Ultrastructural immunodetection of the regulatory factors PAX7 and MyoD in myonuclei of sedentary and trained old mice

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Summary

The plasticity of skeletal muscle and its capability of regeneration drastically decrease during ageing, resulting in a loss of motor units, called sarcopenia. Sarcopenia may depend on a reduced efficiency of muscle tissue regeneration likely due to a decrease in the amount of satellite cells (SC) and a decline in SC myogenic potential.

Previous studies showed that ageing is characterized by a reduction in two important myogenic regulatory factors, PAX7 and MyoD, which are specific markers of SC functional state.

Since some studies suggest that an adapted aerobic physical exercise may be an efficient non-pharmacological approach to mitigate the effect of aging on skeletal muscle, we focused our attention on the possible effects that training may induce on the ultrastructural distribution and relative amount of PAX7 and MyoD in myonuclei of old mice. Since MyoD and PAX7 are expressed in both SCs and myonuclei, ultrastructural immunocytochemistry is a suitable and unique approach for detecting, locating and quantifying these protein factors in the myonuclei of sarcopenic mice before and after mild physical exercise, thus providing basic information to understand their functional modulation in relation to ageing and training.

Our results suggest that, in response to the mild physical exercise, the increase of MyoD in myonuclei is involved in their reactivation; such activation is not mediated by PAX7, which did not increase and seemed to find in the nucleolus its storage site in aged myonuclei.

Key words: ageing, sarcopenia, physical exercise, myonucleus, PAX7, MyoD, ultrastructural immunocytochemistry

Introduction

Skeletal muscle, its regeneration in response to specific conditions and the factors involved in such a tissue activation continue to be an intriguing matter of research.

Following damage, muscle tissue can rapidly repair through the activation of satellite cells (SCs), which are a pool of mitotically quiescent cells (Mouro, 1961) located between the basal lamina and the sarcolemma of myofibers (Anderson and Wozniak, 2004). Upon injury, SCs are capable of initially proliferating to then differentiate into myocytes and fuse either together or with pre-existing myofibers in order to regenerate muscle tissue. The progress of the SC activation is finely regulated by the expression of specific factors, among which the paired box protein 7 (PAX7) and the myogenic differentiation factor D (MyoD). PAX7 is a transcription factor expressed in quiescent as well as activated SCs: it plays a key role in the proliferation phase, while being downregulated when the satellite cells (actually myoblasts, at this step) become committed to muscle differentiation (Kuang and Rudnicki, 2008; Sambasivan and Tajbakhsh, 2007; Tedesco *et al.*, 2010). At this time, the upregulation of MyoD as a potent myogenic master transcription factor takes place and promotes the differentiation of myoblasts into muscle fibers.

Therefore, PAX7 and MyoD are commonly recognized as molecular markers characterizing different phases of SC activation.

The typical plasticity of skeletal muscle and its capability of regeneration drastically decrease during ageing: this results in a loss of motor units, called sarcopenia. Sarcopenia is indeed characterized by low muscle mass and a functional tissue failure. Taking into account that the average human life expectancy has risen, this physiological condition is affecting an increasing portion of population with important healthcare and socio-economic implica-

tions (Cruz-Jentoft *et al.*, 2010). In fact, sarcopenia leads to physical frailty and contributes to a progressive disability and mortality (Burton and Sumukadas, 2010).

Although the mechanisms leading to sarcopenia have not yet been clarified, the accepted hypothesis is that sarcopenia may depend on a decrease in the efficiency of muscle tissue regeneration (Sayer *et al.*, 2013). Actually, under sarcopenic condition the muscle is characterized by a decrease in the amount of SCs (Renault *et al.*, 2002; Garcıa-Prat *et al.*, 2013; Alway *et al.*, 2014) and a reduced SC myogenic potential (Zwetsloot *et al.*, 2013).

Some studies suggest that an adapted aerobic physical exercise may be an efficient non-pharmacological approach to mitigate the effect of ageing on skeletal muscle (Malatesta *et al.*, 2011, Cisterna *et al.*, 2016), at least partially counteracting the age related SC decline. Previous findings indicate that a mild physical exercise can stimulate a reactivation of nuclear activity in SCs, as shown by an increased transcription in SCs as well as in myofibers of trained old mice (Malatesta *et al.*, 2011).

