



Disruption of skeletal muscle mitochondrial network genes and miRNAs in amyotrophic lateral sclerosis

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ARTICLE INFO

Article history:

Received 17 June 2012

Revised 5 August 2012

Accepted 17 August 2012

Available online 4 September 2012

Keywords:

Peroxisome proliferator activator receptor γ

co-activator-1

Mitofusin

Skeletal muscle

MicroRNA

ALS (amyotrophic lateral sclerosis)

ABSTRACT

Skeletal muscle mitochondrial dysfunction is believed to play a role in the progression and severity of amyotrophic lateral sclerosis (ALS). The regulation of transcriptional co-activators involved in mitochondrial biogenesis and function in ALS is not well known. When compared with healthy control subjects, patients with ALS, but not neurogenic disease (ND), had lower levels of skeletal muscle peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) mRNA and protein and estrogen-related receptor- α (ERR α) and mitofusin-2 (Mfn2) mRNA. PGC-1 β , nuclear respiratory factor-1 (NRF-1) and Mfn1 mRNA as well as cytochrome C oxidase subunit IV (COXIV) mRNA and protein were lower in patients with ALS and ND. Both patient groups had reductions in citrate synthase and cytochrome c oxidase activity. Similar observations were made in skeletal muscle from transgenic ALS G93A transgenic mice. *In vitro*, PGC-1 α and PGC-1 β regulated *Mfn1* and *Mfn2* in an ERR α -dependent manner. Compared to healthy controls, miRNA 23a, 29b, 206 and 455 were increased in skeletal muscle of ALS patients. miR-23a repressed PGC-1 α translation in a 3' UTR dependent manner. Transgenic mice over expressing miR-23a had a reduction in PGC-1 α , cytochrome-b and COXIV protein levels. These results show that skeletal muscle mitochondrial dysfunction in ALS patients is associated with a reduction in PGC-1 α signalling networks involved in mitochondrial biogenesis and function, as well as increases in several miRNAs potentially implicated in skeletal muscle and neuromuscular junction regeneration. As miR-23a negatively regulates PGC-1 α signalling, therapeutic inhibition of miR-23a may be a strategy to rescue PGC-1 α activity and ameliorate skeletal muscle mitochondrial function in ALS.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a motor neuron disorder resulting in the progressive degeneration of upper and lower motor neurons, a decline in strength, severe muscle atrophy, respiratory insufficiency and death within 3–5 years after the first symptoms (Pasinelli and Brown, 2006). The primary cause of the more frequent sporadic ALS is presently unknown, while in the 5–10% of familial cases (FALS) approximately 25% are associated with mutations in the CuZn superoxide dismutase (CuZn SOD or SOD1) gene. While the general consensus is that ALS is caused by motor neuron death, the precise factor/s causing motor neuron degeneration in ALS remains equivocal (Brooks et al., 2004; Frey et al., 2000; Kennel et al., 1996).

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Available online on ScienceDirect (www.sciencedirect.com).

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Perturbations in mitochondrial function are considered an important component in the pathogenesis of ALS (Menzies et al., 2002). In transgenic ALS mice harbouring the G93A-mutated SOD1 gene, the increase in motor neuron mitochondrial degeneration precedes paralysis (Kong and Xu, 1998). This suggests that perturbations in mitochondrial biogenesis, structure and/or function, may play a role in motor neuron degeneration. However, expression of the mutated SOD1 gene in neurons or astrocytes does not lead to ALS (Gong et al., 2000; Pramatarova et al., 2001), suggesting that disease onset and/or progression stems from the reciprocal effects of several cellular systems (Clement et al., 2003). In ALS mice degeneration of the neuromuscular junction (Frey et al., 2000; Kennel et al., 1996) and muscle atrophy and degeneration (Brooks et al., 2004; Marcuzzo et al., 2011) precedes neuronal degeneration. This supports the notion that muscle degeneration may lead and/or contribute to neurodegeneration and play a key role in the cause and/or progression of ALS (Dupuis and Echaniz-Laguna, 2010; Wong and Martin, 2010).

Skeletal muscle of ALS patients and ALS mice presents severe atrophy (Leger et al., 2006) and has considerable mitochondrial disruption and dysfunction, indicated by NADH:CoQ oxidoreductase and cytochrome c oxidase deficiency (Vielhaber et al., 2000; Wiedemann et al., 1998), reduced mitochondrial DNA and reduced levels of mitochondria Mn-SOD (Vielhaber et al., 2000). Disruption of the mitochondrial network enhances skeletal muscle atrophy programs (Romanello et al., 2010), suggesting that impaired mitochondrial function may activate signals that trigger muscle atrophy. It is therefore logical to hypothesise that the pathogenesis and progression of ALS may involve perturbations in signalling molecules that are normally required for the healthy maintenance of skeletal muscle mitochondrial biogenesis and function as well as muscle mass.

The transcriptional co-activators, peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) and PGC-1 β positively regulate the mitochondrial network (Finck and Kelly, 2006; Handschin and Spiegelman, 2006) and attenuate muscle atrophy programs (Brault et al., 2010; Sandri et al., 2006). PGC-1 α and PGC-1 β influence mitochondrial biogenesis and function via the induction and activation of several nuclear transcription factors, such as nuclear respiratory factor-1 (NRF-1) (Wu et al., 1999) and estrogen-related receptor alpha (ERR α) (Mootha et al., 2004; Schreiber et al., 2003, 2004) and several gene targets including mitofusin-2 (Mfn2) (Cartoni et al., 2005; Liesa et al., 2008) and cytochrome C oxidase subunit IV (COX IV) (Lelliott et al., 2006; Puigserver et al., 1998). A down regulation of PGC-1 α is observed in conditions associated with mitochondrial dysfunction such as diabetes (Mensink et al., 2007) and ageing (Short et al., 2003) as well as in skeletal muscle atrophy, such as uremia, denervation and cancer cachexia (Sandri et al., 2006). Whether PGC-1 α /1 β and members of their transcriptional program involved in mitochondrial biogenesis and function show altered regulation in atrophied skeletal muscle of ALS patients has not been established.

Recently it has been shown that skeletal muscle development and function can be controlled by microRNAs, short (~20–30 nucleotides [nt]) noncoding ribonucleic acids (RNAs) (reviewed previously (Bartel, 2004)). Presently their known functions are to inhibit protein translation or enhance messenger RNA degradation (Hamilton and Baulcombe, 1999; Reinhart et al., 2000). A suite of muscle-enriched miRNAs (miR-1, 133a, 133b, 206; collectively referred to as myomiRs) (Sempere et al., 2004; Small et al., 2010; van Rooij et al., 2007, 2009) have been identified and shown to regulate muscle proliferation and differentiation (reviewed in (Guller and Russell, 2011)). Recently, miR-206 has been implicated in the regeneration of neuromuscular synapses in ALS mice (Williams et al., 2009). The regulation of skeletal muscle miRNAs has been investigated in several human muscular disorders (Eisenberg et al., 2007; Gambardella et al., 2010; Greco et al., 2009), but not in skeletal muscle from healthy control subjects, patients with ALS

and patients with neurogenic disease (ND) (disease control), (1) expression levels of PGC-1 α and several of their downstream targets involved in mitochondrial biogenesis and function, including ERR α , NRF-1, Mfn1, Mfn2 and COX IV; (2) mitochondrial enzyme activity and (3) expression levels of miRNAs enriched in skeletal muscle, including miR-1, and 206 and those dysregulated in human muscle disease, including miR-9, -23a, -23b, -29a, -29b, -29c, -31 and -455. A functional miRNA/protein relationship was also investigated *in vitro* using reporter assays and *in vivo* using transgenic mice.

