjected to 4 weeks in cage voluntary running. Wheel revolutions per minute were counted to calculate total running distance over the 4-week period [2]. All mice were housed individually and killed at the same time of day after the final running session.

Exercise capacity test was conducted as previously described [20] with minor modifications. Briefly, WT and Tg mouse were placed on a treadmill (Melquest, Japan) at a constant 10° angle. The treadmill was then started at 10 m/min, and the speed was incrementally increased by 1 m/min every 2 min until 24 m/min. Exhaustion was defined as the point where mice were unable to continue running on the treadmill after 10 s of encouragement with a drawing brush.

RNA analysis

Total RNA was extracted from cells and animal tissues using ISOGEN II (WAKO, Osaka, Japan) according to the manufacturer's protocols, and 1 μg of RNA was reverse transcribed using SuperScript III reverse transcriptase (RT; Invitrogen). Oligo-dT was used to generate cDNA, and an aliquot of the RT reaction was used directly for PCR with Ex Taq HS (TaKaRa, Osaka, Japan) and gene-specific primers. Primer sequences for Dicer, Drosha, Exportin-5, and Ago2 were: Primer sequences were: Dicer, 5′-CAC ACG CCT CCT ACC ACT ACA ACA C-3′ and 5′-GGC TGC ATC ATC GGA TAG TAC ACC-3′; Drosha, 5′-CAA ATA CGG ATC GGC AAC TT-3′, and 5′-CAC GGG TCT CTT GGT TTT GT-3′; Exportin-5, 5′-TGG AAG CTC TGG TTC TCG TT-3′ and 5′-GGG TTA CGG AAG ATG GGA TT-3′; and Ago2, 5′-ACA GGG AAA TTG TGG AGC AC-3′ and 5′-GAT GGA AGC CAA ACC ACA CT-3′.

To measure pri- and pre-miR-23a, 500 ng of total RNA was reverse transcribed with ThermoScriptRT (Invitrogen) as previously reported [46]. Briefly, 500 ng of total RNA, 1 μl of 10 mM of specific RT primer (5′-TGG TAA TCC CTG GCA ATG TG-3′) and 2 μl of 10 mM dNTP were mixed, and distilled water was added to a total volume of 12 μl. The mixture was heated at 85 °C for 5 min, then at 57 °C for 5 min. After chilling on ice for 2 min, 4 μl of 5× cDNA synthesis buffer, 1 μl each of 0.1 M DTT, RNase inhibitor (ToYoBo, Osaka, Japan), and ThermoScript RT (15 U/μl) were added to the mixture. The contents were gently mixed and incubated at 57 °C for 60 min and heated at 85 °C for 5 min to terminate the reaction. For quantification of pri- and pre-miR-23a, 1 μl of RT product was amplified by PCR using Ex Taq HS (TaKaRa) in a total reaction volume of 50 μl. Primer sequences for pri- and pre-miR-23a were: pre-miR-23a-F; 5′-CTG GGG TTC CTG GGG AT-3′, and a common reverse primer with the same sequence as the specific RT primer. PCR conditions for pri- and pre-miR-23a were 35 cycles of denaturation at 98 °C for 10 s, annealing at 57 °C for 15 s, and extension at 72 °C for 15 s for extension. PCR products were electrophoresed on 2 % agarose gels containing 5×10–5 % ethidium bromide for 30 min, and fluorescent images were acquired under UV light by LAS3000 (FujiFilm corporation, Tokyo, Japan). Primer sequences for PGC-1α isoforms [35] and GAPDH [46] have been described elsewhere. GAPDH was used as an internal standard.

MicroRNA analysis

The TaqMan MicroRNA Reverse Transcription Kit and TaqMan MicroRNA assays (Applied Biosystems, Foster City, CA) were used according to the manufacturer's protocols for real-time PCR quantification of mature miRNA expression [46]. Product number of TaqMan MicroRNA assay was miR-23a (no. 000399). For miRNA quantification, each reverse RT reaction contained 10 ng of purified total RNA. The reactions were incubated for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C. Real-time PCR reactions for each miRNA were performed in triplicate in a 10-μl reaction mixture that included 1 μl of the RT product. Reactions were carried out on an Applied Biosystems StepOne Plus Real-Time PCR system in 96-well plates at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A small RNA, U6 was used as the endogenous control.

Western blot

Western blotting was performed as described previously [1] with the following antibodies: anti-PGC-1α (AB3242, Millipore), cytochrome *c* oxidase complex IV (COX IV; no. 4844, Cell Signaling Technology), cytochrome *c* (no. 4272, Cell Signaling Technology), γ-tubulin (AK-15, Sigma-Aldrich), and anti-myosin heavy-chain (MyHC) I (BA-F8), IIa (SC-71), and IIb (BF-F3) [40]. The secondary antibodies consisted of a HRP-conjugated anti-rabbit IgG Fab antibody (GE healthcare), an anti-mouse IgG-HRP antibody, an anti-goat IgG-HRP, and an anti-mouse IgM-HRP (Bio-rad).

Immunohistochemistry

Cross-sections from O.C.T.-embedded muscle were fixed with 4 % paraformardehyde in PBS, permeabilized with 0.3 % Triton X, and incubated with the following primary antibodies: anti-myosin heavy-chain (MyHC) I (BA-F8), IIa (SC-71), and IIb (BF-F3); dystrophin (D8043, Sigma-Aldrich); and CD31 antibodies (MCA2388, AbD serotec). Secondary antibodies were DyLight 405-conjugated anti-mouse IgG2b (for MyHC I), DyLight 488-conjugated anti-mouse IgG1 (for MyHC IIa), R-PE-conjugated anti-mouse IgG1 (for dystrophin), DyLight 549-conjugated anti-mouse IgM (for MyHC IIb), and DyLight 488-conjugated anti-rat IgG (for CD31; Jackson Immuno Research). A single anatomical cross-sectional area of the middle portion of each muscle was measured with ImageJ Software.

