Publisher's Disclaimer. E-publishing ahead of print is increasingly important for the rapid dissemination of science. The *Early Access* service lets users access peer-reviewed articles well before print / regular issue publication, significantly reducing the time it takes for critical findings to reach the research community.

These articles are searchable and citable by their DOI (Digital Object Identifier).

The **European Journal of Translational Myology** is, therefore, e-publishing PDF files of an early version of manuscripts that undergone a regular peer review and have been accepted for publication, but have not been through the typesetting, pagination and proofreading processes, which may lead to differences between this version and the final one.

The final version of the manuscript will then appear on a regular issue of the journal.

E-publishing of this PDF file has been approved by the authors.

Eur J Transl Myol 2024 [Online ahead of print]

To cite this Article:

Pietrangelo L, Mancinelli R, Fulle S, et al. **An aged-related structural study of DHPR tetrads in peripheral couplings of human skeletal muscle.** *Eur J Transl Myol* doi: 10.4081/ejtm.2024.13271

An aged-related structural study of DHPR tetrads in peripheral couplings of human skeletal muscle

Laura Pietrangelo,[1,2] Rosa Mancinelli,[3,4] Stefania Fulle,[3,4] Simona Boncompagni[1,3]

[1]Center for Advanced Studies and Technology (CAST), University G. d' Annunzio, Italy;
[2]Department of Medicine and Aging Sciences (DMSI), University G. d' Annunzio, Italy;
[3]Department of Neuroscience, Imaging and Clinical Sciences (DNICS), University G. d' Annunzio, Italy; [4]Interuniversity Institute of Myology (IIM), Perugia, Italy

Abstract

Among the numerous changes that occur in skeletal muscle during aging, the reduced regeneration potential after an injury is largely due to the impaired ability of satellite cells to proliferate and differentiate. Herein, using the freeze-fracture electron microscopy technique, we analyzed both the incidence and size of dihydropyridine receptors (DHPRs) tetrads (4 particles) in cultured myotubes from a young subject (28 years) after 9 days of differentiation and from an old subject (71 years) after 9 and 12 days of differentiation. Compared to young myotubes, at 9 days of differentiation old myotubes exhibited: i) a lower incidence and a smaller size of DHPR clusters and ii) a lower number of complete tetrads. At 12 days of differentiation values of incidence, size and number of complete tetrads in old myotubes were instead comparable with those of young myotubes at 9 days of differentiation. Collectively, these results indicate that in aged myotubes the synthesis process of the proteins involved in the excitation-contraction coupling mechanism, such as the DHPR, is somehow slowed, supporting previous studies evidence of a decrease in the differentiation potential of myotubes from elderly individuals.

Key words: DHPR tetrads; cultured myotubes; excitation-contraction coupling mechanism.

Among the numerous structural and functional changes that occur in the skeletal muscle of the elderly, it has also been demonstrated that aging is accompanied by a decrease in the capacity of muscle tissue to regenerate following injury.[1] Several studies reported that with aging, satellite cells (quiescent mononucleated myogenic cells) diminish their capacity to proliferate and growth.[2,3] Specifically, Pietrangelo *et al.*, 2009 reported that in myoblasts and myotubes derived from satellite cells of elderly subjects, aging causes an impairment to complete differentiation.[4] At the molecular level, it was suggested that the impairment of muscle to regenerate may also be determined by the declining rate of protein synthesis.[5] To further support this hypothesis, we decided to search for the presence of Dihydropyridine Receptors (DHPRs) in myotubes from an old subject and compared them with those of a young subject.

In skeletal muscle, DHPR, an L-type calcium channel of external membranes (plasma membrane and transverse tubules or T-tubules) functions as a voltage sensor that is responsible for initiating the Excitation-Contraction (EC) coupling mechanism.[6-8] According to the mechanical coupling hypothesis, in response to depolarization of external membranes, the DHPR changes its conformation and consequently triggers the Sarcoplasmic Reticulum (SR) calcium release channel (or ryanodine receptor, RyR) opening directly[9] even in the absence of extracellular Ca^{2+}.[10,11] Several ultra-structural studies performed by Clara Franzini-Armstrong over the last 50 years[12] have strongly supported this hypothesis. The functional linkage requires a highly specific association of α1SDHPRs in correspondence of RyR1s (the skeletal muscle-specific isoforms of the two proteins): groups of four DHPRs linked to a single very large cytoplasmic domain of RyR (or foot)[13] define the corners of a square, and constitute a single unit called tetrad.[14-18] Dictating by their link with the tetrameric RyR cannels which are placed in arrays in the junctional sarcoplasmic reticulum (jSR) membrane, tetrads are associated with alternate feet and are placed in ordered arrays in junctional domains of T-tubules and surface membrane.[12,19,20]