Based on this evidence, we focused our attention on the possible effects that physical exercise may induce on the ultrastructural distribution and relative amount of PAX7 and MyoD in the myonuclei. We evaluated their localization on the ribonucleoprotein (RNP)-containing nuclear structures, e.g. perichromatin fibrils (PFs) and interchromatin granules (IGs), and on the nucleolus. The ultrastructural distribution of a molecule can provide basic information on its functional role: thus our investigation may shed light on the expression of these two essential transcription factors in the nuclei of differentiated myofibers, in response to mild physical exercise.

Materials and Methods

Animals and physical training

Four adult (12 months) and eight old (28 months) male mice from the INRCA breed (Ancona, Italy) were used in this study. The INRCA breed is a 40-year established Balb-c mice strain which has been widely used for studies on physiological ageing: these mice have a long life (mean life span 25 months; maximal life span 34 months) (Mocchegiani *et al.*, 2007), and a relatively low incidence of pathologies, in particular tumors (Staats, 1980; Bronson and Lipman, 1993). All animals were bred under controlled environmental conditions with 12 h light/dark cycle, and fed *ad libitum* with a standard commercial chow.

Four old mice were trained by treadmill running (45 min at 9 m/min belt speed, five days a week) for one month (old running group: OR); four old mice (old sedentary group: OS) and four adult animals (adult sedentary group: AS) had only spontaneous free-moving activity in the cage. Ultrastructural analysis is a quite demanding method; therefore the number of investigated animals per group was kept to the minimum required for statistical analysis.

In order to avoid possible interference of acute with chronic effects of physical exercise, the animals were killed three days after the last treadmill session.

Tissue processing

The mice were deeply anaesthetized with pentobarbital (50 mg/Kg i.p.) and then perfused *via* the ascending aorta with a brief prewash with 0.09% NaCl solution followed by a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at 4°C.

For immunocytochemistry, quadriceps femoris muscles were quickly removed and placed for 2 h at 4°C in the same fixation solution. The samples were washed in phosphate buffered saline (PBS), immersed in 0.5 M NH_4Cl in PBS for 45 min at 4°C to block free aldehydes, dehydrated with ethanol, and embedded in LR White resin. Ultrathin sections were collected on Formvar-carbon coated nickel grids and used for the immunocytochemical analyses.

Immunocytochemical analyses

To investigate the fine distribution of PAX7 and MyoD in myonuclei, ultrathin sections of muscle samples were treated with a rabbit polyclonal antibody directed against PAX7 (Abcam, Cambridge, MA, USA) or a mouse monoclonal antibody against MyoD (Abcam). Briefly, the sections were floated for 3 min on normal goat serum diluted 1:100 in PBS and then incubated for 17 h at 4°C with the primary antibodies diluted in PBS containing 0.1% bovine serum albumin (Fluka, St. Louis, MO) and 0.05% Tween 20 (both antibodies were diluted 1:20). After rinsing, sections were floated on normal goat serum, and then reacted for 30 min at room temperature with the secondary goat anti-mouse 6-nm or goat anti-rabbit 12-nm gold-conjugated antibodies (Jackson Immuno Research Laboratories, West Grove, PA) diluted 1:10 in PBS. The sections were rinsed with PBS and water, and air-dried. As controls, some grids were incubated without the primary antibody and then processed as described above.

The sections were weakly stained with 2.5% aqueous solution of uranyl acetate for 2 min and observed in a Philips Morgagni transmission electron micro-