Methods

Subjects

Skeletal muscle biopsies were obtained from fourteen patients diagnosed with ALS (Brooks, 1994), 10 patients diagnosed with neurogenic disease (ND) and 10 age matched healthy control subjects (Table 1). At the time of the biopsy, the duration of illness was 3–48 months. The ND patients consisted of those diagnosed with either SMA type II, post-polio syndrome, motor neuropathy, sensory motor neuropathy or polyneuropathy. The routine histochemical analysis of all biopsies revealed a neurogenic pattern ranging from mild to severe. In all cases, conduction blocks were excluded by sensorimotor nerve conduction studies; laboratory tests ruled out dysproteinemia, hexoaminidase A deficiency, anti-GM1 antibodies, thyroid and parathyroid disorders. The research protocol was approved by the institutional ethics committees and informed consent was obtained from all participants according to the Declaration of Helsinki (BMJ 1991; 302:1194).

Muscle biopsies

For the ALS and ND patients biopsies were taken from the vastus lateralis muscle using either Bergstrom or open biopsy procedures. For the healthy control subjects biopsies were taken from the vastus lateralis muscle using the Bergstrom technique. All biopsies were immediately frozen in liquid nitrogen and stored at -80°C until analysed.

Animals

ALS transgenic mice

Transgenic mice carrying the SOD1 G93A mutation were compared with control wild-type animals carrying a transgene of the human SOD1 gene (WT) (Jackson Laboratories (Bar Harbor, Maine) (Derave et al., 2003)). Thirteen G93A and 10 WT mice were included in the study. Five G93A mice were sacrificed at the age of 90 days (ALS 90) and all other animals were studied at the age of 120 days (ALS 120, n = 8; WT 120, n = 10) (Derave et al., 2003). For the G93A ALS mice 90 and 120 days corresponds to about 65 and 85% respectively, of their life expectancy. All mice were anesthetized by an intraperitoneal infusion of pentobarbitone sodium (50 mg/kg body weight).

miR-23a transgenic mice

The miR-23a transgenic mice have been described (Wada et al., 2011). Genotyping was carried out by PCR and fluorescence microscopy

Table 1
Characteristics of subjects included in the study.

| | Control | ALS | ND |
|---------------|-------------|------------|-------------|
| Patients (n) | 10 | 14 | 10 |
| M/F | 7/3 | 10/4 | 7/3 |
| Age (years) | | | |
| Mean \pm SD | 53 \pm 17 | 59 \pm 9 | 53 \pm 13 |
| Range | 25–73 | 33–79 | 20–71 |

M = male; F = female.

was used to confirm transgene expression in adult skeletal muscles. Skeletal muscle samples (soleus and plantaris) were harvested from the F2 generation of transgenic lines (#345 and #355) and wild-type littermates (C57BL6/J background) at 8 weeks of age.

RNA extraction and real time quantitative PCR

RNA extraction and reverse transcription to cDNA as well as QPCR has been described previously in detail (Cartoni et al., 2005; Leger et al., 2006). To control for any variations due to efficiencies of the reverse transcription and PCR, acidic ribosomal phosphoprotein PO (RPLPO or 36B4) was used as an internal control. PCR primer and probe sequences for human and mouse are shown in Table 2.

Western blotting

Human muscle proteins

Electrophoresis was performed using a 4–12% NuPAGE® Novex Bis-Tris Gel (Invitrogen, Carlsbad, CA) in NuPAGE® SDS MOPS Running Buffer (Invitrogen, Carlsbad, CA). Protein transfer was performed in a Bjerrum buffer containing 50 mM Tris, 17 mM glycine and 10% methanol using PVDF membranes. The membranes were blocked with 5% BSA in PBS, after which they were incubated overnight at 4 °C with the following primary antibodies diluted in 3% BSA in PBS: PGC-1α (Chemicon, Temecula, USA); Mitofusin-1 (MFN-1) (Abnova GmbH, Heidelberg, Germany); MFN-2 (Abnova GmbH, Heidelberg, Germany); NRF-1 (Rockland Immunochemicals Inc. Gilbertsville, USA); COXIV (Santa Cruz Biotechnology, CA, USA). Primary antibodies were

diluted 1:500, 1:1000 for NRF-1 and PGC-1α and 1:10,000 for COXIV. Following washing, the membranes were incubated for 1 h with a goat anti-rabbit IgG antibody labelled with an infrared-fluorescent 800 nm dye (Alexa Fluor® 800, Invitrogen, Carlsbad, CA) or a rabbit anti-mouse IgG antibody labelled with an infrared-fluorescent 680 nm dye (Alexa Fluor® 680, Invitrogen, Carlsbad, CA) diluted 1:5000 in PBS containing 50% Odyssey® blocking buffer (LI-COR Biosciences, Lincoln, USA) and 0.01% SDS. After washing, the proteins were exposed on an Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, USA) and individual protein band optical densities were determined using ImageJ Software (NIH, Bethesda, USA). The blots were normalized against the GAPDH protein (Sigma-Aldrich, Sydney, Australia).

Transgenic ALSG93A mice

Electrophoresis was performed using a 12% SDS PAGE gel in cold (4 °C) buffer containing 25 mM Tris pH 8.8, 192 mM glycine and 10% methanol. After protein transfer the PVDF membranes were blocked with 5% non-fat dry milk in PBS containing 0.05% Tween, then incubated overnight at 4 °C with an anti-PGC-1α antibody (Chemicon, Temecula, USA), diluted 1:5000 in 5% non-fat dry milk in 0.5% Tween-20 in PBS. Following washing the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling, Beverly, MA) at a dilution of 1:2000 for hour, washed for 3×5 min in 0.05% Tween-20 in PBS and treated for 5 min with chemiluminescence substrate (ECL-plus, GE Healthcare, Little Chalfont, UK). Finally, X-ray film was exposed to the PVDF membranes for 1 min. An α-tubulin (Sigma, Basel, Switzerland) was diluted 1:10,000 and used to control for protein loading.

Transgenic miR-23a mice

Western blotting was performed as mentioned previously (Wada et al., 2011) using anti-PGC-1α (Chemicon, Temecula, USA), anti-COX IV, cytochrome-c (Cell Signaling Technologies, Danvers, USA) and anti-γ-tubulin (Sigma, Tokyo, Japan) antibodies.

Mitochondrial enzyme activity

Enzyme activities were measured spectrophotometrically at room temperature. The total activity of COX was assayed by measuring the decrease in absorbance at 550 nm corresponding to the oxidation of ferrocytochrome c by COX using a commercially available kit (Sigma, Sydney, Australia). Citrate synthase activity was measured by following the increase in 5,5'-dithiobis-2-nitrobenzoate at 412 nm (Russell et al., 2002).

Measurement of microRNAs

RNA (10 ng) was reverse transcribed using specific primers for the microRNAs of interest, as per the manufacturer's instructions (Applied Biosystems, Carlsbad, USA). miRNA levels were measured using specific primer and probes sets as per the manufacturer's instructions (Applied Biosystems, Carlsbad, USA) using an MX3000p thermal cycler system. The list of the miRNA sequences is provided in Table 3.

