

# Myoglobin and the regulation of mitochondrial respiratory chain complex IV

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## Key points

- Mitochondrial respiration is regulated by multiple elaborate mechanisms.
- It has been shown that muscle specific O<sub>2</sub> binding protein, Myoglobin (Mb), is localized in mitochondria and interacts with respiratory chain complex IV, suggesting that Mb could be a factor that regulates mitochondrial respiration.
- Here, we demonstrate that muscle mitochondrial respiration is improved by Mb overexpression via up-regulation of complex IV activity in cultured myoblasts; in contrast, suppression of Mb expression induces a decrease in complex IV activity and mitochondrial respiration compared with the overexpression model.
- The present data are the first to show the biological significance of mitochondrial Mb as a potential modulator of mitochondrial respiratory capacity.

**Abstract** Mitochondria are important organelles for metabolism, and their respiratory capacity is a primary factor in the regulation of energy expenditure. Deficiencies of cytochrome *c* oxidase complex IV, which reduces O<sub>2</sub> in mitochondria, are linked to several diseases, such as mitochondrial myopathy. Moreover, mitochondrial respiration in skeletal muscle tissue tends to be susceptible to complex IV activity. Recently, we showed that the muscle-specific protein myoglobin (Mb) interacts with complex IV. The precise roles of mitochondrial Mb remain unclear. Here, we demonstrate that Mb facilitates mitochondrial respiratory capacity in skeletal muscles. Although mitochondrial DNA copy numbers were not altered in Mb-overexpressing myotubes, O<sub>2</sub> consumption was greater in these myotubes than that in mock cells (Mock *vs.* Mb-Flag::GFP: state 4, 1.00 ± 0.09 *vs.* 1.77 ± 0.34; state 3, 1.00 ± 0.29; Mock: 1.60 ± 0.53; complex 2-3-4: 1.00 ± 0.30 *vs.* 1.50 ± 0.44; complex IV: 1.00 ± 0.14 *vs.* 1.87 ± 0.27). This improvement in respiratory capacity could be because of the activation of enzymatic activity of respiratory complexes. Moreover, mitochondrial respiration was up-regulated in myoblasts transiently overexpressing Mb; complex IV activity was solely activated in Mb-overexpressing myoblasts, and complex IV activity was decreased in the myoblasts in which Mb expression was suppressed by Mb-siRNA transfection (Mb vector transfected *vs.* Mb vector, control siRNA transfected *vs.* Mb vector, Mb siRNA transfected: 0.15 *vs.* 0.15 *vs.* 0.06). Therefore, Mb enhances the enzymatic activity of complex IV to ameliorate mitochondrial respiratory capacity, and could play a pivotal role in skeletal muscle metabolism.

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**Abbreviations** COX, cytochrome *c* oxidase; COX IV, cytochrome *c* oxidase subunit IV; IP, immunoprecipitation; Mb, myoglobin; mtDNA, mitochondrial DNA; nucDNA, nuclear DNA;  $P_{O_2}$ , oxygen pressure; *SCO2* (gene), synthesis of cytochrome *c* oxidase 2 (gene); SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VDAC, voltage-dependent anion channel.

## Introduction

Mitochondria produce ATP from ADP by oxidative phosphorylation and serve as a vital power plant of the cell. The chemical reaction pathway involves the critical reduction of molecular oxygen by cytochrome *c* oxidase (COX) or complex IV. Studies have shown that a deficiency in COX can induce severe diseases, such as Leigh syndrome (Lombes *et al.* 1991; Adams *et al.* 1997; Zhu *et al.* 1998) and mitochondrial myopathy (DiMauro *et al.* 1980, 1983). In addition, a mutation in the *SCO2* (synthesis of cytochrome *c* oxidase 2) gene, which enables the assembly of complex IV, leads to fatal infantile cardioencephalomyopathy (Papadopoulou *et al.* 1999). Moreover, cardiac and skeletal muscle mitochondria have distinctive properties regulating respiration via complex IV (Rossignol *et al.* 1999).

COX has then a pivotal role in maintaining cellular homeostasis and health.

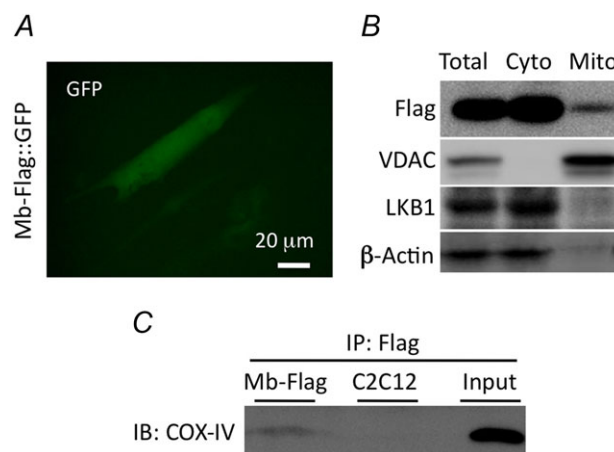
Even though many studies have investigated metabolism during exercise, oxidative metabolism in skeletal muscles still remains poorly understood. A key question focuses on the classic protein myoglobin (Mb), which has oxygen-binding capacity because of the prosthetic haeme (Gros *et al.* 2010). Mb releases its  $O_2$ , when cellular demand exceeds the delivery of the vascular  $O_2$  supply and the intracellular  $P_{O_2}$  drops to a critical level (Molé *et al.* 1999; Ponganis *et al.* 2008; Takakura *et al.* 2010). Although Mb may play important roles in  $O_2$  transport, Mb knockout mice remain viable, fertile and exhibit normal exercise capacity (Garry *et al.* 1998; Flogel *et al.* 2001). The evidence suggests that Mb may have other functions outside of  $O_2$  transport (Shih *et al.* 2014) or the cell has adapted to the missing Mb by eliciting physiological compensations, such as increased capillary density, abundant antioxidant protein expression, and enhanced mitochondrial gene expression (Grange *et al.* 2001; Meeson *et al.* 2001). Our previous studies show that Mb still has an  $O_2$  related function under a defined physiological condition. At the onset of muscle contraction, Mb releases  $O_2$  immediately (Chung *et al.* 2005; Takakura *et al.* 2010), to regulate mitochondrial  $O_2$  consumption in skeletal muscles. However, the mechanism still remains unclear.

Because previous studies have shown mitochondrial localization of Mb (Postnikova *et al.* 2009) and

interaction with COX IV, a subunit comprising respiratory complex IV (Yamada *et al.* 2013), a new hypothesis proposing interaction between mitochondrial protein and Mb (Mito-Mb) has emerged, which identifies the underlying mechanisms for a direct role for Mb in interacting with complex IV to modulate mitochondrial respiration. Therefore, we hypothesized that the direct Mb interaction in the mitochondria (mito-Mb) improves complex IV activity. The present study has investigated the effects of mito-Mb on mitochondrial respiration by manipulating the expression of Mb in cultured skeletal muscle cells. We found that mito-Mb accelerates mitochondrial respiration following the specific activation of complex IV, which indicates a mitochondria-specific regulatory role for Mb.