The EC coupling occurs within specialized structures called Calcium Release Units (CRUs). In myotubes, in which the EC coupling machinery, transverse tubular system included, have poorly developed, functional CRUs are mostly composed by the association between junctional domains of SR with the surface membrane, and they are called peripheral couplings.[14,17,18,20] Peripheral couplings are structurally and functionally equivalent to the junctions between SR and T-tubules (CRUs or triads) which are predominant in mature muscle fibers. For this reason, clusters of EC coupling proteins are predominantly located at the cell periphery.

While RyRs feet are clearly visible in electron microscopy (EM) thin sections,[21] DHPRs are detected in freeze-fracture EM images as clusters of large intra-membranous particles appearing in the cytoplasmic leaflet (the membrane leaflet which is in contact with the cytoplasm) of the fractured surface membrane.[14,20-23]

In here, using the freeze-fracture we examined cultured human myotubes (human developing muscle) obtained upon growth and differentiation of cultured satellite cells (myoblasts precursor cells) from *vastus lateralis* biopsies (see Materials and Methods for details).[4] Specifically, after having identified and confirmed the presence of DHPRs clusters, we compared cluster incidence, size, and completeness of tetrads (4 particles) of myotubes obtained from a biopsy of a young subject (28 years) after 9 days of differentiation (YM9d) with myotubes obtained from a biopsy of an old subject (71 years) after 9 (OM9d) and 12 days (OM12d) of differentiation.

Our observations demonstrated that myotubes derived from the old subject at the earlier culture passage of differentiation (9 days) exhibited a significantly lower incidence of DHPR clusters and a smaller size of the clusters occupying patches of membrane compared to young myotubes at the same time point of differentiation. In addition, in DHPR clusters from OM9d, we detected a lower number of complete tetrads (4 particles) than in those from YM9d. At 12 days of differentiation values of incidence, size, and density of complete tetrads in myotubes from the old subject turned to be comparable with those of young myotubes at 9 days of differentiation.

All together these results suggest a slowing of the synthesis process of the proteins involved in the EC coupling mechanism in aged myotubes and help to support previous studies showing a decrease in the differentiation potential of myotubes derived from elderly individuals.

Materials and methods

Muscle samples

Biopsies from *Vastus Lateralis* (VL) muscles were obtained after informed consent from a young subject (28 years) and of an old subject (71 years), both healthy men undergoing elective orthopedic surgery. Each biopsy sample was collected in Ham's F-10 medium (GIBCO, Invitrogen, Carlsbad, California) supplemented with 50 μg/ml of gentamycin and stored at +4 °C until processing, which was carried out within 24 h of surgery.

Primary culture of myotubes

Muscle biopsies were processed to obtain explants placed in culture as described by Fulle *et al.* 2005.[24] The first mononucleated cells migrated out of the explants within 7 to 13 days from the beginning of culture. After removal of the muscle explants, the satellite cells are grown until they reach confluence in Growth Medium (GM), consisting of HAM's Nutrient Mixture F10 without L-Glutamine medium (GIBCO, Invitrogen) supplemented with 20% Defined Fetal Bovine Serum, US Origin (Euroclone, Pero, MI, Italy), 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin sulphate (Euroclone) and 1% Stable Glutamine (200mM) (Euroclone). Proliferative skeletal myoblasts (mononucleated cells) are induced to fuse into multinucleated myotubes seeded onto ECL-coated dishes. At 2–3 days after plating the medium was replaced with differentiation medium (DM) consisting of DMEM high glucose (Euroclone) supplemented with 5% horse serum (Euroclone), 50 μg/ml of gentamycin (Euroclone), 10 μg/ml of insulin (Sigma-Aldrich, Milan, Italy) and 100 μg/ml of apo-Transferrin (SIGMA). Cells were cultured to induce differentiation until day 9 and/ or 12. See also Pietrangelo *et al.* 2009 for details.

