Muscle-specific integrins in masseter muscle fibers of chimpanzees: an immunohistochemical study

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Abstract: Most notably, recent comparative genomic analyses strongly indicate that the marked differences between modern human and chimpanzees are likely due more to changes in gene regulation than to modifications of the genes. The most peculiar aspect of hominoid karyotypes is that human have 46 chromosomes whereas gorillas and chimpanzees have 48. Interestingly, human and chimpanzees do share identical inversions on chromosome 7 and 9 that are not evident in the gorilla karyotype. Thus, the general phylogeny suggests that humans and chimpanzees are sister taxa; based on this, it seems that human-chimpanzee sequence similarity is an astonishing 99%. At this purpose, of particular interest is the inactivation of the myosin heavy chain 16 (MYH16) gene, most prominently expressed in the masticatory muscle of mammals. It has been showed that the loss of this gene in humans may have resulted in smaller masticatory muscle and consequential changes to cranio-facial morphology and expansion of the human brain case. Powerful masticatory muscles are found in most primates; contrarily, in both modern and fossil member Homo, these muscles are considerably smaller. The evolving hominid masticatory apparatus shifted towards a pattern of gracilization nearly simultaneously with accelerated encephalization in early Homo. To better comprehend the real role of the MYH16 gene, we studied the primary proteins present in the muscle fibers of humans and non-humans, in order to understand if they really can be influenced by MYH16 gene. At this aim we examined the muscle-specific integrins, α7B and β1D-integrins, and their relative fetal isoforms, α7A and β1A-integrins, analyzing, by immunohistochemistry, muscle biopsies of two components of a chimpanzee's group in captivity, an alpha male and a non-alpha male subjects; all these integrins participate in vital biological processes such as maintenance of tissue integrity, embryonic development, cell differentiation, and cell-extracellular matrix interactions. Our results demonstrated a different quantitative composition of integrins, in alpha male in respect to human and non-alpha male, hypothesizing that the MYH16 gene could modify the expression of integrins, influencing, in turn, the phenotype of muscle. In this way, α7A- and β1A-integrin could determine the presence of type II fibers and then they could play a key role in the determination of contraction force. Then, MYH16 gene could be a common interactor of signalling between sarcoglycans and integrins in chimpanzee muscles.

Key words: skeletal muscle, chimpanzee, integrin, sarcoglycan, immunohistochemistry

Introduction

The ancient researches of Huxley [1] and Darwin [2] affirmed that the modern humans and extant chimpanzees share striking similarities. However, in full century later, the molecular methods were used to examine this relationship. Despite the readily apparent physical similarities shared by African apes to the exclusion of modern humans (body hair, knucle-walking, thin tooth enamel), the molecular support for a human-chimpanzee clade is now overwhelming [3].

Most notably, recent comparative genomic analyses strongly indicate that the marked differences between modern human and chimpanzees are likely due more to changes in gene regulation than to modifications of the genes [4]. The most peculiar aspect of hominoid karyotypes is that human have 46 chromosomes whereas gorillas and chimpanzees have 48 [5]. Human chromosome 2 seems to be a fusion of ape chromosomes 12 and 13. However, other Authors speculated

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that this fusion may be very recent and perhaps even accounts for the supposed lack of interbreeding between modern humans and Neanderthals [6].

Interestingly, human and chimpanzees do share identical inversions on chromosome 7 and 9 that are not evident in the gorilla karyotype [7]. Thus, the general phylogeny suggests that humans and chimpanzees are sister taxa; based on this, it seems that human-chimpanzee sequence similarity is an astonishing 99% [8]. Furthermore, a small genetic change could have a dramatic phenotypic impact if it modified the expression patterns of other genes. These regulatory changes are now being identified, including some likely to underlie cognitive differences between chimpanzees and human [4,9].

At this purpose, of particular interest is the inactivation of the myosin heavy chain 16 (MYH16) gene, most prominently expressed in the masticatory muscle of mammals [10]. It has been showed that the loss of this gene in humans may have resulted in smaller masticatory muscle and consequential changes to craniofacial morphology and expansion of the human brain case [10]. In fact, experimental models of masticatory muscle resection or transposition have demonstrated the correlation between cranio-facial morphology and the force of masticatory muscle contraction [11,12].

Besides, powerful masticatory muscles are found in most primates; contrarily, in both modern and fossil member Homo, these muscles are considerably smaller [13]. The evolving hominid masticatory apparatus shifted towards a pattern of gracilization nearly simultaneously with accelerated encephalization in early Homo [14].

The differences between modern humans and chimpanzees are most evident also in gross anatomical comparison which showed that the relative size of individual masticatory muscle homologues contrast remarkably; for example, there are evident differences in the sites of attachment of the masseter and temporal muscles in chimpanzees and human skulls [10]. At the histological level, the difference between human and non-human primate temporalis muscle is demonstrated by staining for type II sarcomeric myosin and interstitial laminin.