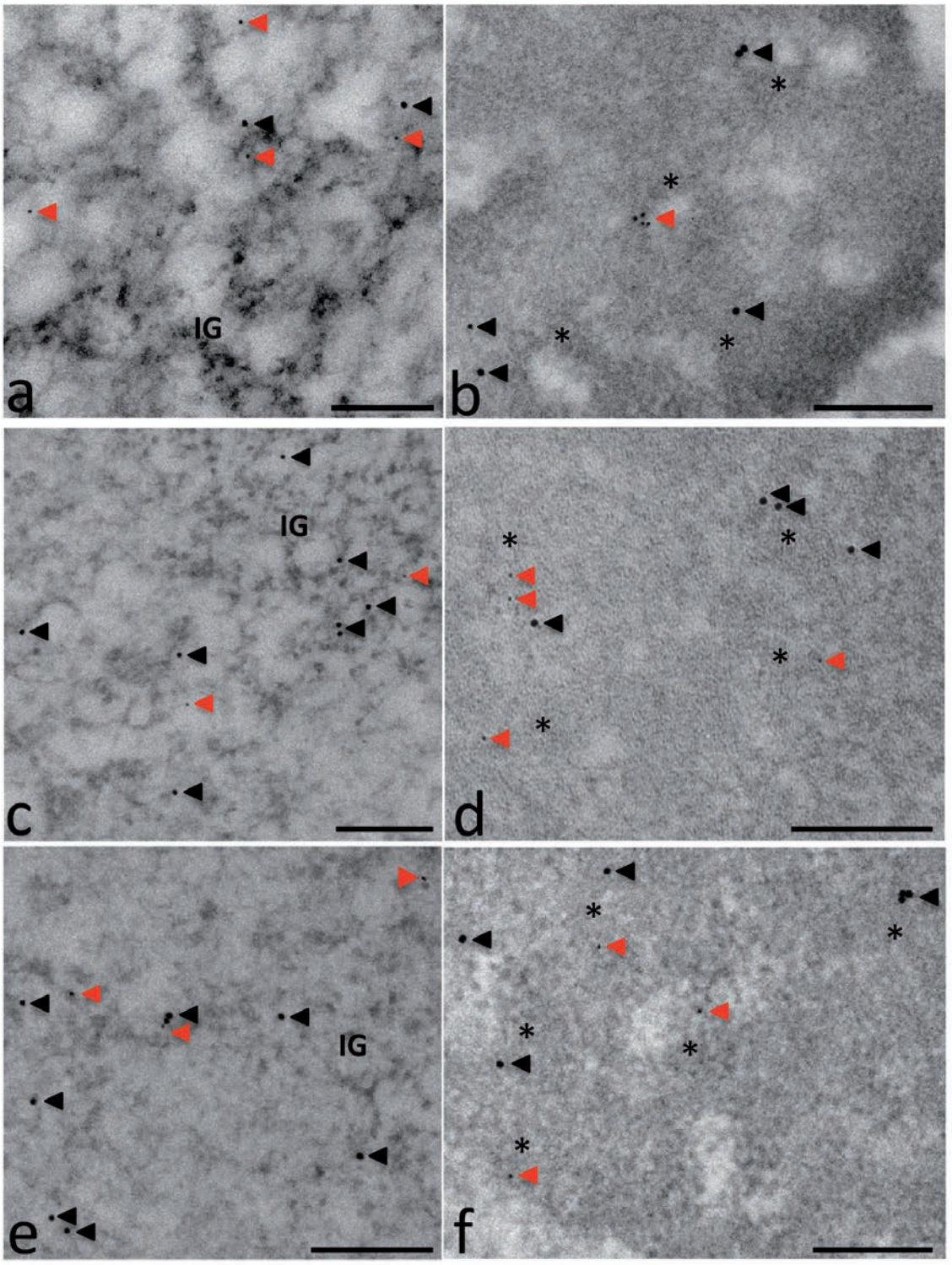

Figure 1. Trasmission electron micrographs of myonuclei from AS (a, d), OS (b, e) and OR (c, f) samples; immunolabelling with anti-PAX7 (large gold grains, black arrowhead) and MyoD (small gold grains, red arrowhead) antibodies. The immunolabelling is present in the nucleoplasm (a-c) on perichromatin fibrils and interchromatin granules (IG) and occurs in the nucleolus (d-f) on DFC (asterisk). Bars: 250 nm.

scope (FEI Company Italia Srl, Milan, Italy) operating at 80kV and equipped with an Olympus Megaview II camera for digital image acquisition.

Quantitative assessment of the immunolabelling was carried out by estimating the gold grain density over selected cellular compartments on sections treated in the same run. The surface area of nucleoplasm, IG clusters and nucleolus was measured on 20 selected electron micrographs (x28,000) from each animal by using ImageJ. Background evaluation was carried out on resin (in the areas devoid of tissue) of immunolabelled samples. Gold grains present over each selected compartment were counted and the labelling density was expressed as number of gold grains/μm^2.