Enzymatic staining

Frozen tissue section was also used for succinate dehydrogenase (SDH) staining as described previously [25].

Transmission electron microscopy

Transmission electron microscopy analysis was performed on soleus muscle longitudinal sections from the miR-23a Tg and WT mice (n=2 for each genotype, 10 weeks of age in C57BL6 background) at the Medical Proteomics Laboratory, University of Tokyo.

Statistics

Data are presented as mean ± standard error of the mean (SEM). Statistical significance (P<0.05) was determined by a Student's t test for comparisons between two groups. Two-way ANOVA was used to analyze data from the exercise experiments. Post hoc analyses following a significant interaction between genotype × exercise or a main effect was performed using t tests. If multiple t test were performed, a Bonferroni adjustment was used.

Results

Expression of miR-23a in skeletal muscle

We quantified miR-23a expression by real-time RT-qPCR and confirmed that miR-23a was highly expressed in skeletal muscle when compared with other tissues such as liver, kidney, brown fat, and spleen (Fig. 1a). We also observed that endogenous miR-23a expression is higher in slow (soleus) muscle than fast (plantaris) muscle. When comparing Tg mice with WT mice, we confirmed that forced expression of miR-23a resulted in an approximate 3- and 6-fold increase respectively, in mature miR-23a levels in slow oxidative soleus and fast glycolytic plantaris skeletal muscle (Fig. 1a). Primary and precursor miR-23a levels were also increased in Tg mice (Fig. 1b). Subsequently, we confirmed mRNA expression levels of several key members of the miRNA processing complex and observed no difference in the levels of Dicer or Drosha but increases in Exportin-5 and Ago2 in both the soleus and plantaris muscles of the Tg when compared with the WT mice (Fig. 1c).

Forced overexpression of miR-23a downregulates PGC-1α and its downstream targets in a slow muscle-specific manner

Western blot analysis demonstrated that PGC-1α and mitochondria respiratory chain proteins, such as COX IV and cytochrome c (Cyt-c) were downregulated in the slow soleus but not in the fast plantaris muscle (Fig. 2a, b). We then determined mRNA expression levels of PGC-1α isoforms. No effect of miR-23a overexpression on PGC-1α isoform mRNAs in the soleus or plantaris muscles of the Tg, when compared with WT mice was observed (Fig. 2c). These data suggest that miR-23a suppresses PGC-1α protein

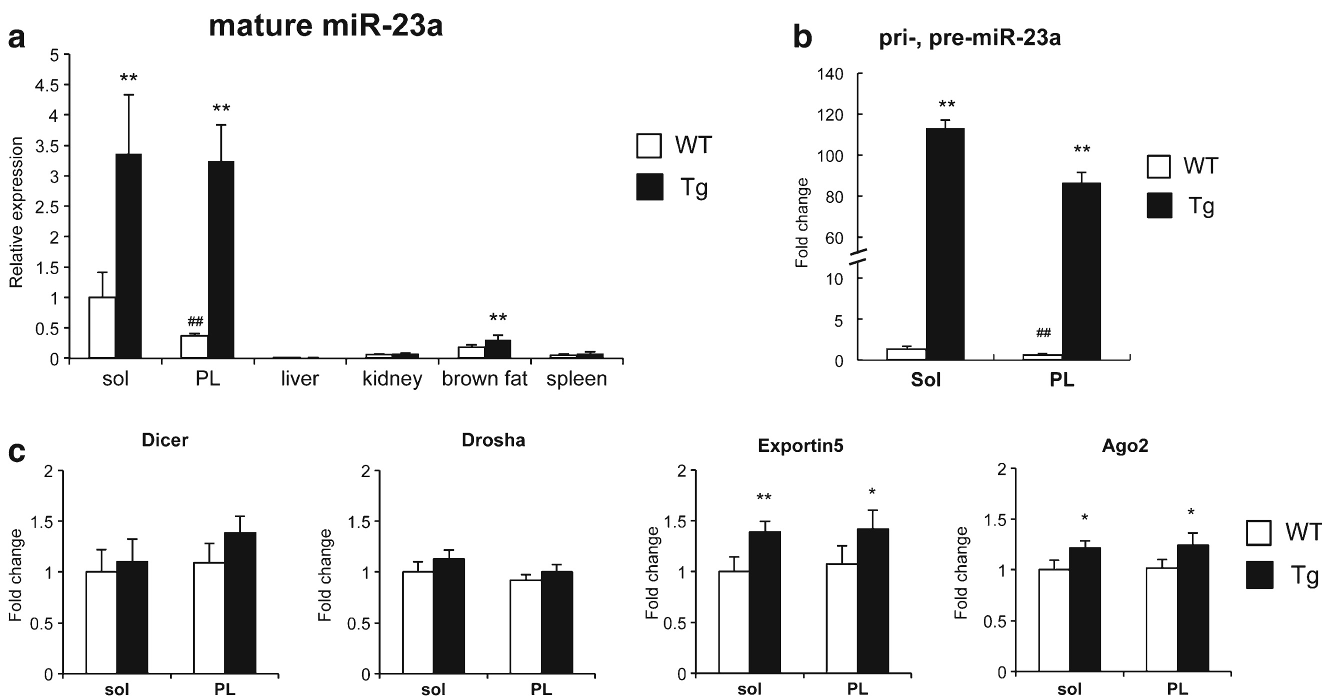

Fig. 1 Expressions of miR-23a and components of miRNA biogenesis machinery in miR-23a Tg mice. **a** Expression of miR-23 in various tissues in miR-23a transgenic (*Tg*) mice (n=5). Values are relative to the soleus muscle of the wild-type (*WT*) mice. **b** Expression of pri- and pre miR-23a was quantified by qRT-PCR (n=5). Values are relative to the soleus muscle of the WT mice. **c** Components of the miRNA biogenesis machinery were measured by RT-PCR (n=6). Data are presented as mean ± SEM. **P<0.01 vs WT control; ##P<0.01 vs WT soleus