To better comprehend the real role of the MYH16 gene, we studied the primary proteins present in the muscle fibers of humans and non-humans, in order to understand if they really can be influenced by MYH16 gene. At this aim we examined the muscle-specific integrins, α7B and β1D-integrins, and their relative fetal isoforms, α7A and β1A-integrins.

Integrins are a family of transmembrane receptor molecules, which participate in vital biological processes such as maintenance of tissue integrity, embryonic development, cell differentiation, and cell-extracellular matrix interactions [15-18]. These proteins also have an important role in transducing mechanical and chemical signals into the cells [17-19]. They are heterodimers composed of two subunits, α and β, both containing extracellular, transmembrane, and cytoplasmic domains [20].

In particular, the β subunits are responsible of interaction with the cytoskeleton, whereas a subunits participate in the determination of the specificity of the ligand binding and signalling. Previously, it has been demonstrated that the β1D integrin displaces the β1A isoform in striated muscle [21].

About α7 subunit, the α7A and α7C isoforms appear to be restricted to skeletal muscle, in contrast to α7B, which is also expressed in nonmuscle cells. In skeletal muscle, α7B is expressed in proliferating, mobile myogenic precursor cells and it is still detected in adult myofibers with restricted localization to neuromuscular junctions and myotendinous junctions [22]. Thus α7A may have a specific role in regenerating muscle during the dynamic adhesion stage, whereas in mature skeletal muscle it appears to have a minor role [20].

Other investigations have demonstrated that the enhanced expression of the α7β1 reduces muscular dystrophy and restore viability in dystrophic mice [22]. On this, recently we examined muscle fibers during muscular inactivity, showing that the fetal isoforms of muscle-specific integrins can be displace the adult isoform due to loss of regulatory effects on gene expression of these protein which could provoke a quantitative modification of transmembrane receptors [23,24].

Besides it was demonstrated a bidirectional signalling between integrins and sarcoglycans [25-28]. This bidirectional signalling, by an common interactor as filamin2, could permit the transduction of mechanical and chemical signalling into the cells and the maintenance of the cell integrity.

On this basis, here we analyzed for first time, the α7A-, α7B- β1A-, and β1D-integrin, by immunohistochemical technique, analyzing muscle biopsies of two components of a chimpanzee's group in captivity, an alpha male and a non-alpha male subjects.

Materials and methods

Tissue samples. Initially, we examined human skeletal muscle biopsies of masseter from two patients who did not have any co-existing neuromuscular pathology, but had undergone orthopaedic surgery for other reasons.

The age of the patients was between 30 and 60 years and all gave their informed consent. The investigation conformed with the principles outlined in the Declaration of Helsinki.

Subsequently, we examined masseter muscle biopsies from two adult males (32 and 30 years old) chimpanzees (*Pan troglodytes*). They were part of a larger social group (7 animals) living in a indoor/outdoor enclosure at the "Zoosafari di Fasano" (BR, Italy).

Muscle biopsies are done using anesthesia and therefore require prior immobilization of the chimpanzee with a chemical agent. The drug to chimpanzees are administered through a process known as "a knockdown" in which pre-loaded syringes are fired through a dart gun. After administration of anesthesia and immobilization of animals, the biopsy are effectuated with excision of cute, subcutaneous tissues and muscle fascia; finally, we used a 6.0 mm biopsy punch. The biopsy specimens were analyzed using immunohistochemistry technique.

Immunohistochemistry. The biopsies were fixed in 3% paraformaldehyde in 0.2 M phosphate buffer, pH 7.4, for 2 hours at room temperature. They were then washed extensively with 0.2 M phosphate buffer, pH 7.4, and then with phosphate-buffered saline (PBS), containing 12% and 18% sucrose. The samples were snap-frozen in liquid nitrogen and 20 μm sections were prepared in a cryostat for their use in a protocol to perform immunofluorescence. The sections were placed on glass slides that were coated with 0.5% gelatin and 0.005% chromium potassium sulphate.

To block non-specific binding sites and to permeabilize the membranes, the sections were preincubated with 1% bovine serum albumin (BSA), 0.3% Triton X-100 in PBS at room temperature for 15 min. Finally, the sections were incubated with primary antibodies. The following primary antibodies were used: anti-α-sarcoglycan diluted 1:100, anti-β-sarcoglycan diluted 1:200, anti-γ-sarcoglycan diluted 1:100, anti-δ-sarcoglycan diluted 1:50, and anti-ε-sarcoglycan diluted 1:100 (all from Novocastra Laboratories); anti-α7B-integrin diluted 1:50, anti-β1D-integrin diluted 1:50, anti-α7A-integrin diluted 1:100, and anti-β1A-integrin diluted 1:50 (synthetic peptides from the COOH terminal region; kindly provided by the laboratory of Professor Tarone, University of Torino). Primary antibodies were detected using Texas Red-conjugated IgG (Jackson ImmunoResearch Laboratories, Inc.). Slides were finally washed in PBS and sealed with mounting medium.