Cell culture experiments

C2C12 muscle cultures

The mouse myoblast cell line C2C12 (ATCC# CRL-1772) was obtained from ATCC (Manassas, VA). C2C12 myoblasts were maintained in DMEM supplemented with 10% FBS, Penicillin–Streptomycin, and Na-Pyruvate (Invitrogen, Carlsbad, CA). Confluent myoblasts were differentiated in DMEM containing 2% horse serum. After four days of differentiation C2C12 myotubes were infected with control or siERRα adenovirus (MOI 200). Forty-eight hours later, the cells were infected with (1) a second dose of either control or siERRα adenovirus, and (2) control (LacZ)

Table 2

Human and mouse primer sequences, and annealing temperatures used for the real-time PCR.

| Gene | Sequence 5'–3' | Temp |
|------------------------------------|--|------|
| hPGC-1α | Sense TCA GTC CTC ACT GGT GGA CA Anti TGC TTC GTC GTC AAA AAC AG | 62 |
| hPGC-1β | Sense CTG CTG GCC CAG ATA CAC TGA Anti ATC CAT GGC TTC ATA CTT GCT | 60 |
| hERRα | Sense TTCTCATCGCTGCTGCTCT Anti CAGCCGCCGACTAGTTG | 64 |
| hNRF-1 | Sense GGT GCA GCA CCT TTG GAG AA Anti CCA GAG CAG ACT CCA GGT CTT C | 60 |
| hMFN1 | sense TGT TTT GGT CGC AAA CTC TG anti CTG TCT GCG TAC GTC TTC CA | 60 |
| hMFN2 | sense ATG CAT CCC CAC TTA AGC AC anti CCA GAG GGC AGA ACT TTG TC | 60 |
| hCOXIV | Sense CAT GTG GCA GAA GCA CTA TGT GT Anti GCC ACC CAC TCT TTG TCA AAG | 60 |
| h36B4 | Sense GTG ATG TGC AGC TGA TCA AGA CT Anti GAT GAC CAG CCC AAA GGA GA | 60 |
| mPGC-1α | Sense AAT GCA GCG GTC TTA GCA CT Anti GTG TGA GGA GGT TCA TCG TT | 62 |
| mPGC-1β | Sense TTC CCA GAA CTG GAT GAA GG Anti TCT GGA ACT GAG GCT GGT CT | 60 |
| mERRα | Sense TGC CAA TTC TGA CTC TGT GC Anti CCA GCT TCA CCC CAT AGA AA | 64 |
| mNRF-1 | Sense CAA CAG GGA AGA AAC GGA AA Anti GCA CCA CAT TCT CCA AAG GT | 60 |
| mMfn1 | Sense GCT GTC AGA GCC CAT CTT TC Anti CAG CCC ACT GTT TTC CAA AT | 60 |
| mMfn2 | Sense GCC AGC TTC CTT GAA GAC AC Anti GCA GAA CTT TGT CCC AGA GC | 60 |
| mCOXIV | Sense ACT ACC CCT TGC CTG ATG TG Anti GCC CAC AAC TGT CTT CCA TT | 60 |
| ChMfn1 proximal ERRE (+ 105 bp) | Sense CTT TCG GGA GGA TGA GAC AG Anti GGT GGA TTC CTA CCT GGA CA | 60 |
| ChMfn2 proximal ERRE (– 437 bp) | Sense TTTTGTGTTGCTGCTCCAG Anti TGT GGC TGC TGT TTA GTT GC | 60 |

h, human; m, mouse; Ch, CHIP.

Table 3
Sequences for the miRNA primers.

| MicroRNA | Sequence | Assay ID |
|----------|--------------------------|----------|
| miR-1 | UGGAAUGUAAAGAAGUAUGUAAU | 002222 |
| miR-23a | AUCACAUUGCCAGGGAUUUCC | 000399 |
| miR-23b | AUCACAUUGCCAGGGAUUUACC | 000400 |
| miR-29a | UAGCACCAUCUGAAAUCCGUUA | 002112 |
| miR-29b | UAGCACCAUUUGAAAUCCAGUGUU | 000413 |
| miR-29c | UAGCACCAUUUGAAAUCCGUUA | 000587 |
| miR-31 | AGGCAAGAUUGCGGCAUAGCU | 002279 |
| miR-133a | UUUGGUCCCUCAACACGUG | 002246 |
| miR-181a | AACAUAUACGUGUGGUGAGU | 000480 |
| miR-455 | UAUGUGCCUUUGGACUACAUCG | 001280 |

or PGC-1 (PGC-1 α or PGC-1 β) adenovirus (MOI 50). We have previously shown that our siERR α adenovirus results in a complete knockdown of the ERR α protein (Cartoni et al., 2005). RNA was harvested 24 h later with RNeasy spin columns (Qiagen, Valencia, CA). cDNA was synthesized from 400 ng RNA and used as template for quantitative PCR detection with SYBR green. qPCR data were normalized against 36B4 levels using the $\Delta\Delta C_t$ method.

Chromatin immunoprecipitation

ChIP experiments were performed as described previously (Cartoni et al., 2005). Briefly, day 7 myotubes overexpressing flag-PGC1 α , flag-PGC1 β or control LacZ adenovirus were crosslinked with 1% formaldehyde in PBS at 37° for 10 min. Samples were lysed and sonicated such that DNA fragments were ~500 bp. Immunoprecipitations were performed with anti-flag M2 monoclonal antibodies (Sigma; Saint Louis, MO) and a 1:1 mixture of protein A and protein G sepharose (Pharmacia; Uppsala, Sweden). The amount of gDNA in each immunoprecipitation was quantified by qPCR using primers listed in Table 2. Data are expressed as relative immunoprecipitation, where values for each IP are first normalized against the “total” or input gDNA for that sample, then expressed relative to the amount of gDNA immunoprecipitated from the LacZ control.

Reporter assay experiments

Mouse PGC-1 α 300 bp-long 3' UTRs, including putative miR-23a/b binding sites, were amplified from mouse cDNA by PCR using Ex Taq HS (TaKaRa, Osaka, Japan). Primer sequences for amplifying the PGC-1 α 3' UTR were 5'-ACC TTT TCA TGC CTT TAG ATG TGA GC-3' (forward) and 5'-GCA AGG GCT CAA ACT AAT CAC TCA CT-3' (reverse). The amplified fragment was cloned downstream of luciferase under the SV40 promoter using the EcoRI and XbaI sites of the pLuc2EXN plasmid (pLuc2-PGC-1 α). We also mutated 4 putative miR-23 binding sites in the mouse PGC-1 α 3' UTR reporter using the following primers (mutated nucleotides are underlined): 1st: CTT TTC ATG CCT TTA GAT GTT TG, 2nd: ATA GCC ATG TAC TAT AAT GTT TT, 3rd: TTC TAA ATT TGT ACC TAT GTT TC, 4th: ACA GAC ATT TTC AAT AAT GTT TA. For overexpression of miR-23a, pCXbG-miR-23a was generated as previously described (Wada et al., 2011). Reporter assay was performed as previously described (Wada et al., 2011). Briefly, HeLa cells were plated into 12 well plates 12 h before transfection. A reporter vector with PGC-1 α 3' UTR (pLuc2-PGC-1 α) was co-transfected with pRL-TK (Promega, Madison, USA) by Lipofectamine 2000 (Invitrogen, Carlsbad, USA). The pCXbG-miR-23a vector was used to overexpress miR-23a. To knock-down miR-23a/b, 20 nM anti-miR-23a biotinylated antisense 2'-O-methyl single strand RNA (2'OMe ssRNA) oligonucleotides was transfected 12 h before harvesting. Cells were harvested with

appropriate buffer 24 h after transfection and luciferase activity was measured using Dual-Luciferase reporter assay kit (Promega, Madison, USA).

Statistics

A one-way ANOVA with a Dunnett C post-hoc test was used for all analyses when comparing control, ALS and ND subjects as well as WT 120, ALS 90 and ALS 120 mice. Unpaired t-tests were used to test for differences between (1), the wild-type and ALS mice; (2), the wild-type and Tg-miR-23a mice; (3) LacZ and PGC-1 α infected cells; (4) LacZ and PGC-1 β infected cells and (5) LacZ and VP16-ERR α infected cells. The level of significance was set at $p < 0.05$. Data are mean \pm standard error of the mean.