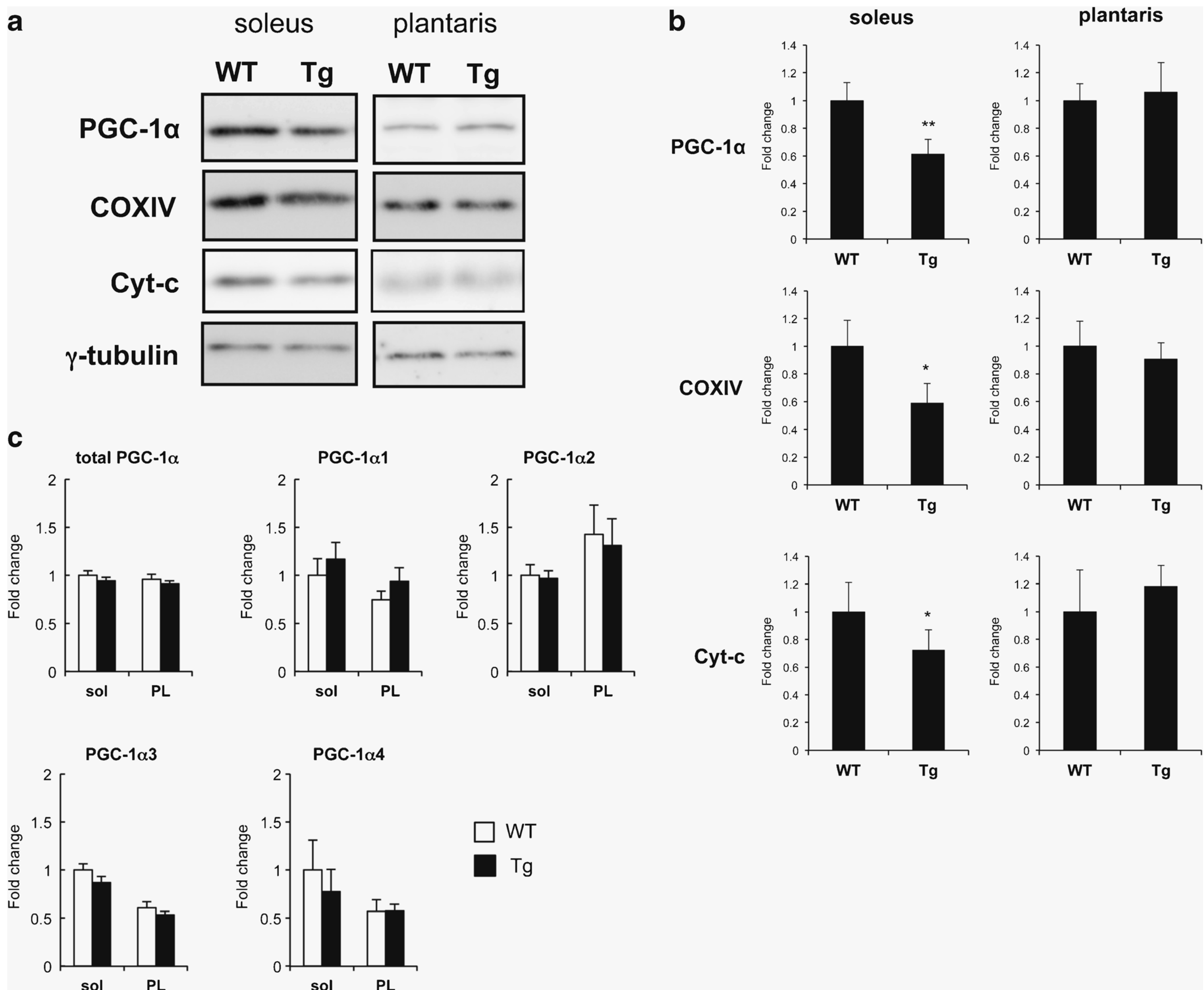

Fig. 2 Forced overexpression of miR-23a downregulates PGC-1α and mitochondrial proteins only in slow muscle. **a** Representative images of PGC-1α and mitochondria respiratory chain proteins from WT and miR-23a Tg mice. **b** Quantitative data for PGC-1α, COX IV and cytochrome *c* proteins from WT and miR-23a Tg mice (n=9). Values are relative to the wild-type (*WT*) mice. **c** PGC-1α isoform mRNA expressions were measured by RT-PCR (n=6). Values are relative to the soleus muscle of the WT mice. Data are presented as mean±SEM. **P<0.01; *P<0.05 vs WT control

expression through translational repression rather than mRNA degradation.

Forced expression of miR-23a did not affect muscle fiber type

Western blot analysis demonstrated no effect of forced overexpression of miR-23a on myosin heavy-chain isoforms (MyHC I, IIa, and IIb) in the soleus or plantaris muscles of the Tg, when compared with WT mice (Fig. 3a). Immunofluorescent imaging of muscle fiber typing was also performed, and again no changes in muscle fiber-type composition in the soleus or plantaris muscles of the miR-23a Tg was observed (Fig. 3b, c), whereas the numbers of MyHC IId/x fibers were slightly decreased in the Tg mice (Fig. 3c). Histological examination revealed reduced SDH staining in type IId/x fibers of soleus muscle (Fig. 3d). Transmission electron microscopy analysis of soleus muscle in the miR-23a Tg mice revealed that mitochondrial structure was generally preserved (Fig. 3d).