Freeze-fracture electron microscopy

Samples and replicas preparation

Myotubes at 9 days of differentiation from the young subject (YM9d) and myotubes at 9 and 12 days of differentiation from the old subject (OM9d and OM12d, respectively) were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) and infiltrated in 30% glycerol, for a minimum of 15 min, frozen in liquid propane, freeze-fractured under vacuum, shadowed with platinum at 45° and replicated with carbon in a Blazer's 400 freeze fracture unit (Balzers, model BFA 400; Balzers S.p.A., Milan, Italy). The replicas were examined in a Phillips 410 electron microscope (Philips Electron Optics, Mahwak, NJ) equipped with a Hamamatsu C4742-95 digital imaging system (Advanced Microscopy Techniques, Chazy, NY).

Quantitative analysis

Column A: the surface densities of DHPR clusters were estimated from freeze-fracture replicas of the cytoplasmic leaflet of the plasmalemma and expressed as average number (in 10 μm^2) ± SD. Micrographs were collected at 17700x magnifications by taking images of the cytoplasmic leaflet of each myotube with a central DHPR cluster. Each image covered about 35 μm^2 of area. 25-39 images were counted for each of the time points. The reason for choosing a non-random approach to data collection is that the groups of DHPR particles are clearly and unequivocally visible only when the shadow is at the appropriate angle, and the replica is of excellent quality. Since these circumstances vary from one area to the other of the replica, counting micrographs collected under the usual criteria of randomness would result in false data. Columns B and C: in micrographs at higher magnifications, the average area of a single DHPR cluster was measured using ImageJ software (v1.54f, National Institute of Health, USA), and the percentage of the total area of micrographs covered by clusters of DHPR was also calculated. Column D: in each single DHPR cluster the ratio of the number of complete tetrads (four particles) to the total number of tetrad-like arrangements (two and three particles) was counted and expressed as percentage ± SD. Column E: in each single DHPR cluster, the incidence of tetrads (four particles) was also evaluated and reported as the average value per area of the cluster (in 1 μm^2). Note: arrays of tetrads tend to be incomplete (lack of particles composing the tetrads) in images from freeze-fractures. It was not possible to establish which tetrads were actually absent and which were missing from the images due to fracturing problems; therefore, results in columns D and E cannot be accurately determined.

Statistical analysis

All data were presented as mean ± SD. Statistical significance was considered for values of $p < 0.05$. Statistical significance was evaluated using a non parametric two-tailed unpaired Student's t-test (Prism 5, GraphPad), or a Chi square test (for percentages) (Excel 365, Microsoft Office).

Results

The freeze-fracture replicas of the surface membrane from young and aged myotubes are distinguished by clusters of DHPR tetrads

Freeze fracture replicas of young and old myotubes had a smooth surface with a uniform distribution of intramembrane particles. In all replicas analyzed, numerous clusters of large intramembrane particles were present. Clusters occupied patches of the membrane of different area sizes.

We identified the clusters as groups of DHPR tetrads based on the following criteria (Figure 1): i) the particles forming them are unusually larger than most other particles in the membrane; ii) several particles are arranged in groups and occupy patches of the membrane which are slightly raised, due to the presence of junctional SR (jSR) underneath; iii) several groups of particles clearly show all four components; iv) the members of the group, even if somewhat incomplete, can be overlaid by an approximately tetragonal grid of dots marking the centre of each tetrad. Despite some distortion in fracturing, resulting in missing units of the tetrads, the disposition in elongated rows was clearly detectable in most of the clusters analysed (Figure 1).

Myotubes cultured from an old subject exhibited a slower onset of DHPR clusters accrual compared to those from the young subject

We freeze-fractured and analysed myotubes from a young (28 years) and an old (71 years) subject at different time points of differentiation. Specifically, we first looked at 9 days of differentiation.

DHPR arrays were found in the surface membrane of all replicas from young and old subjects examined after 9 days of differentiation (Figure 2). However, we immediately noted that in replicas from old myotubes, the incidence of clusters of DHPR particles was more rare and they were much smaller in size (Figure 2). Specifically, while in young myotubes (YM9d), DHPR clusters were clearly visible in each micrograph analyzed as often quite large in size, in old myotubes (OM9d), DHPR clusters were more rarely found, and the particle patch usually covered a smaller area of the plasmalemma (Figure 2).