The sections were then analyzed and images acquired using a Zeiss LSM 5 DUO confocal laser scanning microscope. All images were digitalized at a resolution of 8 bits into an array of 2048 × 2048 pixels. Optical sections of fluorescent specimens were obtained using a HeNe laser (wavelength = 543 nm) and an Argon laser (wavelength = 458 nm) at a 1-min 2-sec scanning speed with up to 8 averages; 1.50 μm-thick sections were obtained using a pinhole of 250. For each reaction, at least 100 individual fibres were examined. Contrast and brightness were established by examining the most brightly labelled pixels and choosing the settings that allowed clear visualization of the structural details while keeping the pixel intensity at its highest (~200). Each image was acquired within 62 sec, in order to minimize photodegradation.

Digital images were cropped and figure montages prepared using Adobe Photoshop 7.0 (Adobe Systems; Palo Alto, CA).

Results

In this report we obtained and studied 20 μm-thick cryosections by acquiring a stack of 16 sections, with a scan step size of 0.8 μm.

Firstly, we analyzed the samples of the human subject, performing immunofluorescence reactions using a single antibody each time. Our results, on longitudinal sections, showed normal intermittent, or periodic, staining patterns for all tested integrins. Moreover, immunostaining of α7A- (Fig. 1A), and β1A-integrin (Fig. 1B) was reduced compared to that of α7B- (Fig. 1 C), and β1D-integrin (Fig. 1D). About sarcoglycans,

it was showed a normal staining pattern of all these tested proteins (Fig. 2).

Secondly, we performed immunostaining on longitudinal sections of masseter muscle fibers from a non-alpha male chimpazee. All of the tested integrin isoforms showed the same behaviour of human samples. In particular, α7A- (Fig. 3A), and β1A-integrin (Fig. 3B) showed normal periodic and reduced staining patterns, whereas immunostaining of α7B- (Fig. 3C), and β1D-integrin (Fig. 3D), showed a normal fluorescence. Also sarcoglycan immunostainings showed the same situation observed in human muscle (Fig. 4).

Finally, we tested the same integrin isoforms in alpha male chimpanzee. Our observations showed the same normal intermittent staining patterns for all tested proteins, but interestingly immunostaining of α7A- (Fig. 5A), and β1A-integrin (Fig. 5B) was clearly increased compared to that of α7B- (Fig. 5C), and β1D-integrin (Fig. 5D) which showed a reduced but detectable fluorescence, and to that of α7A- and β1A-integrin of human and non-alpha male chimpazee. The sarcoglycan subcomplex analysis in the alpha male showed a normal fluorescence of all tested sarcoglycans as well as in the human and non-alpha male muscle fibers (Fig. 6).

Discussion and conclusion

In this study we demonstrated evident differences of protein arrangement between muscle fibers of human and chimpanzees. We also showed clear differences between protein arrangement between muscle fibers of alpha male and non-alpha male chimpanzees of the same group. In particular, by immunofluorescence technique, in human and in non-alpha male we highlighted a clear staining patterns for α7B and β1D integrin, whereas α7A- and β1A-integrin showed a weak staining pattern. Interestingly, in muscle fibers of alpha male we observed an opposite situation showing a very strong fluorescence for α7A and β1A-integrin, and a weak staining pattern for α7B- and β1D-integrin. There are no evident differences in all tested sarcoglycans.