Exercise-induced skeletal muscle adaptation in miR-23a Tg mice

As Tg expression of miR-23a suppressed mitochondrial content in slow muscles, we sought to assess endurance exercise capacity of the mice. Forced overexpression of miR-23a did not influence the spontaneous daily running activity of the mice during 4 weeks of cage voluntary wheel running (Table 1). Following 4 weeks of cage voluntary wheel running, there were no differences in the exercise-induced relative increase in muscle weights of both the

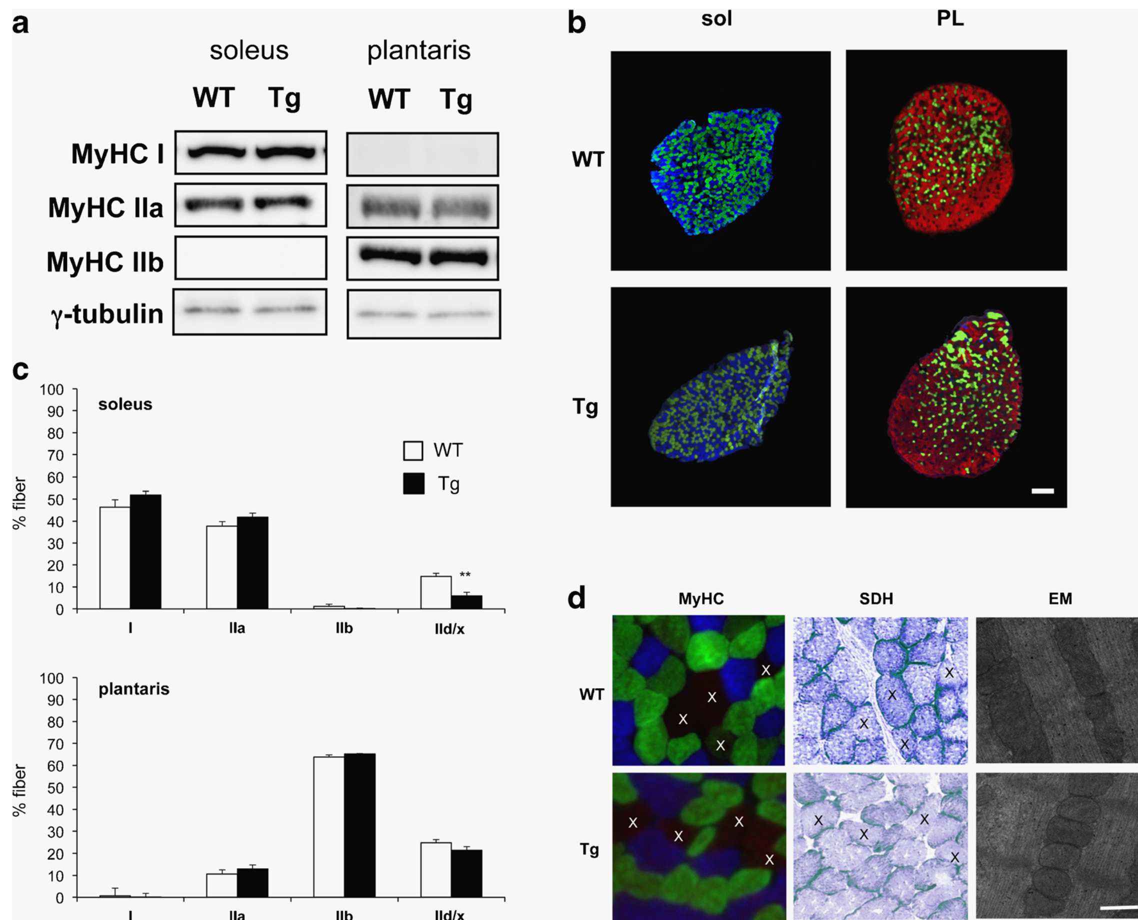

Fig. 3 Forced expression of miR-23a does not influence skeletal muscle fiber type. **a** Representative images of myosin heavy chain (*MyHC*) I, IIa, and IIb in soleus (slow muscle) and plantaris (fast muscle) of WT and transgenic (*Tg*) mice. **b** Representative images of soleus and plantaris muscle frozen sections from WT and miR-23a Tg mice immunostained for MyHC I (*blue*), IIa (*green*), and IIb (*red*). Type IId/x fibers remain unstained. *Scale bar*=200 μm. **c** Quantitation of each fiber type is shown (n=5–6 mice/group). *Open bars*, WT; *black bars*, Tg. **d** Representative images of serial sections with fiber-type staining and succinate dehydrogenase (*SDH*) staining as well as transmission electron microscopy (*EM*) for soleus muscle from WT and miR-23a Tg mice. *X* type IId/x fibers. *Scale bar*=1 μm. Data are presented as mean ± SEM. **P<0.01 vs WT

soleus and plantaris muscle of the WT and miR-23a Tg mice. Immunofluorescence imaging-based fiber typing showed an increase in MyHC IIa fibers in plantaris muscle both in WT and miR-23a Tg after the voluntary running (Fig. 4a). Myosin heavy-chain IIa protein content and markers of mitochondrial content, including PGC-1α, IV, and cytochrome *c* (Fig. 4b) were increased in plantaris muscle of both WT and miR-23a Tg mice following 4 weeks of cage voluntary wheel running. There were no changes in these parameters in the soleus muscle of either mouse following