Results

Measurement of PGC-1 α , PGC-1 β and PGC-1 α -1 β downstream targets involved in mitochondrial biogenesis and function

In ALS patients there was a significant 63, 60 and 45% decrease respectively in PGC-1 α , PGC-1 β and ERR α mRNA levels, when compared to control subjects (Fig. 1A). Of these genes, only PGC-1 α was lower in the ALS compared with the ND patients. While PGC-1 α and ERR α mRNA levels were slightly reduced in the ND patients they were not statistically different from the controls subjects. However, PGC-1 β mRNA was also significantly lower by 70% in the ND patients when compared with the control subjects. NRF-1 mRNA was 60 and 50% lower respectively, in the ALS and ND patients when compared with the control subjects. When compared with the control subjects, Mfn1 mRNA was 73 and 63% lower in the ALS and ND patients, while Mfn2 mRNA was 47% lower in the ALS patients. COXIV mRNA was 63% and 45% lower respectively, in the ALS and ND patients. No significant differences in NRF-1, Mfn1, Mfn2 and COX IV mRNA levels were observed between the ALS and ND patients. With respect to the protein levels of these targets, PGC-1 α protein levels were 42% lower in the ALS patients, when compared with the healthy control subjects (Fig. 1B). In the ND patients PGC-1 α protein levels were 28% lower, when compared with the healthy control subjects, but this tendency did not reach statistical significance ($p = 0.08$). Mfn1 protein levels were 30% lower in the ALS patients, however the 20% reduction observed in the ND patients failed to reach statistical significance ($p = 0.057$). No differences in Mfn2 or NRF-1 protein levels were observed between groups. COXIV protein levels were 60% lower in both the ALS and ND patients, when compared with the healthy control subjects. To control for protein loading all blots were normalized to GAPDH protein, which remained stable across the 3 groups. Additionally, there were no differences in any of the proteins measured when comparing the ALS and the ND patients.

To draw comparisons between patients with ALS and the rodent model of human ALS, similar observations were made in the gastrocnemius muscle of G93A ALS mice. There was an 83% and 75% decrease in Pgc-1 α mRNA levels in the gastrocnemius muscle of G93A ALS mice aged 90 (90 ALS mice) and 120 days (120 ALS mice) when compared with WT mice (Fig. 2A). Pgc-1 β mRNA followed a similar pattern to that of Pgc-1 α , showing a 60 and 46% decrease in 90 ALS mice and 120 ALS mice. This was associated with a 45 and 32% decrease in Err α and a 73 and 45% decrease in Nrf-1 mRNA. Mfn1 was 39 and 48% lower and Mfn2 57 and 50% lower in the 90 ALS mice and 120 ALS mice, when compared with the WT mice ($p < 0.03$) (Fig. 3). PGC-1 α protein levels were also reduced in the soleus, but not the EDL, muscle of the ALS mice (Fig. 2B).

Mitochondrial enzyme activities are reduced in ALS and ND patients

To establish if the reduction in PGC-1 α and PGC-1 β , as well as their downstream targets involved in mitochondrial biogenesis and function,

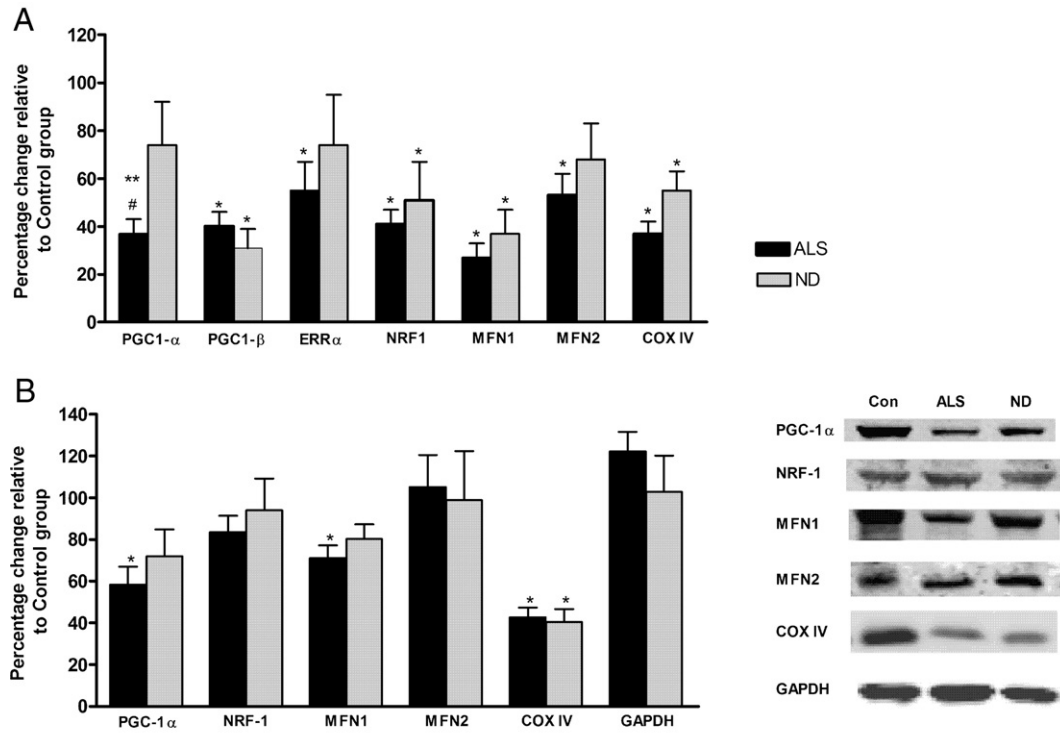


Fig. 1. Regulation of targets involved in mitochondrial biogenesis and function in skeletal muscle of ALS (amyotrophic lateral sclerosis) and ND (neurogenic disease) as percentage if the control subjects (Con). (A) PGC-1 α , PGC-1 β , ERR α , NRF-1, Mfn1, Mfn2 and COX IV mRNA levels (B) PGC-1 α , NRF-1, Mfn1, Mfn2 and COX IV protein levels. Representative densitometry images for all of the proteins measured are shown on the right of Fig. 1B. GAPDH was used as a normalizing protein. *, $p < 0.05$, **, $p < 0.01$ versus Control subjects; #, versus ND patients.

was associated with perturbations in mitochondrial enzyme activity we measured citrate synthase (CS) and cytochrome oxidase (COX) COX activities. When compared with the healthy control subjects, CS activity

was 43 and 49% lower in the ALS and ND patients ($p < 0.05$), while COX activity was 35 and 40% lower in the ALS and ND patients ($p < 0.05$) (Table 4).