## Methods

The experiments in the present study provided two sets of data. One comprises the data for Mb overexpressing myotubes (results shown in Figs 1–3), and the other is



**Figure 1. Characterization of C2C12 myotubes with and without Mb-Flag**

A, GFP fluorescent image of differentiated C2C12 cells expressing both Mb-Flag and the GFP reporter shows a tube-like morphology. B, immunoblots of the Mb-Flag expressed cells probing for localization in the cytosol (LKB1 and  $\beta$ -actin) and in the mitochondria (VDAC). Mb-Flag was detected in all fractions, localized both in the cytosol and the mitochondria. C, immunoprecipitation with using Flag-antibody showed that COX IV was immunoprecipitated with Mb-Flag in stably Mb expressing myotubes.

the data for gene modified Mb: Mb overexpressing or Mb suppressing myoblasts (Figs 4–7). Myotubes were used to investigate the significance of the up-regulation of Mb during the differentiating period. Because endogenous Mb expression in myoblasts is completely quiescent (Kanatous & Mammen, 2010; Singh *et al.* 2014), it is possible to analyse the transient effect of Mb gene modification in myoblasts without the effects of endogenous myoglobin. Thus, myotubes and myoblasts were utilized in the present study.

### Cell culture

C2C12 cells, a cell culture line derived from mice myoblasts, were used for experiments. C2C12 myoblasts can differentiate to form myotubes and produce characteristics of muscle proteins. Cultured myoblasts were grown to confluence in DMEM containing 4.5 mg ml<sup>-1</sup> glucose, 10% fetal bovine serum (FBS), 100 IU ml<sup>-1</sup> penicillin, and 100 µg ml<sup>-1</sup> streptomycin. Confluent myoblast cultures were then exposed to DMEM differentiation medium containing 2% donor bovine serum (DBS) with iron, 100 IU ml<sup>-1</sup> penicillin, and 100 µg ml<sup>-1</sup> streptomycin;

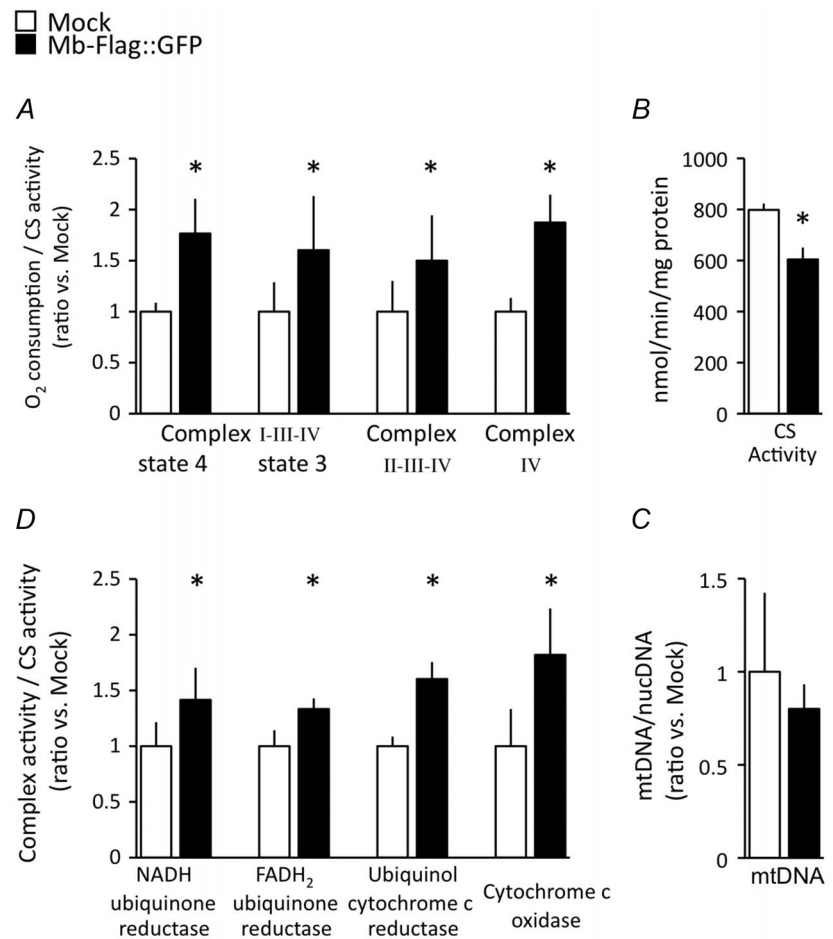
myotubes were formed over a week. Myoblasts and myotubes of cells were then grown at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>, and they were harvested for experiments.

### Plasmid and siRNA design

A pReceiver-M46 plasmid encoding Flag-tagged mouse Mb cDNA with internal ribosomal entry site (IRES)-GFP cDNA (EX-Mm03786-M46, GeneCopoeia, MD, USA) was purchased commercially, and siRNAs were designed to interfere with Mb expression using siDirect (<http://sidirect2.rnai.jp/>). Mb siRNA was selected from the mouse Mb open reading frame (ORF). The sequence of Mb siRNA originated from GATAAGTTTGACAAGTTCA, and was purchased from Nippon Gene (Toyama, Japan). A non-specific siRNA (control) was also purchased from Nippon Gene.

### Transfection and construction of stable cell lines

Plasmids and siRNAs were transfected into C2C12 myoblasts using an Amaxa Nucleofector system (Lonza, Basel, Switzerland) and Ingenio electroporation solution (Mirus,



**Figure 2. Mitochondrial respiratory capacity and volume in myotubes that stably express Mb**

A, mitochondrial O<sub>2</sub> consumption was greater in myotubes that stably express Mb than that in Mock cells in each of 3 electron transfer pathways; *N* = 8, \**P* < 0.05. B, citrate synthetase (CS) activity was suppressed in Mb-overexpressing cells as compared with Mock cells; *N* = 8, \**P* < 0.05. C, copy numbers of mtDNA were not affected by Mb overexpression; *N* = 6. D, the enzymatic activity of all respiratory complexes was improved in myotubes that stably express Mb, potentially indicating an underlying mechanism of enhanced mitochondrial respiration; *N* = 7 and 8, \**P* < 0.05, mean ± SD.

WI, USA) with the program B-032 for high viability, according to the manufacturer's recommendations. Plasmids and siRNA constructs were added at 10  $\mu\text{g}$  and 20 pmol, respectively, per transfection for all transfections. Transiently transfected myoblasts were harvested for experimentation 48 h after transfection.