Table 1 Observations following 4 weeks of voluntary running in WT and Tg mice

Genotype	WT		miR-23a Tg	
Distance run during 4 weeks (km/day)	10.6±0.9		12.0±0.9	
Muscle weight (MW/BW)	Before	After	Before	After
Soleus	0.26±0.01	0.38±0.01**	0.25±0.01	0.36±0.01**
Plantaris	0.53±0.02	0.63±0.02**	0.53±0.01	0.60±0.01**
Endurance exercise capacity (m)	455.2±34.7	1,646.8±479.1#	477.5±30.6	1,661.2±466.2#

Values are means ± SE (n=5 animals/group)

miR-23a Tg miR-23a transgenic mice, *WT* wild-type littermate mice, *MW/BW* muscle weight (mg)/body weight (g), *before* measurements made before 4 weeks of voluntary running, *after* measurements made after 4 weeks of voluntary running

**P<0.01 vs before 4 weeks of voluntary running in the same genotype; # P<0.01 vs before 4 weeks of voluntary running in the same genotype

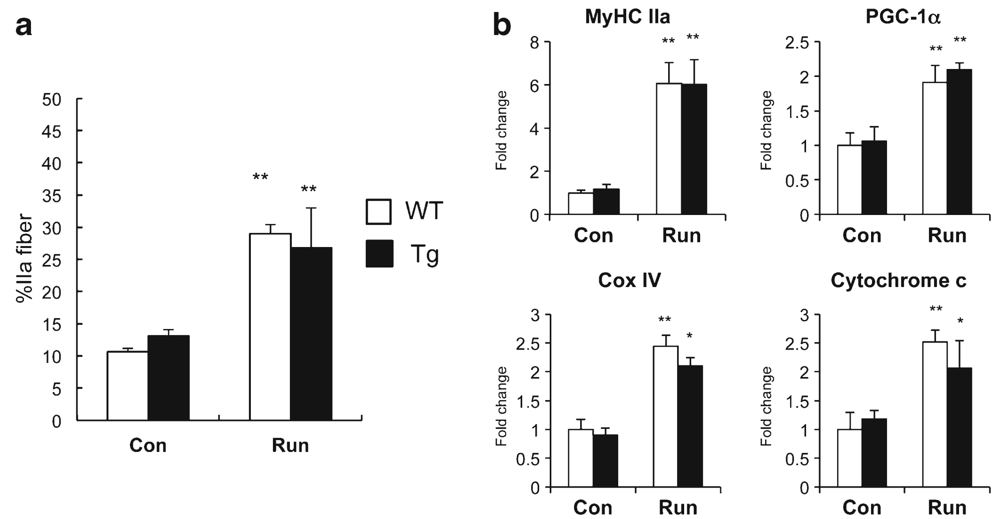

Fig. 4 Forced expression of miR-23a has no impact on endurance exercise-induced muscle adaptation. **a** Quantitation of MyHC IIa fiber in plantaris muscle is shown (n=5–6 mice/group). **b** Relative protein expressions of myosin heavy-chain IIa, PGC-1α, and mitochondria respiratory chain proteins in plantaris muscle from WT and miR-23a Tg mice (n=9). Data are presented as mean ± SEM. *P<0.05; **P<0.01

exercise (data not shown). However, capillary density increased in both the soleus and plantaris muscles of the WT and miR-23a Tg mice (Fig. 5a, b). Finally, there was no difference in the increase in endurance exercise capacity between the WT or the miR-23a Tg mice (Table 1) following 4 weeks of cage voluntary wheel running.

Discussion

This study showed that forced expression of miR-23a in mice downregulates basal PGC-1α protein expression only in the slow soleus muscle, without a major change in their fiber-type composition. We also confirmed that endurance exercise-induced muscle adaptations occurred in fast muscle plantaris muscle of the Tg mice and that this was comparable to the adaptations in the WT littermates.

Interestingly, we found that Tg expression of miR-23a downregulated PGC-1α only in slow muscle (Fig. 2a, b). A previous study showed that double knockout of muscle-enriched miRNAs (miR-499 and miR-208b) led to greater repression of slow myosin expression in soleus than in tibialis anterior [45]. These results may suggest slow muscle has greater capacity in oxidative metabolic gene expression change. Another possibility could be that PGC-1α 3′UTR length may vary with muscle fiber type. In fact, the 3′-sequence of PGC-1α in the EST database shows two putative poly-adenylation sites in the 3′UTR. Further studies should examine whether PGC-1α 3′UTR variants exist without miR-23a target sites and if this influences the sensitivity of PGC-1α to regulation by miRNAs.

MiR-23a can inhibit myogenesis through translational suppression of fast myosin heavy-chain isoforms (MyHC IIa, IId/x, and IIb) in vitro [47]. We have initially expected that forced expression of miR-23a in mice would attenuate myogenesis for fast MyHC muscle fiber formation. Although we found a slight decrease in MyHC IId/x fibers in soleus muscle, it remains unclear why our in vivo data does