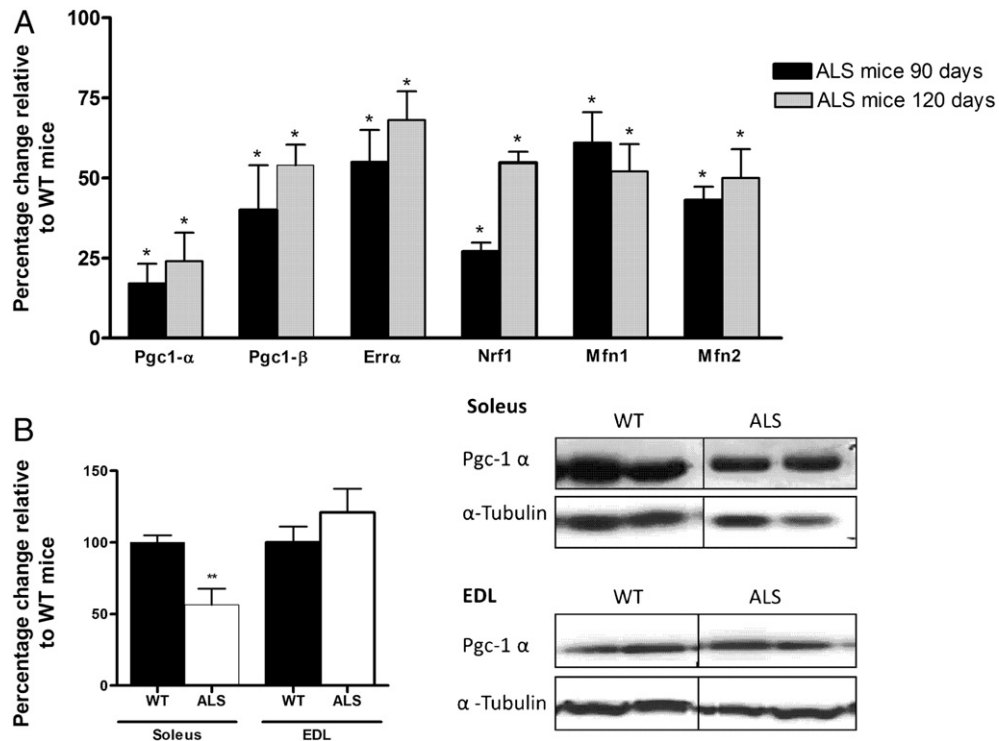


Fig. 2. Regulation of targets involved in mitochondrial biogenesis and function in skeletal muscle of wild type and G93A ALS mice. (A) mRNA levels of *Pgc-1 α* , *Pgc-1 β* , *Err α* , *Nrf-1*, *Mfn1* and *Mfn2*. (B) Protein levels of PGC-1 α in the soleus and EDL muscles of the ALS mice. Representative densitometry images for the proteins measured are shown on the right of Fig. 2B. α -Tubulin was used as a normalizing protein * $p < 0.05$ and **, $p < 0.01$ versus wild type mice.

PGC-1 α and PGC-1 β both regulate Mitofusin-1 (*Mfn1*) and *Mfn2* via ERR α

Mfn2 is a downstream PGC-1 α /ERR α (Cartoni et al., 2005) and PGC-1 β /ERR α (Liesa et al., 2008) target, but little is known about the regulation of *Mfn1*. The adenoviral over expression of PGC-1 α and PGC-1 β in C2C12 myotubes resulted in a 4.5 and 3.0-fold increase respectively in *Mfn1* mRNA and 2.9 and 4.5 increases, respectively in *Mfn2* mRNA. These observations were abolished when endogenous ERR α expression was suppressed by the use of an

adenovirus containing an siRNA sequence against ERR α (Fig. 3A). Expression of a constitutively active form of ERR α , VP16-ERR α also resulted in 7.8 and 6.0-fold increase in *Mfn1* and *Mfn2* mRNA respectively (Fig. 3B). ChIP experiments in mouse C2C12 myotubes showed that there was increased occupancy of PGC-1 α and PGC-1 β at an ERR response element (ERRE) located on the *Mfn1* and *Mfn2* promoters (mouse sequence) (Fig. 3C). Specificity of the PGC-1 α and PGC-1 β ChIP was confirmed by targeting the ERRE-containing region that lies proximal to the *Esrra* promoter (positive control) and a region that lacks ERREs, distal to the *Esrra* promoter (negative control)

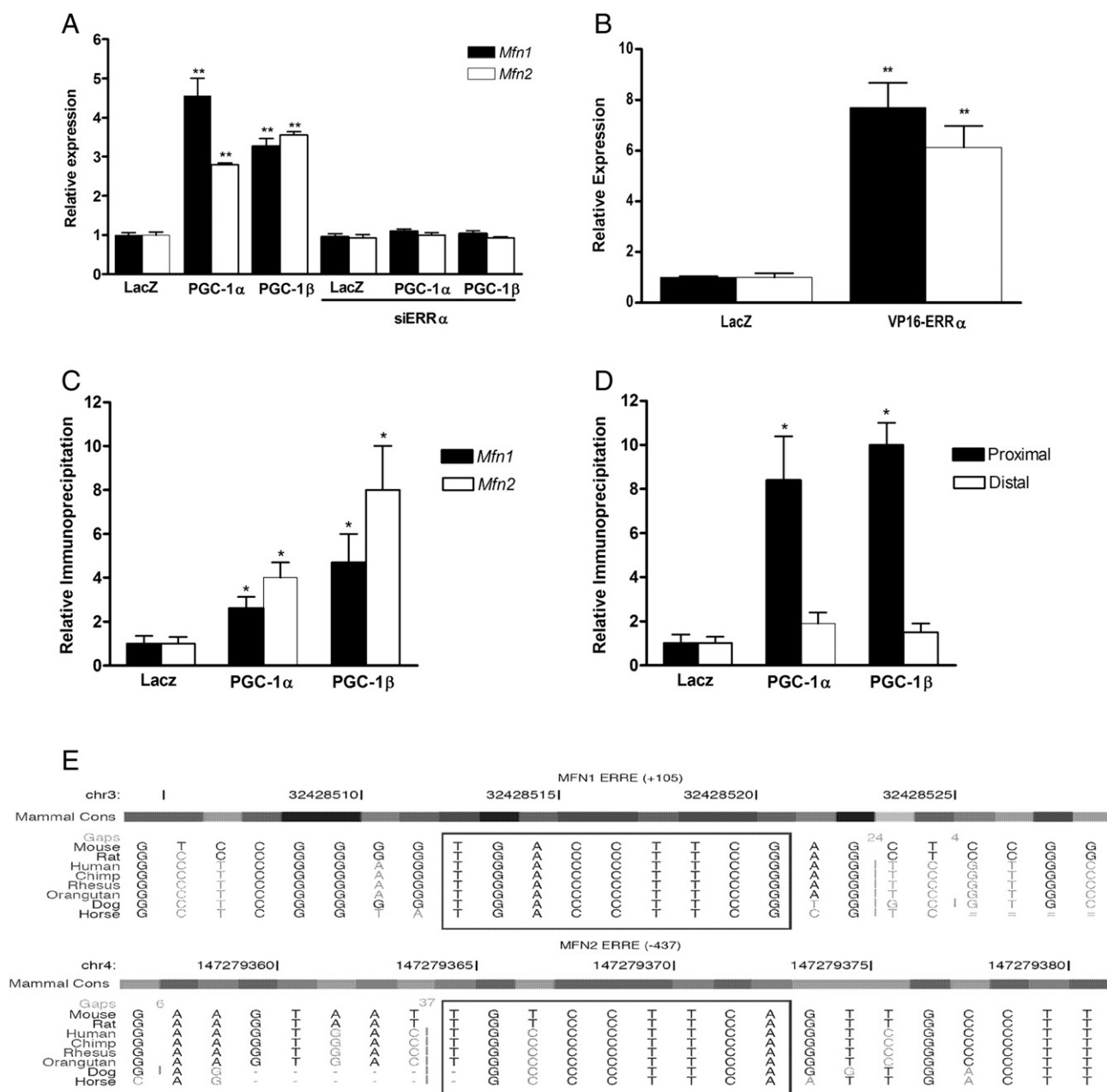


Fig. 3. PGC-1 α and PGC-1 β regulate both *Mfn1* and *Mfn2* in C2C12 myotubes in an ERR α dependent manner. (A) *Mfn1* and *Mfn2* mRNA levels 24 h after over expression of LacZ (control), PGC-1 α or PGC-1 β , in the absence or presence of siRNA against ERR α (siERR α). (B) *Mfn1* and *Mfn2* mRNA levels 24 h after over expression of LacZ (control) or the constitutively active VP16-ERR α . (C) Occupancy of PGC-1 α and PGC-1 β at the ERRE response element located on *Mfn1* and *Mfn2* promoter. (D) Occupancy of PGC-1 α and PGC-1 β at the estrogen-related receptor- α (*Esrra*) proximal (positive control) and distal (negative control) ERREs. (E) University of California, Santa Cruz (UCSC) genome browser screenshot depicting the presence of an ERRE for mouse *Mfn1* and *Mfn2* located respectively +105 and -437 bp from the transcriptional site. These ERREs are well conserved across species. * $p < 0.05$, ** $p < 0.01$, versus the LacZ group. Data are expressed relative to LacZ and are the mean \pm SD of triplicates. All experiments were performed 3 times with 4–6 samples per group.