To generate myotubes that stably express Mb-Flag,  $10 \times 10^6$  C2C12 myoblasts were transfected with 10  $\mu\text{g}$  of plasmid (pReceiver M46) using nucleofector, and they were then plated into 10  $\text{cm}^2$  dishes in culture media containing 1.5  $\text{mg ml}^{-1}$  neomycin (WAKO, Osaka, Japan). GFP positive neomycin-resistant clones were then isolated and analysed for Mb-Flag expression using Western blotting. Neomycine-resistant and Mb-Flag-expressing myoblasts were categorized as the cell line that stably expresses Mb. Neomycine-resistant but not Mb-Flag-expressing cell lines were categorized as 'Mock' and used as a stable cell line control. The GFP images of the myotubes have a resolution of  $1360 \times 1024$  pixels. A CKX41 microscope (Olympus, Japan) was used for observation, and a DP71 camera (Olympus) was used to take fluorescent images. The exposure time was optimized depending on the negative control.

### Cell fractionation

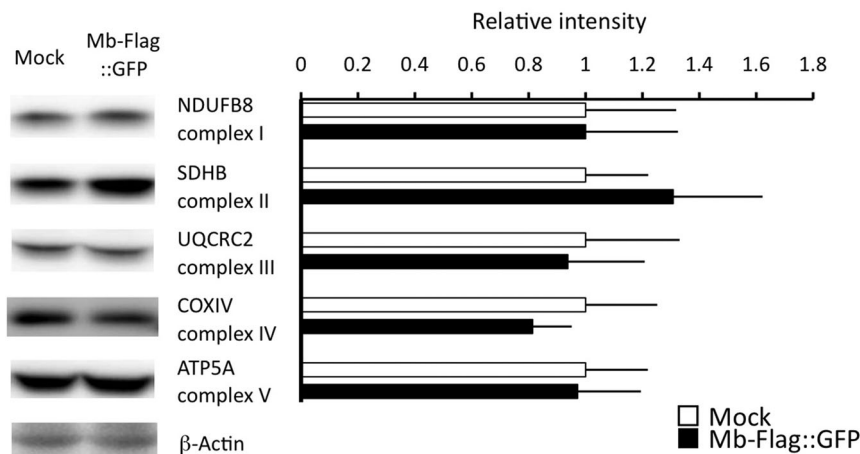
Total cell lysates were homogenized in RIPA buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 4  $\text{mg l}^{-1}$  leupeptin, and 1 mM pepstatin A, using a pestle homogenizer (Nippi, Tokyo, Japan). Homogenates were then successively passed through 27-gauge syringe needles 10 times each and centrifuged at 600 g for 10 min at 4°C to remove debris. Supernatants were then used as total cell lysates in immunoblotting analyses.

Harvested cells were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate,

1 mM DTT, 4  $\text{mg l}^{-1}$  leupeptin, 50  $\text{mg l}^{-1}$  trypsin inhibitor, 0.1 mM benzamidine, and 0.5 mM PMSE, using a pestle homogenizer to obtain crude fractionated samples. These homogenates were also processed using syringe needle mechanical lysis and centrifugation at 600 g for 10 min at 4°C to remove nuclei and debris. Aliquots of supernatants were used as total cell fractions in immunoblots, and the remaining supernatant was centrifuged at 10,000 g for 30 min at 4°C to precipitate crude mitochondrial fractions. The resulting supernatants were used as the cytosolic fraction in immunoblotting experiments, and mitochondrial pellets were washed initially by resuspension in lysis buffer. Suspensions were then centrifuged at 10,000 g for 30 min at 4°C, washed twice in lysis buffer, and resuspended in lysis buffer containing 1% Triton-X 100. Finally, supernatants containing mitochondrial protein fractions were obtained after centrifugation at 1100 g for 20 min at room temperature, and were used in immunoblotting experiments after standardization of protein contents.

### Immunoblotting

Western blot analysis was performed, as previously described (Yamada *et al.* 2013). Samples of equal protein contents were loaded and electrophoresed on SDS-PAGE gels, and proteins were then transferred to PVDF membranes. Subsequently, membranes were incubated in blocking buffer followed by incubation with anti-voltage-dependent anion channel 1 (VDAC-I; ab15895, Abcam, Cambridge, UK), anti- $\beta$ -actin (ab8226, Abcam), anti-LKB1 (ab58786, Abcam), anti-COX-IV (ab14744, Abcam), anti-Flag (no. 2368, Cell Signalling Technology, MA, USA), or anti-mitochondrial respiratory complex (ab110413, Abcam) antibodies at 4°C overnight. After exposure to secondary antibody, chemiluminescent signals were visualized using ECL prime reagents (GE



**Figure 3. Comparisons of mitochondrial respiratory complex expression in myotubes that stably express Mb**

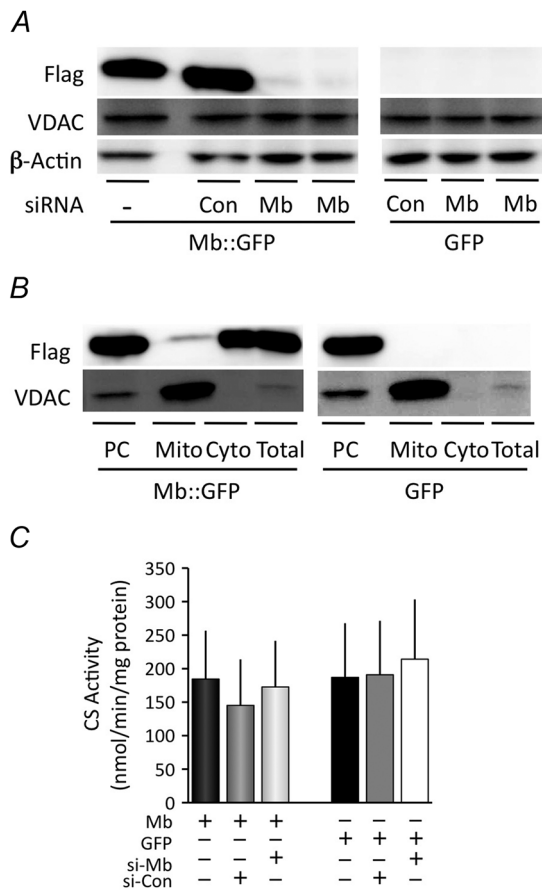
Protein quantities of mitochondrial respiratory complexes were determined in whole cell lysates from Mock or Mb-overexpressing myotubes using immunoblotting of NDUF8 (complex I), SDHB (complex II), UQCRC2 (complex III), COXIV (complex IV), and ATP5A (complex V; ATP synthase) proteins;  $\beta$ -actin expression was determined as a loading control. No significant differences in protein quantities were identified (*t* test,  $P < 0.05$ ,  $N = 5$ , mean  $\pm$  SD), indicating that the number of respiratory complexes was not altered by Mb overexpression.

Healthcare, NJ, USA). Signal intensities were quantified using Image J imaging software (NIH, MD, USA).