Based on this first observation and taking into consideration the results of a previous work by Pietrangelo *et al.,* 2009 (4) showing a slower differentiation capability of aged myoblasts, we decided to look at old myotubes at 12 days of differentiation. We immediately noted that at this later point of differentiation, the visual incidence of clusters of tetrads and the area size of patches of membrane covered by DHPR particles became comparable to that of YM9d (Figure 2).

Results from the visual observation were supported by a detailed quantitative analysis. Incidence of DHPR clusters in the surface membrane was estimated from counts in freeze-fracture replicas (Table 1; see Materials and Methods for details). DHPR clusters are frequently found in myotubes from YM9d (6.4 ± 3.3 / 100 μm^2; Table 1, column A), as many groups of particles were visible in each micrograph analyzed. At the same stage of differentiation, in OM9d the incidence of DHPR clusters per area was significantly decreased (4.4 ± 1.6 / 100 μm^2; Table 1, column A).

We also measured the area of a single DHPR patch on the myotubes surface, and we confirmed that, in old myotubes, the clusters were, on average, significantly smaller than in the young myotubes (0.048 ± 0.022 vs 0.078 ± 0.095 μm^2, respectively; Table 1, column B).

These data, together with the reduced incidence of DHPR clusters per area observed in OM9d replicas was responsible for the decreased percentage of area covered by DHPR patches on the cytoplasmic leaflet of the plasmalemma of old myotubes (1.3 ± 0.5 vs 4.0 ± 3.1, respectively; Table 1, column C).

Notably, at the later stage of differentiation (12 days) the incidence of DHPR clusters on the cytoplasmic leaflet of the plasmalemma of old myotubes became more frequent (7.2 ± 5.7 / 100 μm^2; Table 1, column A), up to levels of the YM9d (6.4 ± 3.3 / 100 μm^2; Table 1, column A). Also the average area size of patches of cluster membrane returned to the value level of that of young myotubes so that the percentage of area covered by DHPR patches in OM12d (0.074 ± 0.057; Table 1, column C) resulted to be similar to that of YM9d (0.078 ± 0.095; Table 1, column C).

Within the DHPR clusters, density of groups of 4 particles (tetrads) was lower in old myotubes

We counted the number of groups of 4 particles (*i.e.* complete tetrads) within each DHPR cluster and reported this value as average percentage of complete tetrads compared to the total number of tetrads (2 or 3 particles) within each analyzed cluster and as average number of complete tetrads per cluster area of 1 m^2 (Table 1, columns D and E respectively; Figure 3).

In OM9d, the percentage and number of complete tetrads were significantly lower than in YM9d (27.7 ± 3.8 vs 49.7 ± 1.8 and 93.7 ± 29.7 vs 119.1 ± 40.0, respectively; Table 1, columns D and E). At 12 days of differentiation, the percentage and number of tetrads within each cluster in myotubes from the old subject reached values very similar to those of YM9d (40.4 ± 6.3 and 99.4 ± 44.7, respectively; Table 1, columns D and E).

Discussion

In here we wanted to study ultra-structural differences of DHPRs presence and arrangement in cultured human myotubes differentiated from biopsies of a young and an old subject. Association of DHPRs particles into arrays of tetrads were found in both young and old myotubes replicas. However, the incidence of DHPRs clusters and size of the membrane patch occupied by the DHPR

proteins is significantly lower in old myotubes at the earlier time point of differentiation (9 days) compared to young myotubes at the same time of differentiation.

Thanks to the enormous contribution of the ultra-structural work of Clara Franzini-Amstrong and collaborators,[12] it was established that, in order to provide sites of direct molecular interaction for EC coupling skeletal muscle activation, the four DHPRs clustered into tetrads in the external membrane (T-tubule or surface membrane), must be precisely associate with alternate feet (RyRs) placed in the facing SR membrane. Thus, in myotubes peripheral couplings (where the SR forms junctions with the surface membrane), the arrangement of tetrads and their organization in ordered arrays is dictated by the specific interaction with the RyR1 arrays in the junctional SR domain (14,25,26). The lack of tetrads as well as the absence of DHPRs alignment in two orthogonal directions, in 1B5s mouse skeletal muscle cell line that carries a null mutation for RyR1 (26) and their reappearance after transfection with cDNA encoding for the RyR1 (26) suggest that in old myotubes the rate of assembling of peripheral couplings (i.e. CRUs, the sites of EC couplings) might be lower than in young myotubes. Thus, in old myotubes at 9 days of differentiation, the lower size of the DHPR clusters area would suggest a smaller area of SR junctional membrane containing RyR arrays. Furthermore, the lower density of tetrads (4 particles) within each cluster would suggest that the arrays of RyRs may not be complete. These latest structural results are in agreement with those published in 2007, demonstrating that, compared to young subjects, in mature fibers from old individuals, there was not only a decreased number of CRUs but also, a high incidence of incomplete triads *i.e.* CRUs in which one or two feet were missing in the gap spanning the space between T-tubule and SR.