Table 4
Mitochondrial enzyme activity.

| | Control | ALS | ND |
|-----|----------|-----------|-----------|
| CS | 245 ± 37 | 140 ± 21* | 125 ± 13* |
| COX | 935 ± 53 | 604 ± 73* | 569 ± 38* |

CS, citrate synthase; COX, cytochrome C oxidase; units for both CS and COX are in $\mu\text{mol}^{-1} \text{min}^{-1} \text{mg protein}^{-1}$; *, $p < 0.05$ versus control.

(primers described in (Villena et al., 2007)) (Fig. 3D). ERRE response elements, located proximal to the Mfn1 and Mfn2 transcriptional start sites, were observed to be well conserved across mouse and humans as highlighted in the box in Fig. 3E.

Measurement of miRNAs

Analyses of miRNAs in skeletal muscle demonstrated that miR-23a, -29b, -206 and -455 were significantly increased in ALS, when compared with the healthy control subjects by 91, 59, 89 and 91%, respectively. miR-31 was increased in both the ALS and ND patients by 101% and 79%, respectively (Fig. 4). Of the miRNAs measured, only miR-23a was significantly higher in the ALS compared to the ND patients.

Establishing a causal relation between miR-23a and PGC-1 α in vitro

As we observed a reduction in PGC-1 α mRNA and protein, as well as several PGC-1 α downstream targets, and an increase in several miRNAs in the ALS muscle samples, we were interested to investigate if a causal relationship existed between PGC-1 α and any of these miRNAs. Using TargetScan5.2 (<http://www.targetscan.org/>) 4 putative binding sites for miR-23a and 23b on the PGC-1 α 3' UTR were identified. The putative miR-23a binding sites in the PGC-1 α 3' UTR were conserved in human and mouse (Fig. 5A). Thus we decided to investigate whether a causal relationship existed between miR-23a and PGC-1 α . The 3' UTR of PGC-1 α mRNA was subcloned downstream of a luciferase reporter. Mutated miR-23a binding sites within the 3' UTR of PGC-1 α mRNA are underlined (Fig. 5A). We co-transfected a miR-23a expression vector (pCXGb-miR-23a) and the reporter vector (pLuc2-PGC-1 α 3' UTR) into HeLa cells, and measured luciferase activity 24 h later. Luciferase activity was markedly reduced when the miR-23a expression vector was co-transfected with pLuc2-PGC-1 α 3' UTR (Fig. 5B). In contrast, luciferase activity was markedly increased when the miR-23a/b expression was knocked down with anti-miR-23 2' OME ssRNA (Fig. 5C). Overexpression or knock-down of miR23a/b did not affect luciferase expression when the putative binding sites for miR23a/b were mutated (Figs. 5B/C).

miR-23a regulates PGC-1 α protein levels in vivo

To investigate whether miR-23a functions to suppress PGC-1 α in vivo, we used a transgenic mouse model with increased expression of

miR-23a. The wild type (WT) littermates and miR-23a transgenic mice did not differ in body weight (Fig. 6A) or muscle weight of slow-twitch soleus and fast-twitch plantaris muscles (Fig. 6B). The protein levels of PGC-1 α , cytochrome c and COX IV were down regulated in the transgenic animals (Tg) compared to the WT littermates (Fig. 6C).

Discussion

The present study aimed to determine the regulation of key transcriptional co-activators and transcription factors involved in mitochondrial biogenesis and miRNAs involved in muscle development and function, in skeletal muscle affected by ALS. Several novel observations were made. Firstly, in ALS patients there was a significant reduction in PGC-1 α mRNA and protein content, as well as in ERR α and Mfn2 mRNA. Secondly, both ALS and ND patients had reductions in PGC-1 β , NRF-1 and Mfn1 mRNA and COX IV protein. Skeletal muscle from transgenic ALS G93A mice had similar reductions in mRNA for *Pgc-1 α* , *Pgc-1 β* and *Pgc-1* downstream targets. In parallel with reduced levels of these key regulators of mitochondrial biogenesis and function, citrate synthase and COX enzyme activities were also lower in ALS and ND patients. Thirdly, miR-23a, -29b, -206 and -455 were increased in the ALS patients while miR-31 was increased in both ALS and ND patients. Only miR-23a was greater in ALS than ND patients. Finally, we demonstrated that miR-23a is a direct repressor of PGC-1 α protein translation and that transgenic mice expressing miR-23a have a reduction in PGC-1 α , cytochrome-b and COX IV protein levels.

Skeletal muscle mitochondrial dysfunction in ALS

In ALS patients NADH:CoQ oxidoreductase and cytochrome c oxidase deficiency and reduced mitochondrial DNA (Vielhaber et al., 2000; Wiedemann et al., 1998), suggest that skeletal muscle mitochondrial dysfunction may play key roles in the pathogenesis of ALS. In an attempt to understand the molecular mechanisms potentially influencing skeletal muscle mitochondrial dysfunction in ALS, we focused on the expression of PGC-1 α and PGC-1 β and several of their downstream targets. PGC-1 α and PGC-1 β maintain mitochondrial mass and function via activating transcription factors, including NRF-1, NRF-2 and ERR α that target the nuclear genome and Tfam, which controls the mitochondrial genome. These transcription factors regulate genes involved in mitochondrial biogenesis, fusion and electron transport chain activity. The downregulation of PGC-1 α , PGC-1 β and many of their targets in skeletal muscle of ALS and ND patients as well as ALS mice, suggest they play a role in the development of mitochondrial dysfunction observed in these groups. Overexpressing PGC-1 α in skeletal muscle protects against denervation-induced muscle atrophy (Sandri et al., 2006), a condition which also presents reduced mitochondrial function (Adhihetty et al., 2007) and improves the muscle phenotype of *mdx* mice (Handschin et al.,

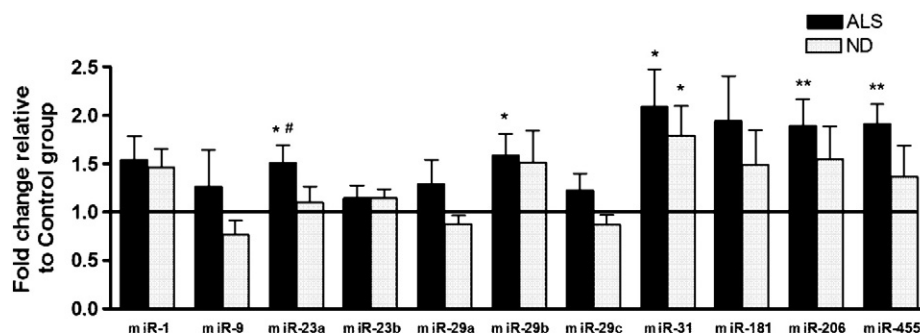


Fig. 4. miRNA analyses in skeletal muscle of Control subjects as well as ALS and ND patients. *, $p < 0.05$, versus healthy control group; #, $p < 0.05$ versus the ND patients.