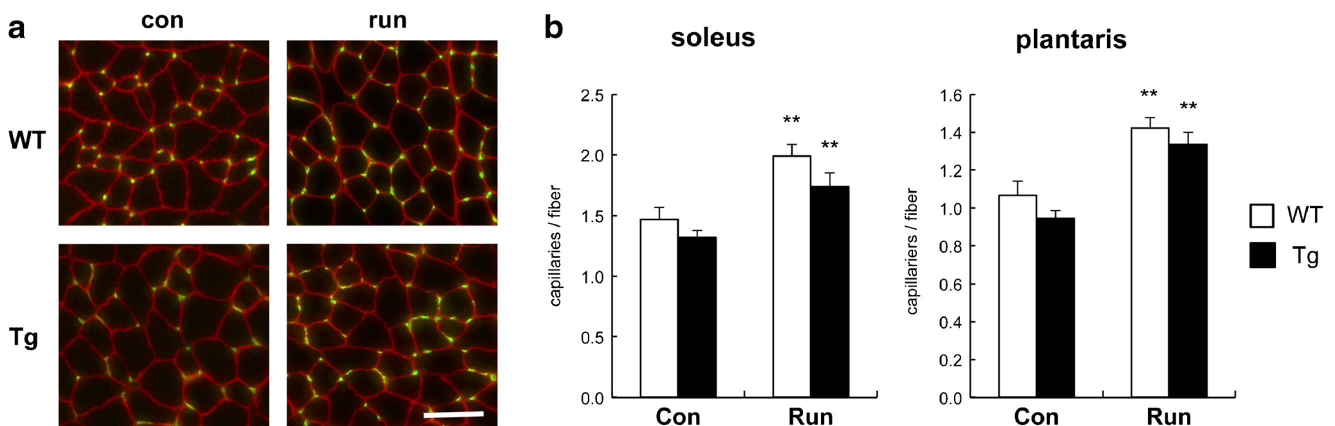

Fig. 5 Forced expression of miR-23a has no impact on endurance exercise-induced angiogenesis. Frozen sections of soleus and plantaris muscles were stained with anti-CD31 antibody. **a** Representative images of plantaris muscle frozen sections from WT and miR-23a transgenic (*Tg*) mice immunostained for CD31 (*green*) and dystrophin (*red*). *Scale bar*= 100 μm. **b** Quantitative data for capillary density in soleus and plantaris muscles from WT and miR-23a Tg mice (n=5–6). Data are presented as mean±SEM. *P<0.05; **P<0.01

not support the in vitro result. It is a possibility that the mild phenotype of the miR-23a Tg mice arises from an insufficient transgene expression. The induction of mature miR-23a in the Tg mice was about 3-fold in slow soleus muscle and 6-fold in fast plantaris muscle, compared with the WT littermates. According to the paper by Wang et al., miR-23a levels were overexpressed by about 5-fold when compared with the negative control in vitro. While this is comparable to that observed in the plantaris muscle in the present study, it is generally recognized that in vitro-cultured myotubes differ considerably from adult myofibers, including the expression profile of MyHC isoforms. In cultured myotubes, the dominant MyHC isoform is embryonic isoform and expressions of MyHC IIa, IIb, or IId/x are very low compared with adult myofiber [26] so that the downregulation of fast myosin heavy-chain isoforms by miR-23a could be prominent in cultured myotubes. Furthermore, miR-23a was transiently overexpressed in vitro while our Tg mouse is a model of chronic miR-23a overexpression. These represent two very different models and therefore comparisons between them are difficult.

To drive the overexpression of miR-23a, we used an expression vector containing the chicken β-actin promoter and cytomegalovirus enhancer as well as the β-actin intron and bovine globin poly-adenylation signal. This construct is able to produce whole body Tg expression from pre-implantation embryo to adult stages [28]. Therefore, it is possible that miR-23a can only transiently downregulate adult MyHC expression in a cell culture model. It should also be noted that although we used conventional Tg mice for miR-23a to gain global expression of the gene, we are not able to rule out the possibility that other regulatory factors functioning in a compensatory manner.

Several miRNAs are reported to change upon endurance exercise to modulate skeletal muscle plasticity [27, 38]. The regulation of these miRNAs following endurance exercise is underexplored. As recently reported, endurance exercise decreases miR-23a expression in skeletal muscle in both rodents and humans [38, 39]. Conversely, a number of studies indicate that endurance exercise induces PGC-1α expression [2, 3, 42]. These results may imply a regulatory relationship between the transcription of PGC-1α and its posttranscriptional control by miR-23a. This notion may contribute to fine tuning of gene expression and muscle adaptation.

Following 4 weeks of cage voluntary wheel running, there was no difference in the relative increase in soleus and plantaris muscle weight between the WT or the miR-23a Tg mice (Table 1). The finding that voluntary running increases muscle mass in soleus and plantaris muscles are in agreement with these previous findings [21, 32]. It is generally recognized that the soleus is not as sensitive as the plantaris muscle to voluntary running-induced metabolic and contractile adaptation [5, 17, 33]. However, as miR-23a Tg mice had a reduction in basal levels of PGC-1α and mitochondria respiratory chain proteins in soleus, but not plantaris muscle, it was of interest to investigate if both muscles were sensitive to voluntary running-induced exercise adaptations.

As well as no effect under basal conditions, forced expression of miR-23a in the fast plantaris muscle did not affect mitochondrial or and contractile adaptations to endurance exercise. These results may imply that the endurance exercise induction of PGC-1α is not targeted by miR-23a. As mentioned previously, there is a possibility that PGC-1α 3′UTR variants without miR-23 target sites exist. Ruas et al. recently reported that several PGC-1α isoforms exist that are induced by exercise [35]. The present study observed no changes in the mRNA levels of these isoforms, but this does not rule out their potential role in skeletal muscle exercise-induced adaptations.

PGC-1α is reported to be essential for exercise-induced angiogenesis in skeletal muscle [9]. We confirmed endurance exercise-induced increase in capillary density in miR-23a Tg mice. Capillary density in the Tg mice tended to decrease compared with that in WT mice, although the difference does not reach statistical significance (P=0.145). This phenomenon is consistent with the changes in muscle properties of miR-23a Tg mice in response to endurance exercise.

