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# Exploring the role of PGC-1 $\alpha$ in defining nuclear organisation in skeletal muscle fibres

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## **ABSTRACT**

Muscle fibres are multinucleated cells, with each nucleus controlling the protein synthesis in a finite volume of cytoplasm termed the myonuclear domain (MND). What determines MND size remains unclear. In the present study, we aimed to test the hypothesis that the level of expression of the transcriptional coactivator PGC-1 $\alpha$  and subsequent activation of the mitochondrial biogenesis are major contributors. Hence, we used two transgenic mouse models with varying expression of PGC-1 $\alpha$  in skeletal muscles. We isolated myofibres from the fast twitch extensor digitorum longus (EDL) and slow twitch diaphragm muscles. We then membrane-permeabilised them and analysed the 3D spatial arrangements of myonuclei. In EDL muscles, when PGC-1 $\alpha$  is over-expressed, MND volume decreases; whereas, when PGC-1 $\alpha$  is lacking no change occurs. In the diaphragm no clear difference was noted. This indicates that PGC-1 $\alpha$  and the related mitochondrial biogenesis programme are determinants of MND size. PGC-1 $\alpha$  may facilitate the addition of new myonuclei in order to reach MND volumes that can support an increased mitochondrial density.

## INTRODUCTION

Skeletal muscle is a complex but highly ordered structure composed of myofibres that can be many centimetres long and a hundred micrometres wide. Such long and large myofibres cannot be supported by only one myonucleus (Edgerton and Roy, 1991), and therefore individual myofibres can encompass hundreds of myonuclei, with each myonucleus controlling the gene products in a finite volume of cytoplasm termed the myonuclear domain (MND) (Hall and Ralston, 1989; Ralston and Hall, 1992). MND sizes are constant during growth or senescence (Gundersen and Bruusgaard, 2008). However, average MND volumes tend to vary between myofibres expressing distinct myosin heavy chain isoforms (Bruusgaard et al., 2003; Bruusgaard et al., 2006). MNDs are smaller in slow, oxidative type I myofibres than in fast, glycolytic type II muscle cells. Despite this clear difference, it remains unclear whether this phenomenon is directly related to the myosin heavy chain isoform composition or to other closely related parameters such as oxidative capacity and mitochondrial content (Tseng et al., 1994). As type I myofibres contain a much higher concentration of mitochondria than type II muscle cells, and as all myonuclei produce mRNA at a similar rate, it has been suggested that smaller MNDs might be a direct consequence of an increased demand for bioenergetic/mitochondrial proteins per se (Moyes and LeMoine, 2005) rather than different myosin heavy chain expression. In the present study, we aimed to experimentally confirm this theory.

Mitochondrial biogenesis and production are controlled by the transcriptional coactivator, peroxisome-proliferator-activated receptor- $\gamma$  coactivator 1- $\alpha$  (PGC-1 $\alpha$ ) (Arany et al., 2005; Lin et al., 2005). Indeed, skeletal muscle-specific PGC-1 $\alpha$  knockout mice (MKO) exhibit decreased number and function of mitochondria (Perez-Schindler et al., 2013) concomitant with a shift from oxidative toward glycolytic myofibres (Handschin et al., 2007), but without any clear shift of the myosin heavy chain isoform composition at the protein level. On the other hand, muscle-specific PGC-1 $\alpha$  over-expressing mice (MCK) have stimulated activation of mitochondrial genes, increased mitochondrial density and oxidative capacity, and again, no major transition in myosin heavy chain expression at the protein level (Perez-Schindler et al., 2013). Hence, in the present study, we tested the hypothesis that PGC-1 $\alpha$  expression is a key regulator of myonuclear organisation. We suggested that MND size would be increased in MKO mice and decreased in MCK mice. We also hypothesised that the response to either modulation might vary between muscle types; therefore we studied myofibres from the

predominately fast-twitch, glycolytic extensor digitorum longus (EDL) muscle, and the diaphragm, which has a high proportion of slow-twitch, oxidative myofibres.