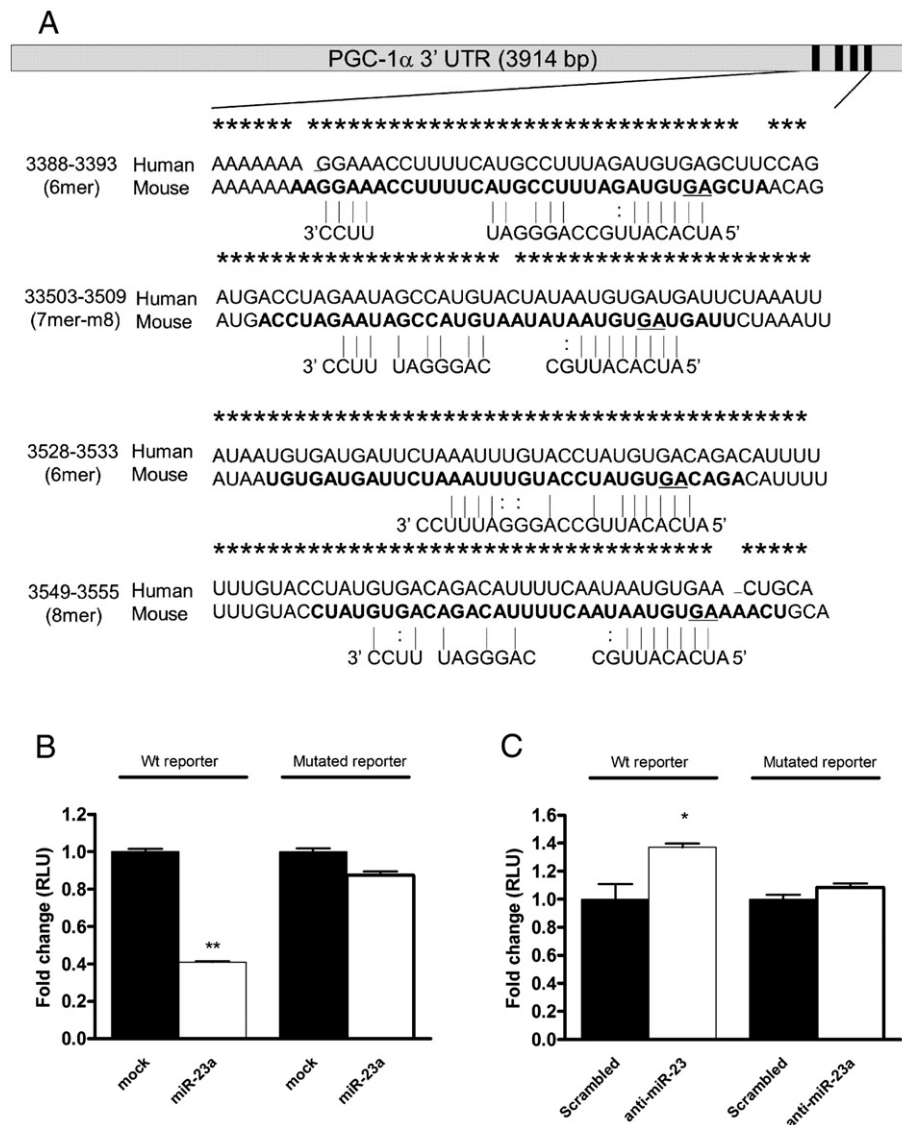


Fig. 5. miR-23a regulates PGC-1 α *in vitro*. (A) Scheme showing the 4 putative miR-23a/b binding sites on the PGC-1 α 3' UTR. The binding sites were identified using the online database, TargetScan (5.2) and show strong conservation across human and mouse. Sites mutated for the reporter experiments are underlined and were replaced with UU sites (underlined). The sequences in bold were cloned down-stream of the luciferase ORF (pLuc2- PGC-1). (B) pLuc2-PGC-1 α was transfected in HeLa cells with either pCXbG-miR-23a expression vector or mock vector. The luciferase activity was significantly inhibited by miR-23a expression. Mutation of the miR-23a/b binding sites blocked the decrease in luciferase activity. (C). pLuc2-PGC-1 α was transfected in HeLa cells with either anti-miR-23a 2'-OME ssRNA or scrambled 2'-OME ssRNA. The luciferase activity was significantly increased by knockdown of miR-23a/b expression. Mutation of the miR-23a/b binding sites blocked the increase in luciferase activity. Renilla luciferase count was used as a reference. Data are presented as mean \pm SEM ($n=6$). * $p<0.05$ vs control; ** $p<0.01$ vs Control.

2007; Selsby et al., 2012). Rescuing the levels of skeletal muscle PGC-1 α , PGC-1 β or their downstream targets that regulate mitochondrial function may delay the progression of ALS. Recently it was shown that transgenic overexpression of PGC-1 α in skeletal muscle of SOD1^{G37R} ALS mice maintains mitochondrial biogenesis and activity, increases endurance capacity, reduces atrophy and improves locomotion (Da Cruz et al., 2012). These improvements in muscle health did not extend survival, demonstrating that muscle is not a primary target of toxicity in SOD1^{G37R} ALS mice. However, these observations do support the notion that increasing PGC-1 α levels in skeletal muscle may improve quality of life.

Several reports, including the present study, demonstrate some level of mitochondrial dysfunction in skeletal muscle of ALS patients (Crugnola et al., 2010; Dupuis et al., 2003; Vielhaber et al., 2000; Wiedemann et al., 1998). Many of these dysfunctions appear not to be ALS specific. Indeed, we show that dysregulation of several targets involved in mitochondrial biogenesis and function were observed in both ALS and ND patients, when compared with control subjects.

This may not be surprising considering that the pathologies of ALS and ND share several commonalities such as muscle atrophy and re-innervation (Dubowitz, 1985). However, examining how changes in these mitochondrial biogenesis targets track with the pathogenesis or progression of ALS would be of clinical interest.

PGC-1 α /1 β regulate *Mfn1* and *Mfn2* via *ERR α* in muscle cells

PGC-1 α regulates *Mfn2*, but not *Mfn1*, in an *ERR α* -dependent manner in human SAO2 cells (Cartoni et al., 2005). Our observation of a downregulation in PGC-1 α , PGC-1 β , *ERR α* , *Mfn1* and *Mfn2*, in the skeletal muscle of ALS and ND patient groups and ALS mice, tempted speculation of a more intimate relationship between these molecules in muscle. In C2C12 myotubes, PGC-1 α and PGC-1 β both regulated *Mfn1* and *Mfn2* in an *ERR α* -dependent manner. *Mfn1* and *Mfn2*, located on the outer mitochondrial membrane, are involved in mitochondrial fusion (Koshiba et al., 2004), a process controlling mitochondrial morphology (Bleazard et al., 1999) and metabolism

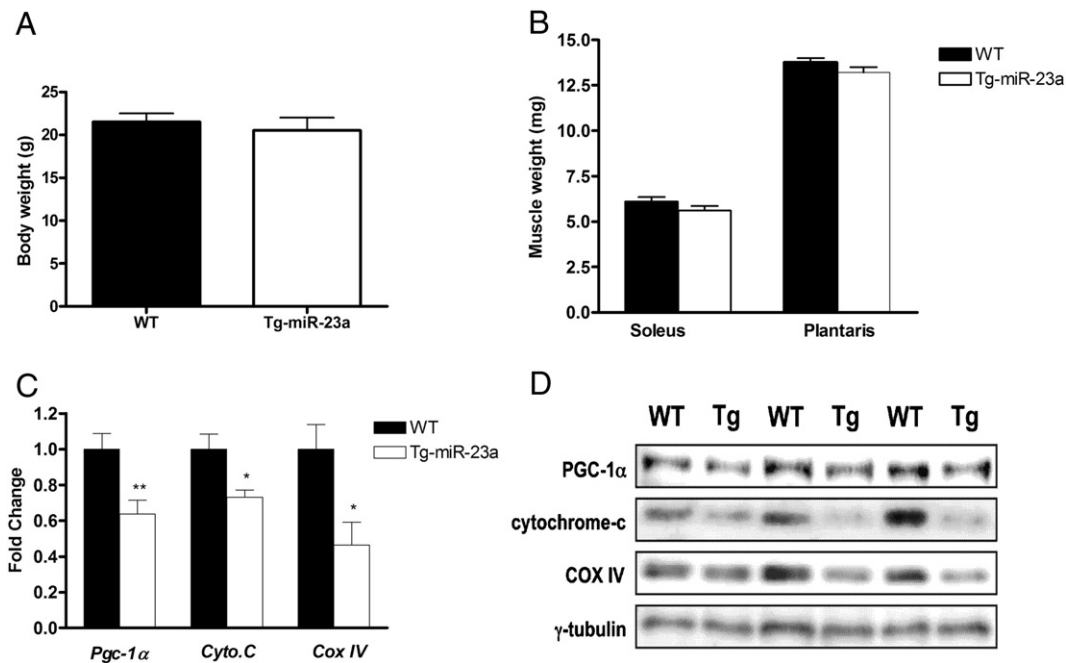


Fig. 6. miR-23a regulates PGC-1 α and markers of mitochondrial biogenesis *in vivo* in miR-23a transgenic mice. (A) Body weight and (B) muscle weight of soleus and plantaris in the miR-23a transgenic (Tg) mice and wild-type littermates. (C), densitometry analysis shows that PGC-1 α , cytochrome c and cytochrome c oxidase subunit IV (COX IV) were decreased in soleus muscle of the miR-23a transgenic mice. (D) Representative images of protein levels of PGC-1 α , cytochrome c and cytochrome c oxidase subunit IV (COX IV). γ -Tubulin was used to control for protein loading. Data are presented as mean \pm SEM ($n = 6-7$). * $p < 0.05$ versus wild type mice; ** $p < 0.01$ versus wild type mice.