### Immunoprecipitation

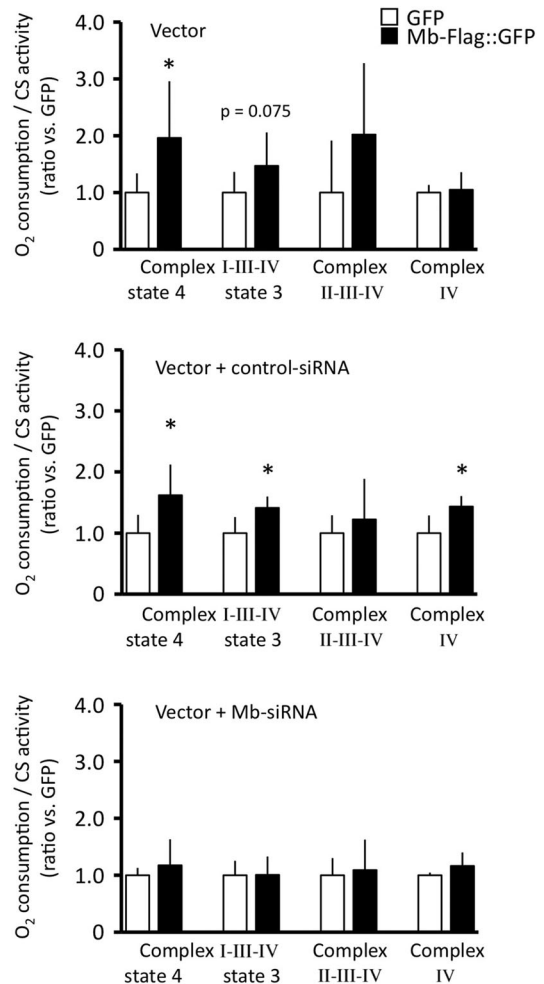
Mitochondrial fractions were obtained, as described above for immunoblotting. Proteins of the mitochondrial fraction were then divided into 1 ml aliquots of PBS, and Dynabeads (Invitrogen, CA, USA) were added and

incubated at 4°C for 1 h. Dynabeads were then collected using magnets, and normal mouse immunoglobulin G (nIgG; sc2343-B2111, Santa Cruz Biotechnology, CA, USA) was added to supernatants and incubated at 4°C overnight. Samples were then centrifuged at 7000 g for 30 s at 4°C, and aliquots of supernatant were reacted with complexes of Dynabeads and primary antibody against Flag at 4°C overnight. Beads and antibodies complexes



**Figure 4. Characterization of myoblasts transiently expressing Mb**

A, Mb-Flag expression was determined using immunoblotting. Transient modification of Mb gene expression in myoblasts was achieved by transfection with an Mb vector, a control siRNA, and an Mb siRNA. Mb-Flag expression was confirmed in Mb vector-transfected and Mb vector plus control siRNA-transfected myoblasts, whereas it was significantly decreased in Mb vector and Mb siRNA-transfected myoblast. VDAC and  $\beta$ -actin protein expression was determined as loading controls, and it was not affected by gene modifications even in GFP vector-transfected myoblasts. B, Mb-Flag was detected in all fractions, indicating mitochondrial localization of transiently expressed Mb. No Mb-Flag protein was detected in any fraction of GFP-transfected myoblasts. C, CS activity did not show any significant differences in each gene modified cell. These results indicated that transient gene modifications did not influence the capacity to produce respiratory substrates;  $N = 6$ , mean  $\pm$  SD.



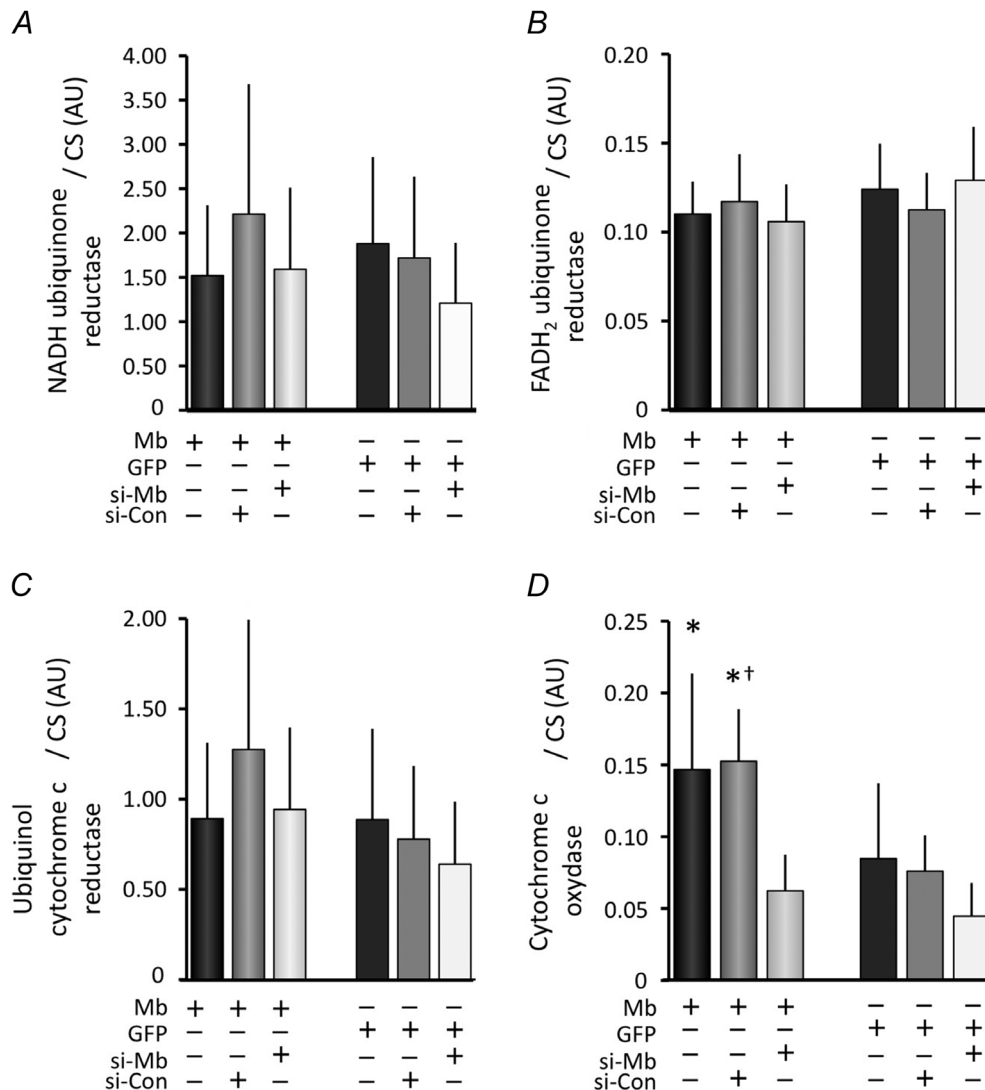
**Figure 5. Comparison of O<sub>2</sub> consumption in myoblasts transiently modified with Mb gene**