## **MATERIALS AND METHODS**

### **Animals**

PGC-1 $\alpha$  muscle-specific over-expressing mice (MCK) and PGC-1 $\alpha$  muscle-specific knockout mice (MKO) were generated as previously described (Perez-Schindler et al., 2013). Seven-month old MCK, MKO and wild-type (WT) mature adult mice were sacrificed by CO<sub>2</sub> inhalation followed by cervical dislocation (four animals per genotype). EDL and diaphragm (DIA) muscles were then dissected. We focused our attention on these two specific muscles known to be glycolytic (EDL) or oxidative and rich in mitochondria (DIA) (Schiaffino and Reggiani, 2011). The Animal Experimentation Ethics Committee of The University of Basel approved all animal procedures.

### **Relaxing Solution**

Relaxing solution contained 4 mM Mg-ATP, 1 mM free Mg<sup>2+</sup>, 20 mM imidazole, 7 mM EGTA, 14.5 mM creatine phosphate, and KCl to adjust the ionic strength to 180 mM and pH to 7.0. The concentration of free Ca<sup>2+</sup> was 10<sup>-9.00</sup> M.

### **Myofibre Permeabilisation**

Muscle samples were placed in relaxing solution at 4°C. Bundles of approximately 50 myofibres were dissected free and then tied with surgical silk to glass capillary tubes at slightly stretched lengths. They were then treated with skinning solution (relaxing solution containing glycerol; 50:50 v/v) for 24 hours at 4°C, after which they were transferred to -20°C (Frontera and Larsson, 1997).

### **Myonuclear organisation of single myofibres**

On the day of experiment (within two weeks after the permeabilisation procedure), bundles were detached from the capillary tubes, transferred to a relaxing solution, and single myofibres were dissected. Arrays of approximately nine myofibres were prepared at room temperature (RT). For each myofibre, both ends were clamped to half-split copper meshes designed for electron microscopy (SPI G100 2010C-XA, width, 3 mm), which had been glued to cover slips (Menzel-Gläser, 22 x 50 mm, thickness 0.13-0.16 mm). Myofibres were mounted at a fixed sarcomere length of  $\approx$ 2.20  $\mu$ m. This was a prerequisite for exact determination of myonuclear spatial organisation as it allowed accurate comparisons between myofibres (Cristea et al., 2010; Qaisar et al., 2012).

At RT, arrays were subsequently subjected to actin staining (1:100 Alexa Fluor Phalloidin 488, Molecular Probes, A12379) and myonuclear staining (1:1000 DAPI, Molecular Probes, D3571). Images were taken using a confocal microscope (Zeiss Axiovert 200, objective x20) attached to a CARVII imager (BD Biosciences) and Coolsnap HQ camera (Photometrics). To visualise myofibres in 3D, stacks of 100 images were acquired (1  $\mu\text{m}$  z increments) and analysed with a custom-made Matlab programme. Further staining was achieved using an antibody to satellite cell marker Pax7 (primary antibody: DSHB, AB 528428; secondary antibody: Alexa Fluor® 488, Novex, A-11001); this stain excluded the presence of satellite cells in skinned fibres, which might confound counts of myonuclei.

### **Statistical analysis**

Data are presented as mean  $\pm$  standard error of the mean (SEM). A total of 252 myofibres were isolated and tested. Because many myofibres were studied for each muscle, animal and genotype, a specific model was used to statistically analyse the data (Ochala et al., 2011). This model is based on an analysis of variance (ANOVA) including the following factors: “genotype”, “muscle” and “animal” (where “animal” was nested within “genotype”). The only interaction terms that were judged to be of importance and therefore included were that between “genotype” and “muscle”. JMP software (SAS Institute) was used for the generation of this model.

## RESULTS

### **Increase in myonuclei number and decrease in MND size when PGC-1 $\alpha$ is over-expressed in EDL but not in diaphragm**

We counted the number of myonuclei per fibre length and observed that, in the EDL muscle, the overall number of nuclei per mm fibre length was significantly greater in MCK and MKO than in WT (Table 1). On the contrary, in the diaphragm muscle, no significant difference was detected (Table 1).