(Bach et al., 2003). Additionally, Mfn2 tethers the endoplasmic reticulum to the mitochondria, thereby affecting mitochondrial calcium uptake (de Brito and Scorrano, 2008). While our cell-based observations do not show that the loss of PGC-1 α /PGC-1 β /ERR α in ALS or ND muscle is responsible for reductions in Mfn1 and Mfn2, they provide a rationale for such regulation. Clinically, reductions in human skeletal muscle Mfn2 is associated with impaired mitochondrial function in obesity and type 2 diabetes (Bach et al., 2003, 2005; Gastaldi et al., 2007). In mice the double deletion of skeletal muscle PGC-1 α and PGC-1 β results in a 60–70% reduction in Mfn1 and Mfn2 mRNA levels and mitochondrial dysfunction (Zechner et al., 2010). Deletion of both Mfn1 and Mfn2 in mouse skeletal muscle causes mitochondrial dysfunction, compensatory mitochondrial proliferation, mtDNA depletion, high levels of mtDNA mutations and muscle atrophy (Chen et al., 2010).

miRNA regulation in ALS patients

miRNAs-23a, -29b, -206 and -455 were increased in ALS while miR-31 was increased in both the ALS and ND patients. These observations are similar to those made in ALS G93A mice with increases in miR-206, -23a and -29b also observed (Williams et al., 2009). In ALS mice miR-206 upregulation is required for the regeneration of neuromuscular synapses (Williams et al., 2009). miR-206 is increased in patients with myotonic dystrophy type 1 (DM1) (Gambardella et al., 2010), Miyoshi myopathy and polymyositis and in Duchenne Muscular Dystrophy (DMD) (Eisenberg et al., 2007). The 2-fold upregulation of miR-455 in ALS patients is similar to that observed in patients with FSHD (facioscapulo-humeral muscular dystrophy), LGMD2A (limb girdle muscular dystrophy type 2A) and NM (nemaline myopathy) (Eisenberg et al., 2007). miR-455 is increased in C2C12 myotubes following treatment with the catabolic agent TWEAK (Panguluri et al., 2010); suggesting involvement in muscle wasting. The upregulation of miR-29b in the ALS patients is similar to that observed in ALS G93A mice (Williams et al., 2009) and patients with FSHD and LGMD2B (Eisenberg et al., 2007). In contrast, miR-29b is decreased in other myopathies including DMD, NM (Eisenberg et al., 2007; Greco et al., 2009) and DM1 (Perbellini et al.,

2011). Increased miR-29b levels during human myotube differentiation and in regenerating mouse muscle (Wang et al., 2008) suggest a role in muscle regeneration. miR-31 was upregulated in ALS and ND patients, as observed DMD patients and regenerating muscle (Greco et al., 2009). Loss-of-function/gain-of-function experiments in proliferating vascular smooth muscle cells confirmed a pro-proliferative role for miR-31 (Liu et al., 2011). The upregulation of miR-31 in ALS and ND may be an attempt to regenerate and delay skeletal muscle atrophy.

As seen in ALS mice (Williams et al., 2009) miR-23a expression was increased in ALS patients, when compared with both the healthy control and the ND patients. While the role of miR-23a in skeletal muscle is not well understood, we have shown it suppresses atrogen-1 and MuRF1 translation and protects against glucocorticoid-induced muscle wasting (Wada et al., 2011). As miR-23a binding sites were identified on the PGC-1 α 3-UTR we were interested in investigating their potential relationship. We extend the roles of miR-23a and show that miR-23a regulates PGC-1 α protein expression *in vitro* and in transgenic miR-23a mouse skeletal muscle. There was also a reduction in PGC-1 α targets involved in mitochondrial biogenesis and function including COX IV and cytochrome-c. These observations suggest that in skeletal muscle miR-23a inhibition of PGC-1 α has consequences for mitochondrial biogenesis and activity.

A reduction in skeletal muscle mitochondrial function in ALS may involve miR-23a inhibition of PGC-1 α and its downstream signalling. With this in mind, several important questions arise. Firstly, what causes the up-regulation of miR-23a in skeletal muscle of ALS patients? Secondly, does long-term miR-23a over expression in skeletal muscle contribute to muscle degeneration and the onset of neurodegeneration in ALS, as hypothesized recently (Dupuis and Echaniz-Laguna, 2010; Wong and Martin, 2010)? Exercise is a potent enhancer of PGC-1 α (Russell et al., 2005; Wallace et al., 2011) and mitochondrial biogenesis (Holloszy and Booth, 1976), while inactivity has the opposite effects (Oishi et al., 2008; Rimbart et al., 2004). Patients with early-stage ALS show reduced VO₂ peak, without muscle loss (Mezzani et al., 2012), suggesting that de-conditioning and impaired exercise capacity precedes muscle loss in ALS. While situations of reduced physical activity, such in ALS, show increased miR23a levels (Williams et al., 2009), we have

observed that moderate cycling exercise in 7 healthy males reduces miR-23a by 50% (unpublished observations) with a paralleled increase in PGC-1 α (Wallace et al., 2011). It is possible that the signaling program causing dysregulation of miRNA-23a/PGC-1 α in ALS is, in part, initiated or exacerbated by reduced skeletal muscle contractile activity. Fibre type changes can potentially contribute to observed differences in mRNA and protein levels in diseased muscle. Acknowledging this is important since PGC-1 α is known to be enriched in oxidative muscle (Russell et al., 2003). However, we have previously measured and compared muscle fibre type in the present healthy controls subjects and ALS patient cohort and did not find any differences in fibre type between groups (Leger et al., 2006). Therefore it appears that the observed differences obtained in the present study are not fibre type-dependent.

Conclusion

A reduction in mitochondrial function in ALS and patients with ND is associated with reductions in PGC-1 α and PGC-1 β and their downstream mediators of mitochondrial biogenesis and function. In ALS, there is an increase in several miRNA species potentially involved in muscle atrophy or attempted regeneration. Specifically, the increase in skeletal muscle miR-23a and associated decrease in PGC-1 α protein levels in ALS potentially has a causal effect on skeletal muscle mitochondrial dysfunction. Our observation that miR-23a directly targets and reduces skeletal muscle PGC-1 α protein levels, and impairs skeletal muscle mitochondrial function in mice, supports this notion and identifies a novel therapeutic target to potentially ameliorate skeletal muscle function and pathogenesis of ALS.

Acknowledgments

This work was funded in part by the Association Française contre le Myopathies (AFM; 11879), the Fonds National Suisse de la Recherche Scientifique (3200B0-105936) and grants-in-Aid for young investigators (A) (21680049 to TA) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Takeda Science Foundation (2009); APR was supported by a National Health and Medical Research Council Biomedical Career Development Award (479536); MBH, and AK were supported by a National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases grant (DK064951); SW is supported by the Japan Society for the Promotion of Science. We also wish to thank the Eurobiobank and Telethon (GUP 07001) for their support and provision of human tissue.

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