The integrins transmit signals by organizing the cytoskeleton, thus regulating cell shape and internal cellular architecture. In fact, integrin cytoplasmic domains interact with cytoskeletal components [29,30]. In this view, appropriate cell shape and cytoskeletal organization can regulate the biosynthetic capabilities of the cell, and thus contribute to cell growth or differentiation [31,33]. Furthermore, integrins are deemed to be true receptors capable of giving rise to biochemical signals within the cell [18]. Previously it is demonstrated that high levels of α7A were detected in differentiating myotubes, but in proliferating myoblast only the α7B isoform was present
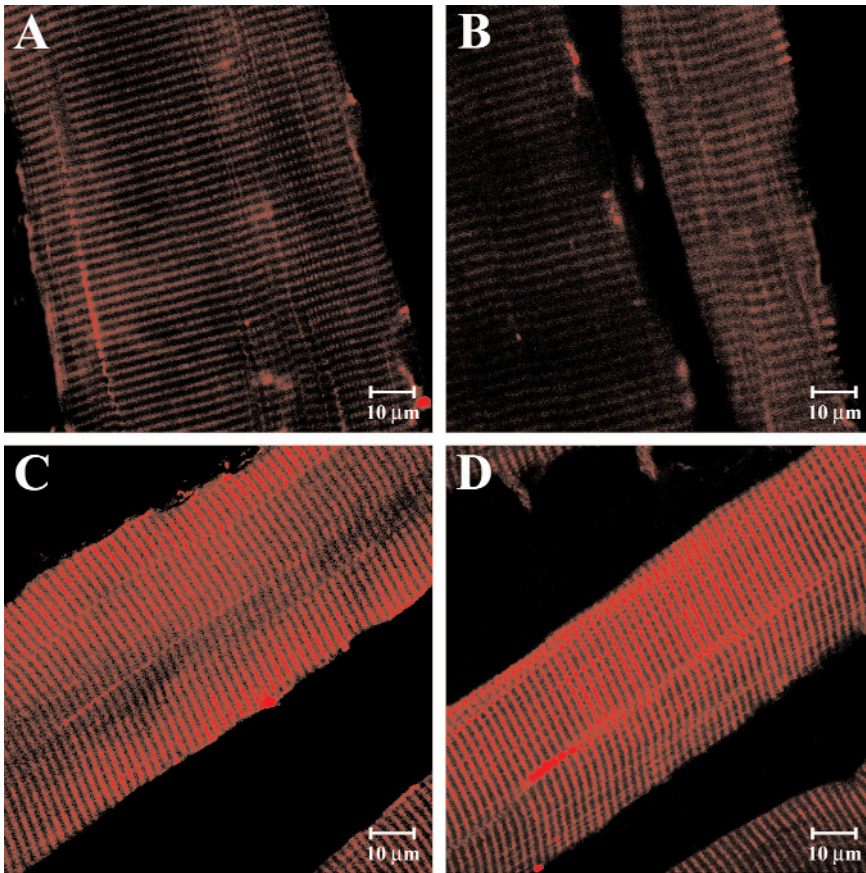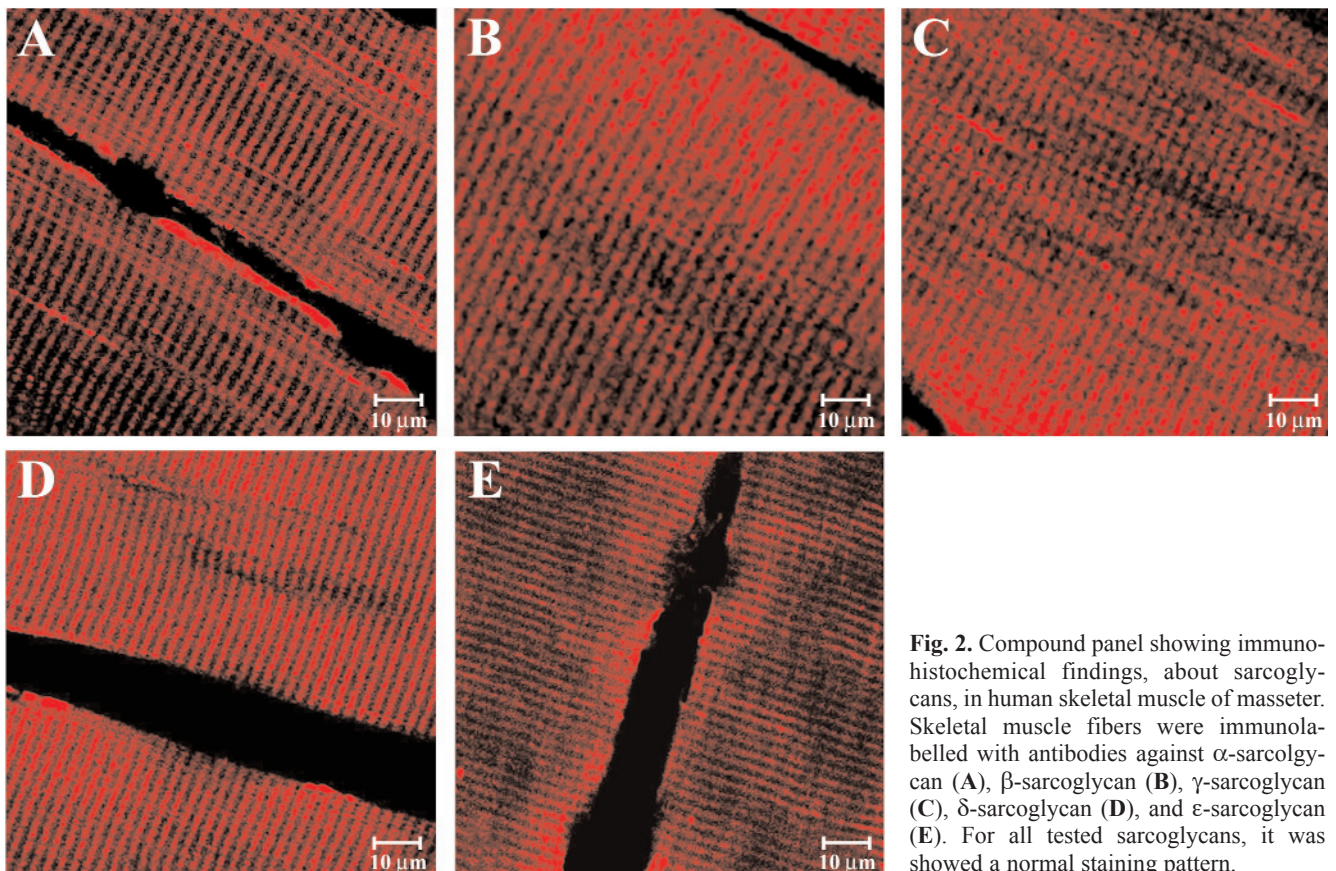
Fig. 1. Compound panel showing immuno-histochemical findings, about integrins, in human skeletal muscle of masseter. Skeletal muscle fibers were immunolabelled with antibodies against α7A- (**A**), β1A- (**B**), α7B- (**C**), and β1D-integrin (**D**). Immunostaining of α7A- and β1A-integrin was reduced compared to α7B- and β1D-integrin.