A, transient Mb expression after Mb vector transfection (Mb+, si-RNA-) improved O<sub>2</sub> consumption in myoblasts; State 4 respiration via the complexes I, III and IV was improved, and state 3 respiration showed a slight enhancement in Mb-overexpressing cells ( $P = 0.075$ ). B, Mb overexpression following transfection with Mb vector and control siRNA (Mb+, si-Con+) also improved O<sub>2</sub> consumption in myoblasts, and state 4, state 3 and complex IV-dependent respiration was significantly promoted. C, mitochondrial O<sub>2</sub> consumption in Mb suppressed model by Mb vector and Mb siRNA transfection (Mb+, si-Mb+) did not show a significant difference compared with O<sub>2</sub> consumption in myoblasts transfected with GFP vector and Mb siRNA (GFP+, si-Mb+). Consequently, mitochondrial respiration is regulated in an Mb-dependent manner;  $N = 5-8$ , mean  $\pm$  SD; \* $P < 0.05$  vs. GFP at the same respiratory state.

were prepared before adding samples. A 60  $\mu\text{l}$  aliquot of beads and 10  $\mu\text{g}$  of Flag antibodies were incubated in a tube to make bead-antibody complexes. Subsequently, pellets were collected using magnets and washed with PBS. Immunoprecipitates were eluted from Dynabeads by incubation in Flag peptide solution (500  $\text{mg ml}^{-1}$ ) at room temperature for 1 h, and immunoprecipitated (IP) supernatants were analysed using SDS-PAGE and immunoblotting. Anti-Flag and anti-COXIV antibodies were used for Mb-Flag immunoprecipitation and detection, respectively.

### Mitochondrial respiration

Measurements of mitochondrial respiration were performed, as described previously (Kuznetsov *et al.* 2008). Mitochondrial oxygen consumption was monitored using a Oxygraph-2k (Oroboros, Innsbruck, Austria) at 37°C in a 2 ml thermostatically controlled chamber equipped with polarographic oxygen sensors in respiration buffer containing 0.5 mM EGTA, 3 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 20 mM taurine, 10 mM  $\text{KH}_2\text{PO}_4$ , 20 mM Hepes, 1  $\text{g l}^{-1}$  BSA, 60 mM potassium-lactobionate,



**Figure 6. Comparisons of the enzymatic activity of respiratory chain complexes**

Transient Mb gene modification did not significantly affect enzymatic activities of the NADH-ubiquinone reductase, FADH<sub>2</sub>-ubiquinone reductase, ubiquinol-cytochrome c reductase (A–C). However, cytochrome c oxidase activity was significantly greater in Mb-overexpressing myoblasts (Mb+, si-Con+) than that in GFP-expressing myoblasts (GFP+, si-Con+). In addition, the complex IV activity in Mb-overexpressing myoblasts (Mb+, si-RNA–) and (Mb+, si-Con+) was significantly higher than that in Mb suppressed myoblasts (Mb+, si-Mb+). These data indicate that transient Mb overexpression could improve cytochrome c oxidase activity and the termination of Mb overexpression by siRNA could compromise the activation of cytochrome c oxidase capacity (D);  $N = 5$  and 6, \* $P < 0.05$  vs. (Mb+, si-Mb+), † $P < 0.05$  vs. (GFP+, si-Con+), mean  $\pm$  SD.

110 mM mannitol, and 0.3 mM dithiothreitol (pH was adjusted to 7.1 using 5 N KOH). This buffer was sterilized by filtration through a 0.45  $\mu$ m filter. Malate (5 mM) and glutamate (10 mM) were then added to induce state 4 respiration, which is dependent on the complexes I, III and IV. ADP (1 mM) was added to the chambers to induce state 3 respiration, and after inhibition with 0.5  $\mu$ M rotenone, 10 mM succinate was added to activate respiration via complexes II, III, and IV. Subsequently, antimycin A (5  $\mu$ M) was added to the chambers to stop respiration, and COX IV-dependent O<sub>2</sub> consumption was monitored after the addition of 0.5 mM TMPD and 2 mM ascorbate. TMPD was added after addition of ascorbate to the chamber to avoid oxidation, and chemical background fluxes in the chamber due to autoxidation of TMPD/ascorbate were determined before the experiment, in the absence of biological material, and used for corresponding correction.

### Mitochondrial enzyme activity

Enzymatic activities were assessed using a spectrophotometer on the basis of a previously described protocol (Spinazzi *et al.* 2012). Oxidation of NADH (10 mM) by complex I (NADH-ubiquinone reductase) was recorded using the ubiquinone analogue decylubiquinone (10 mM) as an electron acceptor. Oxidation of succinate (400 mM) by complex II (FADH<sub>2</sub>-ubiquinone reductase) was determined using decylubiquinone as an electron acceptor. Oxidation of decylubiquinol (10 mM) by complex III (ubiquinol-cytochrome *c* reductase) was determined using oxidized cytochrome *c* as an electron acceptor. Complex IV (cytochrome *c* oxidase) activity was determined using reduced cytochrome *c* as a substrate and O<sub>2</sub> (dissolved oxygen in cuvettes) as an acceptor

(Rossignol *et al.* 1999). Citrate synthetase (CS) activity was measured using a spectrophotometer, as previously described (Spinazzi *et al.* 2012). All enzymatic activities were measured at 37°C in final volumes of 1 ml, and they were expressed in nanomoles of the product formed per minute per milligram of mitochondrial protein, and normalized to CS activity.

### Mitochondrial DNA (mtDNA) copy number

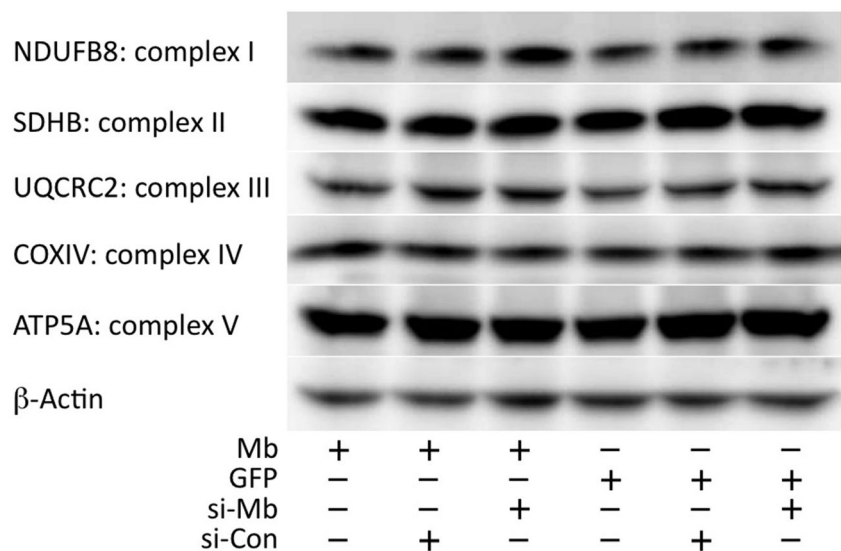
MtDNA copy numbers were determined using real-time PCR, as previously reported (Philp *et al.* 2011). Total DNA was isolated using DNeasy blood and tissue kits (Qiagen, Venlo, Netherlands), and real-time PCR was performed using Platinum SYBR Green qPCR Supermix-UDG (Takara Bio, Tokyo, Japan). Subsequently, RT-PCR was performed using specific primers for mitochondrial (cytochrome *b* Fwd, 5'-TTCGCAGTCATAGCCACAG-3'; Rev, 5'-AGATGAAGTGGAAAGCGAAG-3') or nuclear ( $\beta$ -globulin Fwd, 5'-TGCCATCCCATCACAACAAG-3'; Rev, 5'-GCCAATACACAGGTCACAGAG-3') encoded genes. Relative mtDNA contents were calculated according to the CT method with genomic DNA as an internal standard using the 7500 System SDS Software (Version 1.2.1.22, Applied Biosystems, CA, USA).