Statistics

Results for each measured parameter evaluated were pooled according to the experimental groups and the means±standard error of the mean (SE) values were calculated. Statistical analysis of the results was performed by the Kruskal Wallis test; moreover, in order to determine which pairs of samples tended to differ, the Mann Whitney test was used (significance was set at P≤0.05).

Results

The ultrastructural organization of the myonuclei was similar in AS, OS and OR mice. Subsarcolemmal myonuclei were typically elongated with one or more roundish nucleoli. A few heterochromatin clumps were associated with the nuclear envelope and the nucleolus; the RNP-containing components, which are active in the synthesis and maturation of mRNA, occupied the nucleoplasmic area. In detail, PFs spread from the borders of condensed chromatin and IGs are recognized as clusters of small granules connected by thin fibrils (Thiry, 1995). The distribution of PAX7 and MyoD was similar in all animals: both transcription factors occurred in the nucleoplasm on PFs and IGs (Figure 1a-c). In the nucleolus, they were detected in one of the nucleolar components i.e., the dense fibrillar component (DFC), whereas the fibrillar centers (FCs) and the granular component (GC) were devoid of signal (Figure 1d-f).

The semiquantitative analysis showed a significant decrease (p=0.005) of PAX7 immunolabelling in the nucleoplasmic area of OS in comparison with the AS samples. Such a reduction was as well observed in OR mice (Figure 2a).

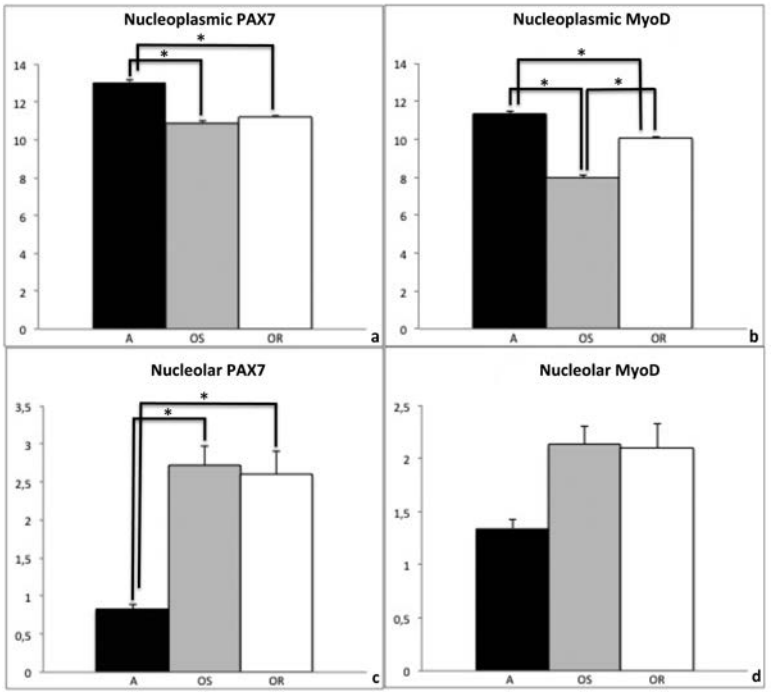

Figure 2. Quantitative evaluation of anti-PAX7 and MyoD labelling density on nucleoplasmic (a-b) and nucleolar area in AS, OS and OR mice (mean±SE). Mann Whitney test reveals significant differences and the columns identified by asterisks are significantly different from each other.

Similarly, as shown in Figure 2b, the nucleoplasmic immunolabeling for MyoD was significantly lower in OS than in AS ($p<0.001$) and OR ($p=0.011$) mice. This demonstrates that the labelling density for MyoD was partially recovered in OR mice ($p=0.032$), although it did not reach the AS values.

The immunolabelling on IGs did not significantly differ among the three samples for either PAX7 (mean values±SE: AS 1.91±1.2; OS 2.98±0.77; OR 2.77±0.74) or MyoD (mean values±SE: AS 1.79±0.96; OS 1.56±0.25; OR 3.28±0.87).