 Collectively, these results would suggest that in old myotubes the synthesis process of the proteins involved in the EC coupling mechanism, such as the DHPR, is somehow slowed. In mature fibers, a significant decrease in the amount of DHPRs in the transverse tubule membrane was detected with advancing age in rats by the laboratory of Delbono in 1997[27] and a partial impairment of the EC

coupling mechanism due to a partial uncoupling between RyRs and DHPRs has been proposed (EC uncoupling theory). The resulting inefficient activation of contraction has been proposed as one of the important factors in the age-related decline of muscle performance.[27] However, at least in myotubes, we also observed a recovery, even if at a slower rate, of the protein synthesis of old individuals compared to that observed in young people as a longer period of differentiation (12 days) values from all parameters analyzed are very similar to those of young myotubes at 9 days of differentiation. We haven't looked at young myotubes at 12 days of differentiation; therefore, it remains difficult to establish whether young myotubes would have retained the differences observed at 9 days with the old myotubes.

Conclusions

Satellite cells (*i.e.* to myo-stem cells) are responsible for skeletal muscle mass maintenance.[28-30] However, the capacity of satellite cells to repair muscle tissue following injury is reduced in the elderly.[31-34] It has been shown that aging negatively impacts the proliferation and differentiation capability of muscle satellite cells.[4-24]

The ultra-structural results presented in this study suggest that in myotubes differentiated from aged myoblasts (*i.e.,* satellite cells), the synthesis process of the proteins involved in the EC coupling mechanism, such as the DHPR, is somehow slowed, supporting previous evidence of a decrease in the differentiation potential of myotubes from elderly individuals.[2,3]

In conclusion, the ultra-structural results observed *in vitro* in myotubes differentiated from the old subject biopsy suggest that also *in vivo* the differentiation potential of satellite cells could be at a lower rate, thus compromising their capability to repair muscle tissue after injury.

Corresponding author: Simona Boncompagni, Center for Advanced Studies and Technology;, University G. d'Annunzio, Chieti, I-66100 Italy.
E-mail: simona.boncompagni@unich.it
ORCID: 0000-0001-5308-5069

Co-authors
Laura Pietrangelo
E-mail: laura.pietrangelo@unich.it
ORCID: 0000-0003-1197-7813

Stefania Fulle
E-mail: stefania.fulle@unich.it
ORCID: 0000-003-4557-9127

Rosa Mancinelli
E-mail: rosa.mancinelli@unich.it
ORCID: 0000-0002-1538-7784

Conflict of interest

The authors declare no conflicts of interest.

Ethics approval

Muscles are obtained from healthy untrained patients who underwent elective orthopedic surgery, after informed consent. The study is conformed with the Helsinki Declaration of 1964, as revised in 2013, concerning human and animal rights.

Informed consent

All patients participating in this study signed a written informed consent form for participating in this study.

Patient consent for publication

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

References

1.   Wiedmer P, Jung T, Castro JP, et al. Sarcopenia - Molecular mechanisms and open questions. Ageing Res Rev 2021;65:101200.

2.   Domingues-Faria C, Vasson MP, Goncalves-Mendes N, et al. Skeletal muscle regeneration and impact of aging and nutrition. Ageing Res Rev 2016;26:22-36.

3.   Yamakawa H, Kusumoto D, Hashimoto H, Yuasa S. Stem cell aging in skeletal muscle regeneration and disease. Int J Mol Sci 2020;21:1830.

4.   Pietrangelo T, Puglielli C, Mancinelli R, et al. Molecular basis of the myogenic profile of aged human skeletal muscle satellite cells during differentiation. Exp Gerontol 2009;44:523-31.

5.   Cade WT, Yarasheski KE. Metabolic and molecular aspects of sarcopenia. In: Runge MS, Patterson C (eds.), Principles of Molecular Medicine. Springer; 2006.