MiRNA biogenesis is a complex process requiring coordination of pri-miRNA transcription, their cleavage by endonucleases, exportation from nucleus to cytoplasm, additional cleavage, then incorporation into the RISC complex [4]. Forced expression of miR-23a under control of chicken β-actin promoter increases mRNA for two key components, miRNA export protein, Exportin-5, and Ago2. Pri- and pre-miR23a levels were significantly increased in the skeletal muscle of Tg mice, suggesting that the upregulation of these genes most likely reflects the accumulation of pri- and pre-miR-23a in the Tg mice. Mechanisms regulating the miRNA biogenesis machinery in Tg animals are unclear, and this should be a focus for future investigation.

In summary, this study provides evidence that forced expression of miR-23a in mice decreases basal markers of mitochondrial biogenesis such as PGC-1α, COX IV, and cytochrome *c* in slow muscle. By contrast, miR-23a Tg mice showed no change in their fiber-type composition and endurance exercise-induced muscle adaptation in fast muscle. This suggests that miR-23a plays a limited role in regulating the skeletal muscle adaptation to endurance exercise.

Acknowledgments The authors thank Dr. Hiroshi Sagara (The University of Tokyo) and Akisa Tobimatsu for excellent technical support. The authors also thank Dr. Stefano Schiaffino (Venetian Institute for Molecular Medicine) for comments on EM images. This study was supported in part by Grants-in Aid for Young Investigators (A; 21680049 to T. A.) and for Scientific Research (B; 25282198 to T. A.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Takeda Science Foundation. S. W., K. A. and J. H. P. were supported by Japan Society of the Promotion of Science (JSPS).

References

1. Akimoto T, Okuhira K, Aizawa K, Wada S, Honda H, Fukubayashi T, Ushida T (2013) Skeletal muscle adaptation in response to mechanical stress in p130Cas−/−mice. Am J Physiol Cell Physiol 304: C541–C547
2. Akimoto T, Pohnert SC, Li P, Zhang M, Gumbs C, Rosenberg PB, Williams RS, Yan Z (2005) Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway. J Biol Chem 280:19587–19593
3. Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, Holloszy JO (2002) Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB J 16: 1879–1886
4. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297
5. Brown M, Ross TP, Holloszy JO (1992) Effects of ageing and exercise on soleus and extensor digitorum longus muscles of female rats. Mech Ageing Dev 63:69–77
6. Carrer M, Liu N, Grueter CE, Williams AH, Frisard MI, Hulver MW, Bassel-Duby R, Olson EN (2012) Control of mitochondrial metabolism and systemic energy homeostasis by microRNAs 378 and 378. Proc Natl Acad Sci U S A 109:15330–15335
7. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 38:228–233
8. Chin ER, Olson EN, Richardson JA, Yang Q, Humphries C, Shelton JM, Wu H, Zhu W, Bassel-Duby R, Williams RS (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes Dev 12:2499–2509
9. Chinsomboon J, Ruas J, Gupta RK, Thom R, Shoag J, Rowe GC, Sawada N, Raghuram S, Arany Z (2009) The transcriptional coactivator PGC-1alpha mediates exercise-induced angiogenesis in skeletal muscle. Proc Natl Acad Sci U S A 106:21401–21406
10. Finck BN, Kelly DP (2006) PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J Clin Invest 116: 615–622
11. Geng T, Li P, Okutsu M, Yin X, Kwek J, Zhang M, Yan Z (2010) PGC-1alpha plays a functional role in exercise-induced mitochondrial biogenesis and angiogenesis but not fiber-type transformation in mouse skeletal muscle. Am J Physiol Cell Physiol 298: C572–C579
12. Goto M, Terada S, Kato M, Katoh M, Yokozeki T, Tabata I, Shimokawa T (2000) cDNA Cloning and mRNA analysis of PGC-1 in epitrochlearis muscle in swimming-exercised rats. Biochem Biophys Res Commun 274:350–354
13. Grueter CE, van Rooij E, Johnson BA, DeLeon SM, Sutherland LB, Qi X, Gautron L, Elmquist JK, Bassel-Duby R, Olson EN (2012) A cardiac microRNA governs systemic energy homeostasis by regulation of MED13. Cell 149:671–683
14. Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286: 950–952
15. Handschin C, Chin S, Li P, Liu F, Maratos-Flier E, Lebrasseur NK, Yan Z, Spiegelman BM (2007) Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1alpha muscle-specific knock-out animals. J Biol Chem 282:30014–30021
16. Holloszy JO (1967) Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. J Biol Chem 242:2278–2282
17. Ikeda S, Kawamoto H, Kasaoka K, Hitomi Y, Kizaki T, Sankai Y, Ohno H, Haga S, Takemasa T (2006) Muscle type-specific response of PGC-1 alpha and oxidative enzymes during voluntary wheel running in mouseskeletal muscle. Acta Physiol (Oxf) 188:217–223
18. Katzmarzyk PT, Janssen I (2004) The economic costs associated with physical inactivity and obesity in Canada: an update. Can J Appl Physiol 29:90–115
19. Kim HK, Lee YS, Sivaprasad U, Malhotra A, Dutta A (2006) Muscle-specific microRNA miR-206 promotes muscle differentiation. J Cell Biol 174:677–687
20. Kinugawa S, Wang Z, Kaminski PM, Wolin MS, Edwards JG, Kaley G, Hintze TH (2005) Limited exercise capacity in heterozygous manganese superoxide dismutase gene-knockout mice: roles of superoxide anion and nitric oxide. Circulation 111:1480–1486
21. Li P, Akimoto T, Zhang M, Williams RS, Yan Z (2006) Resident stem cells are not required for exercise-induced fiber-type switching and angiogenesis but are necessary for activity-dependent muscle growth. Am J Physiol Cell Physiol 290:C1461–C1468
22. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433:769–773
23. Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature 418:797–801
24. McCarthy JJ, Esser KA, Peterson CA, Dupont-Versteegden EE (2009) Evidence of MyomiR network regulation of beta-myosin heavy chain gene expression during skeletal muscle atrophy. Physiol Genomics 39:219–226
25. Nachlas MM, Tsou KC, de Souza E, Cheng CS, Seligman AM (1957) Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. J Histochem Cytochem 5:420–436
26. Naumann K, Pette D (1994) Effects of chronic stimulation with different impulse patterns on the expression of myosin isoforms in rat myotube cultures. Differentiation 55:203–211
27. Nielsen S, Scheele C, Yfanti C, Akerstrom T, Nielsen AR, Pedersen BK, Laye MJ (2010) Muscle specific microRNAs are regulated by endurance exercise in human skeletal muscle. J Physiol 588:4029–4037
28. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y (1997) 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407: 313–319
29. Pogozelski AR, Geng T, Li P, Yin X, Lira VA, Zhang M, Chi JT, Yan Z (2009) P38Gamma mitogen-activated protein kinase is a key regulator in skeletal muscle metabolic adaptation in mice. PLoS One 4:e7934
30. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 92:829–839
31. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403:901–906
32. Riedy M, Moore RL, Gollnick PD (1985) Adaptive response of hypertrophied skeletal muscle to endurance training. J Appl Physiol 59:127–131
33. Rodnick KJ, Henriksen EJ, James DE, Holloszy JO (1992) Exercise training, glucose transporters, and glucose transport in rat skeletal muscles. Am J Physiol 262:C9–C14
34. Rowe GC, El-Khoury R, Patten IS, Rustin P, Arany Z (2012) PGC-1α is dispensable for exercise-induced mitochondrial biogenesis in skeletal muscle. PLoS One 7:e41817
35. Ruas JL, White JP, Rao RR, Kleiner S, Brannan KT, Harrison BC, Greene NP, Wu J, Estall JL, Irving BA, Lanza IR, Rasbach KA, Okutsu M, Nair KS, Yan Z, Leinwand LA, Spiegelman BM (2012) A PGC-1α isoform induced by resistance training regulates skeletal muscle hypertrophy. Cell 151:1319–1331