As previously observed (Qaisar et al., 2012), a linear correlation existed between the volume of MNDs and myofibre cross-sectional area (CSA) in all muscles and genotypes (Figure 1). Thus, instead of maintaining a constant MND volume, larger fibres are known to possess larger MNDs, suggesting that nuclei can be pushed to a certain capacity (physiological “ceiling”) before new nuclei need to be incorporated. To account for this relationship, (1) the MND to myofibre CSA ratio was calculated (Table 1), and (2) a scatter plot of MND vs CSA was created (Figure 1). Thus, in the EDL muscle, at any given CSA, MND was significantly smaller in MCK than in WT. In the diaphragm muscle, we did not observe any significant difference.

The average MND provides valuable information on the average volume controlled by each myonucleus; however, it does not estimate the spatial arrangement of myonuclei. To precisely define the latter, we calculated nearest neighbour distances (NNs) using the 3D coordinates of individual myonuclei within each single myofibre. At any given CSA, in the EDL muscle, NN was significantly smaller in MCK than in WT, in agreement with the smaller observed MND (Table 1). In the diaphragm muscle, we did not observe any difference (Table 1). To evaluate the variability in the spatial arrangement of myonuclei, we used the standard deviation related to NNs within each single myofibre (SD-NN) (Qaisar et al., 2012). In the present study, SD-NN was not affected by the various genotypes (Table 1), which suggests that the regularity of spacing was not affected when PGC-1 $\alpha$  levels were modulated.



Overall, this data demonstrates that in the EDL, overexpression of PGC-1 $\alpha$  (MCK mice) results in more myonuclei per volume, and smaller internuclear distances. In the diaphragm, however, modulation of PGC-1 $\alpha$  resulted in few, if any differences.

### **Change in myonuclear shape when PGC-1 $\alpha$ is over-expressed or absent in both EDL and diaphragm**

We measured a number of morphological parameters of individual nuclei (Figure 1). In the EDL muscle, the aspect ratio was significantly smaller in MCK than in WT, indicating that nuclei were rounder (Table 2). In the diaphragm muscle, on the other hand, the aspect ratio and area were significantly greater in MKO than in WT suggesting that nuclei were bigger and longer (Table 2). Overall our results show that PGC-1 $\alpha$  affects nuclear shape as well as organisation throughout the length of muscle fibres.

## **DISCUSSION**

In the present study, we aimed to investigate whether PGC-1 $\alpha$  directly regulates MND volume in myofibres. To achieve this, we studied two transgenic mouse models with varying skeletal muscle expression of the transcriptional coactivator PGC-1 $\alpha$  known to be a key mediator of mitochondrial biogenesis and number (Handschin et al., 2007; Perez-Schindler et al., 2013). Our results demonstrate that a relation between these two entities exists but is more complex than initially hypothesised.

### **PGC-1 $\alpha$ regulates MND size in EDL muscle**

The most common method used to count the number of myonuclei and estimate the MND size is muscle cross-sections. This method is accompanied by a number of technical limitations which question the reliability of 2D data (Qaisar and Larsson, 2014). Here, we applied a 3D approach to precisely define the spatial arrangement of myonuclei.

In EDL muscle where PGC-1 $\alpha$  is over-expressed, we observed rounder nuclei (Table 2) as well as an increase in the number of myonuclei and subsequent decreases in MND volume and NN (Table 1, Figure 1). Hence, this substantiates what others have suggested without any experimental evidence (Moyes and LeMoine, 2005), that signalling pathways responsible for mitochondrial number define MND size independently of myosin heavy chain composition. Interestingly, the incorporation of new myonuclei, to reach MND volumes that would optimise the supply of gene products destined for mitochondria, was not associated with any significant myonuclear disorganisation (SD-NN, Table 1). This probably means that inter-nuclear communication and coordination of bioenergetic and contractile protein expression is not deteriorated (Table 2). Even though we believe that all the above changes are directly related to mitochondrial density, it is not totally excluded that PGC-1 $\alpha$  has a direct effect on the nuclei themselves, rather than acting through the medium of mitochondrial number.