### Statistical analysis

All data are expressed as means  $\pm$  standard deviations (SD). Multiple comparisons of variables were performed using one-way ANOVA, and Turkey–Kramer *post hoc* tests were conducted if significant differences were identified by ANOVA. Student's *t* tests were used to identify pairwise differences between groups and differences were considered significant when  $P < 0.05$ .

**Figure 7. Comparisons of mitochondrial respiratory complex expression in transiently modified myoblasts with Mb gene**

NDUFB8 (complex I), SDHB (complex II), UQCRC2 (complex III), COXIV (complex IV), and ATP5A (complex V; ATP synthase) proteins were detected in whole cell lysates from genetically modified myoblasts, and  $\beta$ -actin expression was used as a loading control.



## Results

### Characterization of stable cell lines

Profiles of stable cell lines were assessed using imaging and biochemical analyses. Myotubes that stably expressed Mb had tube-like morphology and showed green fluorescence from the reporter GFP (Fig. 1A). Mb-Flag expression in myotubes was confirmed by immunoblotting using a Flag antibody, and localization in mitochondrial fractions was observed. LKB1,  $\beta$ -actin and VDAC protein expressions were used as cytosolic and mitochondrial markers, respectively (Fig. 1B), and COXIV was detected in immunoprecipitates of mitochondrial Mb-Flag-expressing cells, but not in C2C12 cells (Fig. 1C). Even though COXIV also interacts with endogenous Mb in Mb-Flag-expressing and 'Mock' myotubes, Mb-Flag does not express in Mock (data not shown). Therefore, the amount of Mb that interacts with COXIV should be increased in myotubes stably overexpressing Mb as compared with Mock. These data indicate that mitochondrial Mb-Flag interacts with COXIV in myotubes that stably express Mb.

### Mitochondrial respiratory capacity in stable cell lines

In subsequent experiments, we analysed the respiratory capacity of mitochondria in Mb-overexpressing myotubes to test the hypothesis that interactions of Mito-Mb with complex IV facilitate mitochondrial respiration. Mb overexpression enhanced mitochondrial respiratory capacity, but it did not influence the number of mitochondria in myotubes. Mitochondrial O<sub>2</sub> consumption relative to CS activity was elevated in Mb-overexpressing myotubes (Fig. 2A), and was mediated by electron transfer from glutamate and malate via complexes I, III and IV (means  $\pm$  SD of raw data for O<sub>2</sub> consumption/CS activity in myotubes are represented as follows: state 4 respiration of Mb-Flag::GFP,  $0.07 \pm 0.01$ , Mock,  $0.04 \pm 0.01$ ; state 3 respiration of Mb-Flag::GFP,  $0.26 \pm 0.09$ ; Mock,  $0.16 \pm 0.05$ ), from succinate via complexes II, III and IV (Mb-Flag::GFP,  $0.23 \pm 0.07$ ; Mock,  $0.15 \pm 0.05$ ), and from ascorbate and TMPD via complex IV-dependent respiration (Mb-Flag::GFP,  $0.60 \pm 0.09$ ; Mock,  $0.32 \pm 0.04$ ) (Fig. 2A).

Mb-overexpressing myotubes do not significantly increase the amount of mitochondrial enzyme but do increase the activities of respiratory enzymes (Fig. 2C and D). Specifically, the activities of NADH-ubiquinone reductase, FADH<sub>2</sub>-ubiquinone reductase, ubiquinol-cytochrome *c* reductase, and cytochrome *c* oxidase increase in Mb-overexpressing cells (Fig. 2D). However, CS activity decreases significantly. mtDNA copy numbers also do not change in Mb-overexpressing myotubes (means  $\pm$  SD of raw data for mtDNA copy number are represented as follows; Mb-Flag::GFP,

$(30.7 \times 10^3) \pm (5.1 \times 10^3)$ ; Mock,  $(38.4 \times 10^3) \pm (16.3 \times 10^3)$ ) (Fig. 2B and C).

These data indicate that Mb overexpression enhances mitochondrial respiratory capacity per mitochondria, and then decreases the substrate supply for mitochondrial respiration via the TCA cycle. This observation suggests that the efficiency of ADP to ATP conversion per energy substrate has improved in Mb-overexpressing myotubes. Moreover, the enhanced mitochondrial respiratory complex activities do not correlate with any increase in the quantity of respiratory chain complexes (Fig. 3), which remain stable in these experiments (means  $\pm$  SD of raw data for immunoblotting (protein of interest/actin) are represented as follows; NDUF8: Mb-Flag::GFP,  $0.52 \pm 0.17$ ; Mock,  $0.52 \pm 0.17$ ; SDHB: Mb-Flag::GFP,  $1.10 \pm 0.26$ ; Mock,  $0.84 \pm 0.19$ ; UQCRC2: Mb-Flag::GFP,  $0.57 \pm 0.16$ ; Mock,  $0.61 \pm 0.20$ ; COXIV: Mb-Flag::GFP,  $1.27 \pm 0.21$ ; Mock,  $1.56 \pm 0.39$ ; ATP5A: Mb-Flag::GFP,  $1.18 \pm 0.27$ ; Mock,  $1.22 \pm 0.27$ ). In summary, Mb overexpression appears to improve the efficiency of ATP production in skeletal muscle cells and suggests that Mb contributes to enhancing an effective mitochondrial respiratory capacity.

### Characterization of transient Mb expression in myoblasts

Experiments in myotubes stably overexpressing Mb can show adaptation effects during the proliferation and/or differentiation of muscle cells. Thus, to exclude these adaptive factors from the investigations of Mb molecular function in mitochondrial respiration, transient gene modifications were performed in C2C12 myoblasts.

Transient modification of Mb gene expression in myoblasts was achieved by transfection with an Mb-Flag vector, a control siRNA and an Mb siRNA. Mb-Flag expression was confirmed in genetically modified myoblasts using immunoblotting with anti-Flag antibody.