36. Russell AP, Feilchenfeldt J, Schreiber S, Praz M, Crettenand A, Gobelet C, Meier CA, Bell DR, Kralli A, Giacobino JP, Dériaz O (2003) Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and peroxisome proliferator-activated receptor-alpha in skeletal muscle. Diabetes 52:2874–2881
37. Russell AP, Wada S, Vergani L, Hock MB, Lamon S, Léger B, Ushida T, Cartoni R, Wadley GD, Hespel P, Kralli A, Soraru G, Angelini C, Akimoto T (2012) Disruption of skeletal muscle mitochondrial network genes and miRNAs in amyotrophic lateral sclerosis. Neurobiol Dis 49C:107–117
38. Russell AP, Lamon S, Boon H, Wada S, Guller I, Brown EL, Chibalin AV, Zierath J, Snow RJ, Stepto NK, Wadley GD, Akimoto T. (2013) Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short term endurance training. *J Physiol.* 2013; (in press)
39. Safdar A, Abadi A, Akhtar M, Hettinga BP, Tarnopolsky MA (2009) miRNA in the regulation of skeletal muscle adaptation to acute endurance exercise in C57Bl/6 J male mice. PLoS One 4:e5610
40. Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, Vianello M, Gundersen K, Lømo T (1989) Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. J Muscle Res Cell Motil 10:197–205
41. Schiaffino S, Reggiani C (2011) Fiber types in mammalian skeletal muscles. Physiol Rev 91:1447–1531
42. Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T, Tabata I (2002) Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. Biochem Biophys Res Commun 296:350–354
43. Terada S, Tabata I (2004) Effects of acute bouts of running and swimming exercise on PGC-1alpha protein expression in rat epitrochlearis and soleus muscle. Am J Physiol Endocrinol Metab 286:E208–E216
44. Thyfault JP, Cree MG, Zheng D, Zwetsloot JJ, Tapscott EB, Koves TR, Ilkayeva O, Wolfe RR, Muoio DM, Dohm GL (2007) Contraction of insulin-resistant muscle normalizes insulin action in association with increased mitochondrial activity and fatty acid catabolism. Am J Physiol Cell Physiol 292:C729–C739
45. van Rooij E, Quiat D, Johnson BA, Sutherland LB, Qi X, Richardson JA, Kelm RJ Jr, Olson EN (2009) A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. Dev Cell 17:662–673
46. Wada S, Kato Y, Okutsu M, Miyaki S, Suzuki K, Yan Z, Schiaffino S, Asahara H, Ushida T, Akimoto T (2011) Translational suppression of atrophic regulators by microRNA-23a integrates resistance to skeletal muscle atrophy. J Biol Chem 286:38456–38465
47. Wang L, Chen X, Zheng Y, Li F, Lu Z, Chen C, Liu J, Wang Y, Peng Y, Shen Z, Gao J, Zhu M, Chen H (2012) MiR-23a inhibits myogenic differentiation through down regulation of fast myosin heavy chain isoforms. Exp Cell Res 318:2324–2334
48. Wende AR, Schaeffer PJ, Parker GJ, Zechner C, Han DH, Chen MM, Hancock CR, Lehman JJ, Huss JM, McClain DA, Holloszy JO, Kelly DP (2007) A role for the transcriptional coactivator PGC-1alpha in muscle refueling. J Biol Chem 282:36642–36651
49. Wu H, Rothermel B, Kanatous S, Rosenberg P, Naya FJ, Shelton JM, Hutcheson KA, DiMaio JM, Olson EN, Bassel-Duby R, Williams RS (2001) Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. EMBO J 20:6414–6423
50. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ, Srivastava D (2007) Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell 129:303–317
51. Zhao Y, Samal E, Srivastava D (2005) Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature 436:214–220