Fig. 2. Compound panel showing immuno-histochemical findings, about sarcogly-cans, in human skeletal muscle of masseter. Skeletal muscle fibers were immunola-belled with antibodies against α-sarcolgy-can (**A**), β-sarcoglycan (**B**), γ-sarcoglycan (**C**), δ-sarcoglycan (**D**), and ε-sarcoglycan (**E**). For all tested sarcoglycans, it was showed a normal staining pattern.
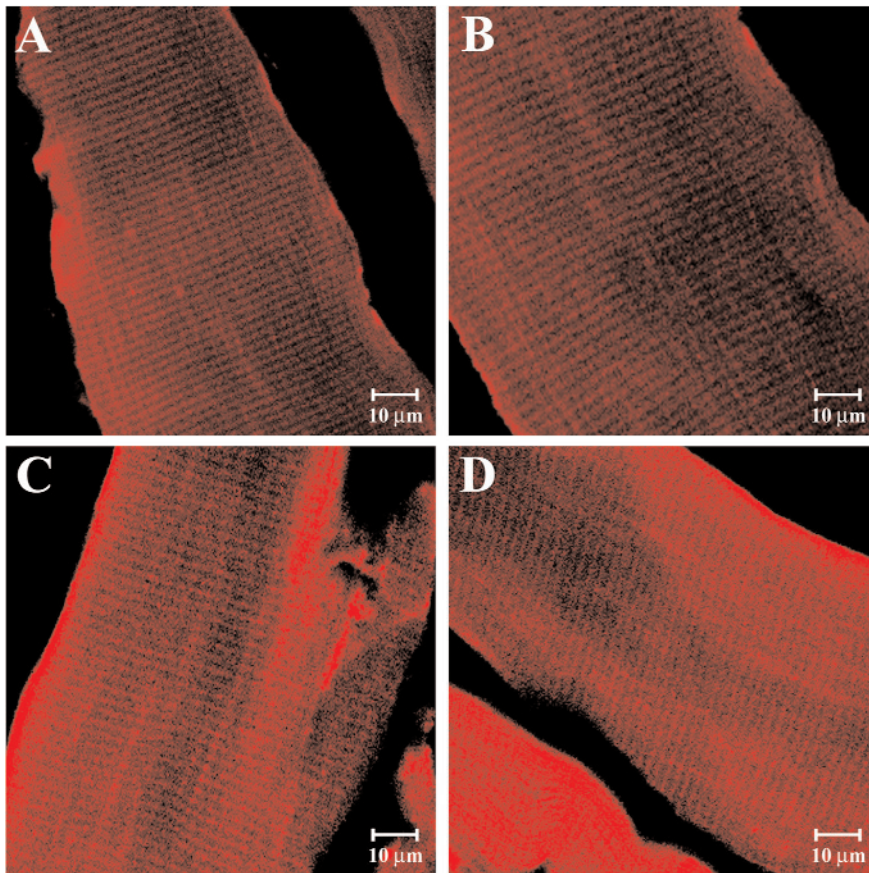
Fig. 3. Compound panel showing immuno-histochemical findings, about integrins, in skeletal muscle fibers of masseter in non-alpha male chimpanzee. Skeletal muscle fibers were immunolabelled with antibodies against α7A- (**A**), β1A- (**B**), α7B- (**C**), and β1D-integrin (**D**). Immunostaining of α7A- and β1A-integrin was reduced in comparison to α7B- and β1D-integrin.
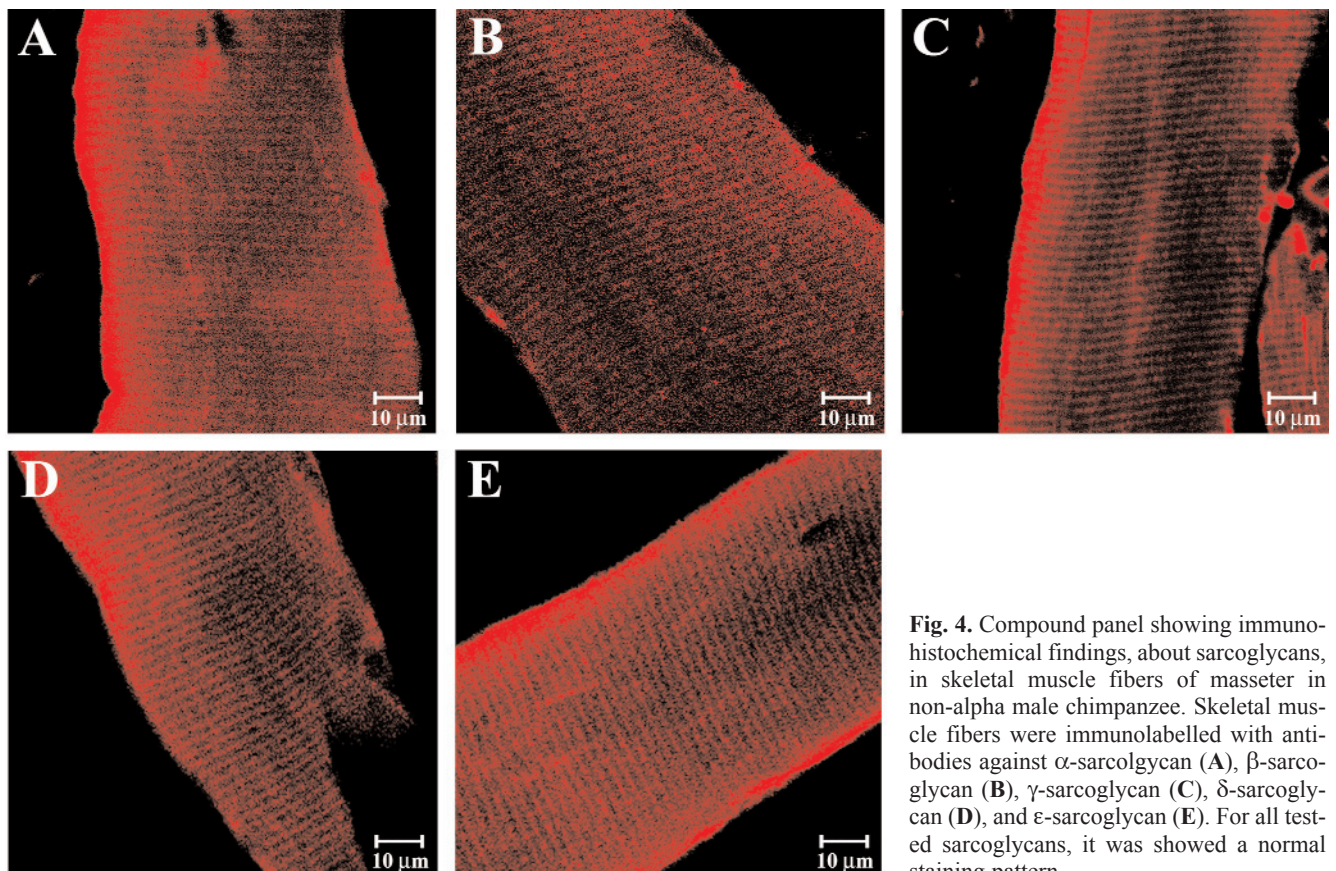