The left panel of Fig. 4A shows four lanes from an immunoblot for Flag, VDAC and  $\beta$ -actin in Mb-Flag transfected cells under the following conditions: (1) without any siRNA, (2) with control siRNA: si-Con, (3) with Mb siRNA; si-Mb. The right panel shows cells transfected with GFP vector without any siRNA, with si-Con and with si-Mb. The left panel shows that Mb siRNA transfection suppresses Mb-Flag expression. GFP with or without siRNAs transfected myoblasts showed no blot of Mb-Flag. VDAC and  $\beta$ -actin protein expression were used as loading controls. Gene modifications do not effect either VDAC or  $\beta$ -actin protein, even in GFP vector-transfected myoblasts. Transient expression of Mb-Flag was detected in mitochondrial fractions, indicating the mitochondrial localization of Mb in myoblasts (Fig. 4B). Endogenous Mb does not express in WT C2C12 at all (data not shown). Accordingly, it was possible for us to observe the exogenous



**Table 1. Amount of mitochondrial subunits in each genotype (myoblasts)**

Vector siRNA	Mb-Flag::GFP —	GFP —	Mb-Flag::GFP Control	GFP Control	Mb-Flag::GFP Mb	GFP Mb
Protein/actin						
NDUFB8	0.86 ± 0.31	0.94 ± 0.20	0.99 ± 0.42	0.92 ± 0.23	1.01 ± 0.43	1.10 ± 0.30
SDHB	1.30 ± 0.51	1.50 ± 0.26	1.38 ± 0.45	1.63 ± 0.46	1.32 ± 0.36	1.41 ± 0.57
UQCRC2	0.89 ± 0.15	1.01 ± 0.47	1.01 ± 0.21	1.13 ± 0.52	0.99 ± 0.26	0.98 ± 0.39
COXIV	1.04 ± 0.39	1.06 ± 0.30	1.12 ± 0.30	1.29 ± 0.42	0.90 ± 0.38	1.32 ± 0.22
ATP5A	1.36 ± 0.51	1.34 ± 0.33	1.38 ± 0.35	1.50 ± 0.47	1.40 ± 0.44	1.53 ± 0.43

Values are means ± SD ( $n = 5$  and  $6$ ); units are arbitrary units (AU); 6 genotypes – Mb-Flag::GFP, GFP, Mb-Flag::GFP + control-siRNA, GFP + control-siRNA, Mb-Flag::GFP + Mb-siRNA and GFP + Mb-siRNA – are assessed by immunoblotting. Amount of protein expression of mitochondrial respiratory complexes NDUFB8 (= complex I), SDHB (= complex II), UQCRC2 (= complex III), COXIV (= complex IV) and ATP5A (= complex V) was determined, and was not found to differ significantly (ANOVA). Therefore, the amount of protein expression was not altered in the respiratory complexes that were subjected to gene modification in this study.

Mb-Flag effect specifically with the gene-modified myoblasts that we constructed. The CS activity does not change significantly in myoblasts with a Mb vector transfection with and without si-Con and si-Mb (Fig. 4C).

### Mitochondrial respiratory capacity in myoblasts of each genotype

O<sub>2</sub> consumption was determined to assess mitochondrial respiratory capacity in myoblasts with transient Mb gene modifications. In these experiments, Mb overexpression increased state 4 mitochondrial O<sub>2</sub> consumption by a factor of 2. Mb vector-transfected myoblasts showed an accelerated state 3 O<sub>2</sub> consumption compared with that shown by GFP vector-transfected myoblasts ( $P = 0.075$ ) (mean ± SD of raw data for O<sub>2</sub> consumption/CS activity of myoblasts transfected with Mb-Flag::GFP or GFP are represented as follows; state 4 respiration of Mb-Flag::GFP, 0.27 ± 0.14; GFP, 0.14 ± 0.05; state 3 respiration of Mb-Flag::GFP, 0.48 ± 0.19; GFP, 0.33 ± 0.12; complex 2-3-4, Mb-Flag::GFP, 0.09 ± 0.06; GFP, 0.05 ± 0.04; complex 4, Mb-Flag::GFP, 0.92 ± 0.27; GFP, 0.88 ± 0.12).

Control siRNA-transfected myoblasts do not alter the effect of increased Mb expression. State 3, 4 still shows the similar Mb induced VO<sub>2</sub> enhancement. Complex IV-dependent O<sub>2</sub> consumption also increases (mean ± SD of raw data for O<sub>2</sub> consumption / CS activity of myoblasts transfected with Mb-Flag::GFP + control siRNA or GFP + control siRNA are represented as following; state 4 respiration of Mb-Flag::GFP: 0.31 ± 0.10, GFP: 0.19 ± 0.06, state 3 respiration of Mb-Flag::GFP: 0.44 ± 0.06, GFP: 0.31 ± 0.08, complex 2-3-4, Mb-Flag::GFP: 0.08 ± 0.04, GFP: 0.06 ± 0.02, complex 4, Mb-Flag::GFP: 0.79 ± 0.09, GFP: 0.55 ± 0.16).

However, Mb siRNA abolishes the Mb induced VO<sub>2</sub> enhancement (mean ± SD of raw data for O<sub>2</sub> consumption / CS activity of myoblasts transfected with Mb-Flag::GFP + Mb siRNA or GFP + Mb siRNA

are represented as following; state 4 respiration of Mb-Flag::GFP: 0.24 ± 0.09, GFP: 0.20 ± 0.03, state 3 respiration of Mb-Flag::GFP: 0.45 ± 0.14, GFP: 0.45 ± 0.11, complex 2-3-4, Mb-Flag::GFP: 0.11 ± 0.05, GFP: 0.10 ± 0.03, complex 4, Mb-Flag::GFP: 0.90 ± 0.18, GFP: 0.78 ± 0.04) (Fig. 5). These data indicate that mitochondrial respiratory capacity is regulated by Mb concentration, suggesting that Mb is a key modulator of skeletal muscle O<sub>2</sub> consumption.

### Enzymatic activity in myoblasts of each genotype

Because muscle mitochondrial respiration increased with transient Mb overexpression, we determined the activities of respiratory chain complexes in each cell type to test the hypothesis that respiratory enzyme activity was affected by these genetic modifications. Transient Mb overexpression does not affect key respiratory chain enzymes in complex I to III (NADH ubiquinone reductase, FADH<sub>2</sub> ubiquinone reductase, and Ubiquinol cytochrome *c* reductase) but markedly increased complex IV activity (Fig 6A-D). Moreover, Mb vector and control siRNA-transfected myoblasts had significantly higher complex IV activity than that of GFP vector and control siRNA-transfected cells. Myoblasts that were transfected only with the Mb vector also showed slight increases in complex IV activity compared with that in GFP vector-transfected myoblasts. Specifically, the simultaneous transfection of Mb vector and Mb-specific siRNA abolished the effect of Mb overexpression on complex IV activity, indicating the direct regulation of complex IV by Mb concentrations (Fig. 6D).