The nucleolar labelling for PAX7 was significantly higher ($p=0.026$) in both OS and OR than in AS mice (Figure 2c), whereas no statistical difference was found for the MyoD immunolabelling, although it showed a trend similar to PAX7 (Figure 2d).

Discussion

PAX7 and MyoD are two important myogenic regulatory factors, which play an essential role in skeletal muscle plasticity, in addition to being recognized as specific markers of the SC functional state (Tedesco *et al.*, 2010; Berkes and Tapscott, 2005).

It is worth noting that a reduction in PAX7 and MyoD takes place during ageing, consistently with the muscle loss (Always *et al.*, 2001; Drummond *et al.*, 2011). Moreover, increasing evidence suggests that muscle atrophy in sarcopenia may be due to a decline in potential of SCs to activate, proliferate and differentiate into motor units (Garcia-Prat *et al.*, 2013; Always *et al.*, 2014). Taking into account that the SC myogenic potential depends on the modulated expression of PAX7 and MyoD, it is worth recalling that a mild physical exercise has been demonstrated to increase the amount of these two transcription factors in SC-derived myoblasts from old mice (Cisterna *et al.*, 2016).

Our results suggest that the mild physical exercise may even have effects on MyoD expression in myofibres. In fact, the nucleoplasmic immunolabeling for MyoD was lower in OS than in AS samples, but significantly increased in OR mice. Therefore, although MyoD did not reach the levels of the AS samples our findings indicate a reactivation of myonuclear activity, as suggested by a rise in transcriptional factors. Interestingly, Malatesta and co-workers have already documented that the exercise can stimulate the synthesis, maturation and export of RNAs to the cytoplasm in aged myonuclei (Malatesta *et al.*, 2011). Accordingly, we observed that in the nucleoplasm MyoD localizes on PFs, which represent the morphological expression of mRNA transcription and processing (Fakan *et al.*, 1984). Some MyoD was also found in the nucleolus, where it showed a trend to increase in OS and OR mice, although the difference with the AS group did not reach statistical significance.

MyoD has been already found to be expressed in myonuclei, although its role remains unclear (reviewed in Legerlotz and Smith, 2008), and an increase in its content has been shown after denervation (Chen *et al.*, 2002; Hyatt *et al.*, 2006), suggesting a possible nuclear reactivation essential for the myonuclei to react to such a critical condition.

PAX7 nucleoplasmic immunolabelling significantly decreased in OS, consistently with a reduced nuclear activity, but remained low in OR, suggesting that physical exercise does not affect this transcription factor. In both samples PAX7 has been observed to accumulate in the nucleolus, which could represent a storage site for this factor, as well as for MyoD. In fact, in addition to its typical role in rRNA synthesis and ribosomal subunit assembly, the nucleolus is thought to be involved in non-ribosomal functions (reviewed in Cisterna and Biggiogera, 2010). In the nucleolus, both PAX7 and MyoD preferentially localize in the DFC, which is recognised to be the active component in rRNA transcription and processing (Hernandez-Verdun, 2006; Raska *et al.*, 2004; Olson *et al.*, 2002): this is plausible, since the conformation of the DFC should guarantee a permanence and/or transit of molecules without risk of being trapped.

We did not observe any accumulation of either PAX7 or MyoD in IGs, which normally serve as storage/assembly/modification compartments for factors engaged in the transcriptional and processing mechanisms (Misteli and Spector, 1997). The immunolabelling for both factors was quite scarce on IGs, suggesting that these RNP-containing structures may possibly be sites for the functional modification of PAX7 and MyoD, but not their accumulation sites.

In conclusion, our results suggest that MyoD is involved in the reactivation of myonuclei as a consequence of the stimulated response of skeletal muscle to the mild physical exercise; such activation is not mediated by PAX7, which did not increase after physical exercise and seemed to find in the nucleolus its storage site in aged myonuclei.

As already recalled, MyoD and PAX7 are expressed in both SCs and myonuclei: this makes it impossible to discriminate their presence and expression levels in either cell type by biochemical techniques. Ultrastructural immunocytochemistry proved to be a suitable and unique approach for detecting, locating and quantifying MyoD and PAX7 in the myonuclei of sarcopenic mice before and after mild physical exercise, thus providing basic information to understand their functional modulation in relation to ageing and training.

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