Fig. 4. Compound panel showing immuno-histochemical findings, about sarcoglycans, in skeletal muscle fibers of masseter in non-alpha male chimpanzee. Skeletal muscle fibers were immunolabelled with antibodies against α-sarcolgycan (**A**), β-sarcoglycan (**B**), γ-sarcoglycan (**C**), δ-sarcoglycan (**D**), and ϵ-sarcoglycan (**E**). For all tested sarcoglycans, it was showed a normal staining pattern.
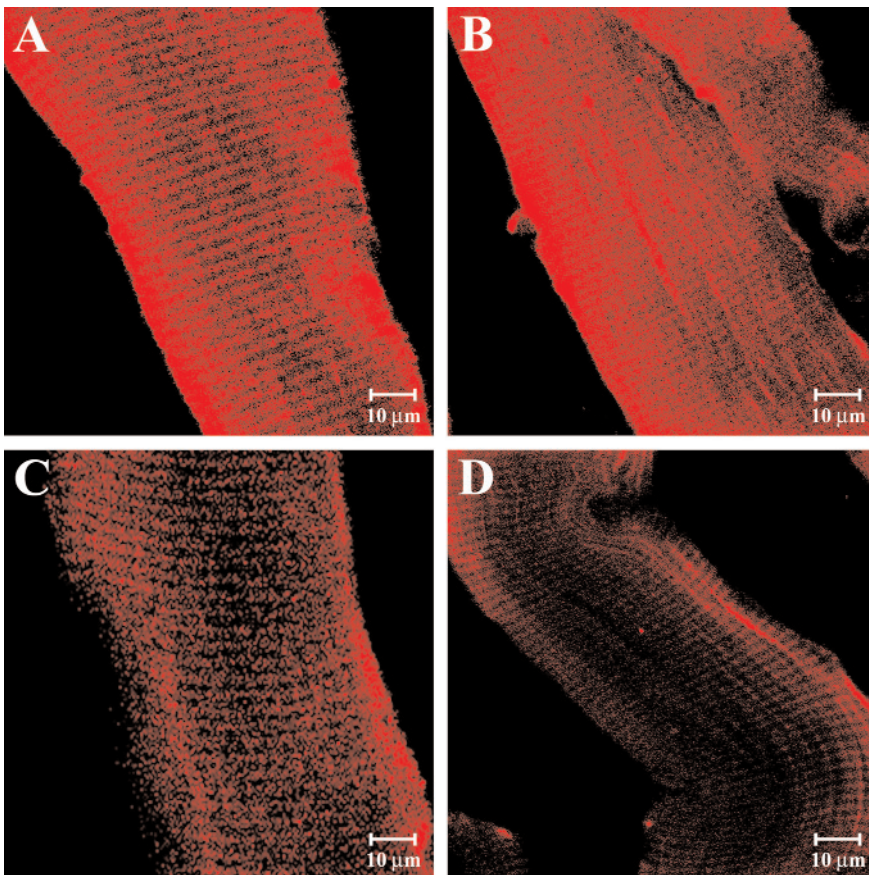