In summary, these data indicate that Mito-Mb modulates complex IV activity. Moreover, because transient gene modifications did not alter the protein quantities of any respiratory complexes in myoblasts (Fig. 7 and Table 1), the activation of complex IV can be attributed to the direct functional effects of Mb on complex IV. Since

Table 2. List of raw data of the present study

	Myotubes				Myoblasts				
	Mb-Flag::GFP	Mock	Vector si-RNA	Mb-Flag::GFP	GFP	Mb-Flag::GFP control	GFP control	Mb-Flag::GFP Mb	GFP Mb
	O <sub>2</sub> consumption/CS activity								
Complex 1-3-4	0.07 ± 0.01	0.04 ± 0.01	0.27 ± 0.14	0.14 ± 0.05	0.31 ± 0.10	0.19 ± 0.06	0.24 ± 0.09	0.20 ± 0.03	
State3	0.26 ± 0.09	0.16 ± 0.05	0.48 ± 0.19	0.33 ± 0.12	0.44 ± 0.06	0.31 ± 0.08	0.45 ± 0.14	0.45 ± 0.11	
Complex 2-3-4	0.23 ± 0.07	0.15 ± 0.05	0.09 ± 0.06	0.05 ± 0.04	0.08 ± 0.04	0.06 ± 0.02	0.11 ± 0.05	0.10 ± 0.03	
Complex 4	0.60 ± 0.09	0.32 ± 0.04	0.92 ± 0.27	0.88 ± 0.12	0.79 ± 0.09	0.55 ± 0.16	0.90 ± 0.18	0.78 ± 0.04	
mtDNA/nucDNA	30.7*10 <sup>3</sup>	38.4*10 <sup>3</sup>	—	—	—	—	—	—	—
	± 5.1*10 <sup>3</sup>	± 16.3*10 <sup>3</sup>							
	Protein/actin								
NDUFB8	0.52 ± 0.17	0.52 ± 0.17	—	—	—	—	—	—	—
SDHB	1.10 ± 0.26	0.84 ± 0.19	—	—	—	—	—	—	—
UQCRC2	0.57 ± 0.16	0.61 ± 0.20	—	—	—	—	—	—	—
COXIV	1.27 ± 0.21	1.56 ± 0.39	—	—	—	—	—	—	—
ATP5A	1.18 ± 0.27	1.22 ± 0.27	—	—	—	—	—	—	—

our data that stated in graphs were relative value, raw data for each experiment were summarized in Table 2.

## Discussion

In this study, we showed that mitochondrial respiration is regulated by the muscle-specific protein Mb in skeletal muscle cells. Specifically, Mb overexpression in myotubes led to enhanced respiratory capacities of muscle mitochondria. Moreover, Mb-overexpressing myotubes consumed O<sub>2</sub> faster, and they had increased respiratory complex activities (complexes I to IV). However, CS activity decreases in these myotubes, indicating that mitochondria in Mb-overexpressing myotubes produce less substrate for respiration (NADH and FADH<sub>2</sub>). However, the increased O<sub>2</sub> consumption relative to CS activity suggests an enhanced respiratory capacity, consistent with more H<sup>+</sup> was pumped into the inter membrane space and a more efficient ATP production. These data support the interpretation that mitochondrial Mb enhances mitochondrial respiratory capacity during the differentiation of skeletal muscle cells.

In a previous study, the improved mitochondrial respiratory capacity in differentiating-C2C12 cells led to a shift in energy dependence from anaerobic glycolysis to mitochondrial ATP production (Leary *et al.* 1998). The observed increase in the expression of nuclear encoded mitochondrial genes correlates with Mb enhancement both in mRNA and protein levels (Kim *et al.* 1995; Garry *et al.* 1996; Yan *et al.* 2001). Moreover, the onset of Mb expression also correlates with the start of differentiation (Kanatous & Mammen, 2010; Singh *et al.* 2014), suggesting that Mb is required to improve mitochondrial respiration during the differentiation of muscle cells. (In the present study, however, we confirmed Mb-Flag expression in myoblasts of stable cell lines (data not shown). Thus, our stable cell line model have distinct expression pattern of Mb from WT C2C12. For this reason, several adaptations were observed in this model, which is discussed below.

Traditionally, Mb has been considered an O<sub>2</sub> binding protein that is localized in the cytosol and releases O<sub>2</sub> under hypoxic conditions. On the other hand, the present data indicate that Mb is localized in the mitochondria where it interacts directly with complex IV and plays a distinct role in the regulation of mitochondrial O<sub>2</sub> consumption. Our previous study also have unveiled that Mb binds to COXIV and not other mitochondrial components e.g. cytochrome *c*, in rodent skeletal muscle tissue (Yamada *et al.* 2013), suggesting that the role of Mb in skeletal muscle mitochondria would interact specifically with complex IV. In transiently Mb gene modified myoblasts, Mb activation of the complex IV activity correlates that is due to the functional acceleration

of complex IV. Mb has been shown to participate in electron transfers with other haeme-containing proteins (Yamazaki *et al.* 1964; Wu *et al.* 1972; Livingston *et al.* 1985).

It is worth noting that myotubes and myoblasts resulted in different consequences of respiratory enzymatic activities. Whereas complex IV activity was improved distinctively in myoblasts that transiently expressed Mb, enzymatic activity of 4 respiratory complexes were upregulated and CS activity was compromised in differentiated stable cell lines. These differences reflect the effects of long-term incubation for differentiation and the times of Mb expression. Previous study revealed that Sirt1 down-regulation in C2C12 myotubes induced decrease of PGC1 alpha gene expression, and meanwhile CS activity also decreased in the myotubes (Gerhart-Hines *et al.* 2007). On the contrary, muscle specific PGC1- $\alpha$  transgenic mice revealed up-regulation of CS activity (Calvo *et al.* 2008). These evidences suggest that regulation of CS activity might be dependent upon PGC1- $\alpha$  expression. Decrease of CS activity in Mb-overexpressing myotubes might be caused by down-regulation of PGC1- $\alpha$ . However, the number of mitochondrial respiratory subunits was not altered in any gene modified models (Figs 3 and 7), which means mitochondrial biogenesis was not affected by Mb gene modification. Given our present data of transient Mb expression model, mitochondria could consume O<sub>2</sub> faster than WT in myoblasts of stable cell lines, which may have induced excessive consumption of energy substrates (lipids and carbohydrates). This accelerated energy expenditure would have caused the situation that was close to starvation, which can induce macroautophagy (He & Klionsky, 2009; Mizushima & Komatsu, 2011). Accordingly, damaged mitochondria may have been culled, and highly functional mitochondria that showed accelerated complex activity (complex I-IV) would have been selected in myotubes during differentiating periods. In addition, increase of mitophagy flux induces loss of CS activity (Gegg *et al.* 2010; Su *et al.* 2015). That might be the reason why stable cell lines have revealed improved complex activities and decreased CS activity. It would be intriguing to investigate whether Mb overexpression induces mitophagy.

We conclude that Mb that is highly abundant in myocytes specifically regulated the activity of complex IV in skeletal muscles. These data indicate that Mb is a central modulator of skeletal muscle mitochondrial respiration and skeletal muscle oxidative capacity.

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## Additional information

### Competing interests

None of the authors has any conflict of interest.

### Author contribution

T.Y., K.M. and T.J. contributed to the conception and design of this experiment. T.Y., H.T., T.H., Y.K., N.I. and K.M. contributed to the data collection and analysis. T.Y., H.T., Y.F. and K.M. contributed to the data interpretation. T.Y., H.T., T.J., T.K., R.I., Y.F., Y.K., N.I. and K.M. contributed to the drafting and critical revision of the article. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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