### **EDL and diaphragm muscles respond differently to varying levels of PGC-1 $\alpha$**

In diaphragm muscle, on the other hand, PGC-1 $\alpha$  over-expression did not lead to any noticeable changes in MND (Table 1, Figure 1). The reasons are unclear. One potential explanation lies in the design of the transgenic line overexpressing PGC-1 $\alpha$ . This was driven by the creatine kinase promoter, which is more potent in glycolytic (e.g. EDL) compared to

oxidative (e.g. diaphragm) muscles (Arnold et al., 2014; Lin et al., 2002). Another potential explanation could originate from the fact that EDL and diaphragm have distinct morphological, metabolic and contractile properties. Indeed, because of various functional demands (Schiuffino and Reggiani, 2011), respiratory myofibres have a greater overall mitochondrial volume density (Gamboa and Andrade, 2010) and smaller average MND size (Verheul et al., 2004) when compared with limb muscle fibres. Since the oxidative diaphragm muscle requires more mitochondria, it may depend more heavily on PGC-1 $\alpha$  expression for its normal functioning, compared to the glycolytic EDL muscle; this may explain the varying responses to overexpression and knock out of this gene, in different muscle types.

In addition, Redshaw and co-workers have shown that satellite cell proliferation and fusion rates differ between diaphragm and limb muscles of mammals (Redshaw et al., 2010). Satellite cells originating from the diaphragm proliferate less but differentiate more (Redshaw et al., 2010). This may allow an increased incorporation of new myonuclei into adult respiratory muscle. The volume of MNDs may then already be optimal to meet the high demands of the contractile and bioenergetic properties of this muscle. Increasing PGC-1 $\alpha$  content in the diaphragm of MCK mice may not have the ability to further reduce MND size, since the MND sizes are already much lower in these oxidative muscles.

## **Conclusions**

We propose that PGC-1 $\alpha$  and mitochondrial levels contribute to the regulation of MND size in glycolytic limb muscles. Across species, PGC-1 $\alpha$  content and mitochondrial biogenesis has been shown to decrease in response to the ageing process or to various disease states in e.g. skeletal and cardiac muscle. Over-expressing PGC-1 $\alpha$  confers therapeutic benefits by notably improving mitochondrial biogenesis and number in various tissues and in cells. Therefore, others have hypothesised that currently available pharmacological up-regulators of PGC-1 $\alpha$  could provide significant benefits in terms of muscle function (Dillon et al., 2012). However, according to our current findings, such positive effects could be limited to glycolytic limb muscles and be less marked in oxidative respiratory muscles, which are also known to be heavily affected during biological ageing and muscle disorders such as muscular dystrophies and congenital myopathies (Lindqvist et al., 2013).

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## **AUTHOR CONTRIBUTIONS**

CH and JO contributed to the conception and design of the work

JR, AP, YL, BC, CH and JO did the acquisition, analysis, and interpretation of data

JR, AP, YL, BC, CH and JO drafted the work and revised it critically

JR, AP, YL, BC, CH and JO approved the final version to be published

JR, AP, YL, BC, CH and JO agreed on all aspects of the work

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## FIGURES

### **Figure 1: Typical myonuclei and isolated myofibres**

Single myofibres were isolated from age-matched MCK (PGC-1 $\alpha$  muscle-specific over-expression), MKO (PGC-1 $\alpha$  muscle-specific knockout) and WT (wild-type) rodents. These were then stained for actin (Alexa Fluor Phalloidin 488, green) and myonuclei (DAPI, blue). Scatterplots of MND volume versus myofibre CSA for cells isolated from MCK (red, PGC-1 $\alpha$  muscle-specific over-expression), MKO (green, PGC-1 $\alpha$  muscle-specific knockout) and WT (blue, wild-type) rodents. All regression lines demonstrated a statistically significant correlation ( $p < 0.05$ ).