Fig. 5. Compound panel showing immuno-histochemical findings, about integrins, in skeletal muscle fibers of masseter in alpha male chimpanzee. Skeletal muscle fibers were immunolabelled with antibodies against α7A- (**A**), β1A- (**B**), α7B- (**C**), and β1D-integrin (**D**). Immunostaining of α7A-, and β1A-integrin was clearly increased compared to that of α7B-, and β1D-integrin which showed a reduced but detectale fluorescence, and to that of α7A- and β1A-integrin of human and non-alpha male chimpanzee.
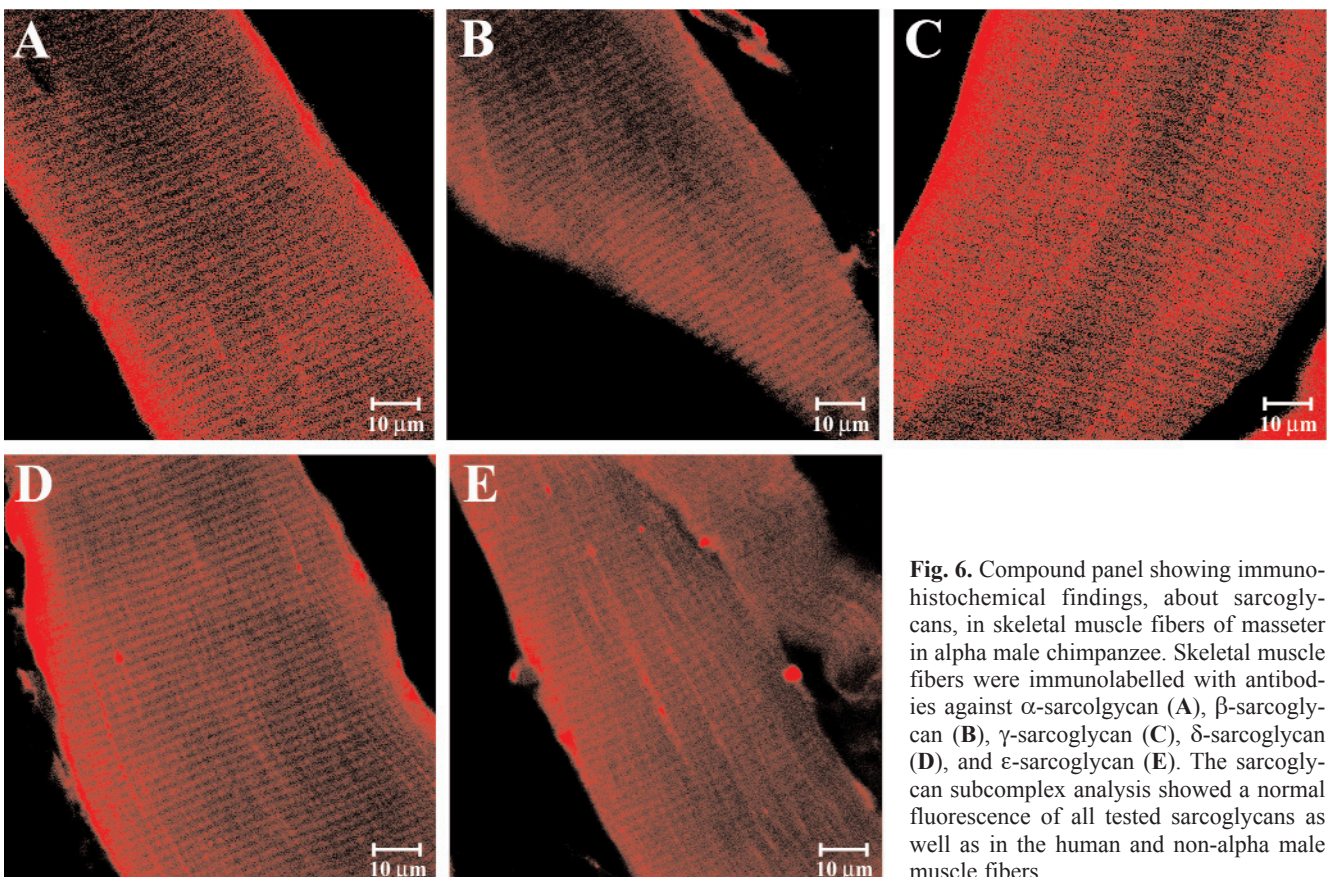

Fig. 6. Compound panel showing immuno-histochemical findings, about sarcogly-cans, in skeletal muscle fibers of masseter in alpha male chimpanzee. Skeletal muscle fibers were immunolabelled with antibodies against α-sarcolgycan (**A**), β-sarcogly-can (**B**), γ-sarcoglycan (**C**), δ-sarcoglycan (**D**), and ε-sarcoglycan (**E**). The sarcogly-can subcomplex analysis showed a normal fluorescence of all tested sarcoglycans as well as in the human and non-alpha male muscle fibers.

[34]. Thus, the progression from myoblast to myotubes is paralleled by increase in α7A; this suggests that α7, isoform switching may be an important mechanism in skeletal muscle differentiation [35].

About isoform of β1-integrin, it is showed that the β1D-integrin chain is restricted to differentiated skeletal and cardiac muscle [36,37]; in contrast, the β1A chain is present in a wide variety of cell types including myogenic precursor cells [22]. The β1D cytoplasmic domain may act to arrest the progression of myoblast proliferation and alter subcellular localization [20]; therefore, increased β1D expression may increase the interaction between the extracellular matrix, sarcolemma, and the cell cytoskeleton, stabilizing muscle integrity [22]. In this way, the shift from β1A and increased β1D-integrin reflects fewer mononuclear cell infiltrates and increased stability of muscle fibers [22].

Previously, it has been demonstrated that the loss of MYH16 gene, implied in jaw musculature regulation, in humans may have resulted in smaller masticatory muscle and consequential changes to craniofacial morphology with consequential expansion of the human brain case [10]. Besides, it was showed that protein expression in muscle fibers is highly plastic and it is unlikely that the inactivation of MYH16 would have associated with dramatic changes in hominin masticatory mechanics [38]. Based on this, powerful masticatory muscles are found in most primates, including chimpanzees and gorillas; in contrast, masticatory muscles are considerably smaller in both modern and fossil members of Homo [13].

On this basis, here, we hypothesized that the different quantitative composition of integrins, observed in muscle fibers of alpha-male, could be influenced by MYH16 gene. Then, in our opinion it is intriguing to hypothesize that the MYH16 gene, more expressed in chimpanzees and loss in human muscles, could modify the expression of integrins, influencing, in turn, the phenotype of muscle.

In this way, α7A- and β1A-integrin could determine the presence of type II fibers and then they could play a key role in the determination of contraction force. Then, MYH16 gene could be a common interactor of signalling between sarcoglycans and integrins in chimpanzee muscles.

This hypothesis, highlighting the influence of the gene expression by different social condition, as well as the different role of chimpanzee in the same group, open a new line of research in order to better comprehend the real mechanism on the basis of the evolution.

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