6.   Tanabe T, Beam KG, Powell JA, Numa S. Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. Nature 1988;336:134–9.

7.   Adams BA, Tanabe T, Mikami A, et al. Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs. Nature 1990;346:569-72.

8.   Rios E, Brum G. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. Nature 1987;325:717–20.

9.   Schneider MF, Chandler WK. Voltage dependent charge movement of skeletal muscle: a possible step in excitation-contraction coupling. Nature 1973;242:244–6.

10.   Rios E, Ma JJ, Gonzalez A. The mechanical hypothesis of excitation-contraction (EC) coupling in skeletal muscle. J Muscle Res Cell Motil 1991;12:127–35.

11.   Schneider MF. Control of calcium release in functioning skeletal muscle fibers. Annu Rev Physiol 1994;56:463–84.

12.   Franzini-Armstrong C. An updated view of the structural basis for dihydropyridine receptors-ryanodine receptors direct molecular interaction in skeletal muscle. Eur J Transl Myol 2024;34:12476.

13.   Franzini-Armstrong C. Studies of the triad: I. Structure of the junction in frog twitch fibers. J Cell Biol 1970;47:488–99.

14.   Block BA, Imagawa T, Campbell KP, Franzini-Armstrong C. Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. J Cell Biol 1988;107:2587–600.

15.   Franzini-Armstrong C. Simultaneous maturation of transverse tubules and sarcoplasmic reticulum during muscle differentiation in the mouse. Dev Biol 1991;146:353-63.

16.   Takekura H, Sun X, Franzini-Armstrong C. Development of the excitation-contraction coupling apparatus in skeletal muscle: peripheral and internal calcium release units are formed sequentially. J Muscle Res Cell Motil 1994;15:102–18.

17.   Takekura H, Bennett L, Tanabe T, et al. Restoration of junctional tetrads in dysgenic myotubes by dihydropyridine receptor cDNA. Biophys J 1994;67:793-803.

18.   Protasi F, Franzini-Armstrong C, Allen PD. Role of ryanodine receptors in the assembly of calcium release units in skeletal muscle. J Cell Biol 1998;140:831–42.

19. Protasi F, Franzini-Armstrong C, Flucher BE. Coordinated incorporation of skeletal muscle dihydropyridine receptors and ryanodine receptors in peripheral couplings of BC3H1 cells. J Cell 1997;137:859–70.

20. Franzini-Armstrong C, Kish JW. Alternate disposition of tetrads in peripheral couplings of skeletal muscle. J Muscle Res Cell Motil 1995;16:319-24.

21. D Appelt 1, B Buenviaje, C Champ, C Franzini-Armstrong. Quantitation of 'junctional feet' content in two types of muscle fiber from hind limb muscles of the rat. Tissue Cell 1989;21:783-94.

22. Franzini-Armstrong C. Freeze-fracture of frog slow tonic fibers. Structure of surface and internal membranes. Tissue Cell 1984;16:647-64.

23. Takekura H, Shuman H, Franzini-Armstrong C. Differentiation of membrane systems during development of slow and fast skeletal muscle fibres in chicken. J Muscle Res Cell Motil 1993;14:633-45.

24. Fulle S, Di Donna S, Puglielli C, et al. Age-dependent imbalance of the antioxidative system in human satellite cells. Exp Gerontol 2005;40:189-97.

25. Protasi F. Structural interaction between RYRs and DHPRs in calcium release units of cardiac and skeletal muscle cells. Front Biosci 2002;7:d650-8.

26. Protasi F, Takekura H, Wang Y, et al. RYR1 and RYR3 have different roles in the assembly of calcium release units of skeletal muscle. Biophys J 2000;79:2494–508.

27. Renganathan M, Messi ML, Delbono O. Dihydropyridine receptor-ryanodine receptor uncoupling in aged skeletal muscle. J Membr Biol 1997;157:247–53.

28. Dayanidhi S, Lieber RL. Skeletal muscle satellite cells: Mediators of muscle growth during development and implications for developmental disorders. Muscle Nerve 2014;50:723-32.

29. Snijders T, Nederveen JP, McKay BR, et al. Satellite cells in human skeletal muscle plasticity. Front Physiol 2015;6:283.

30. Pallafacchina G, Blaauw B, Schiaffino S. Role of satellite cells in muscle growth and maintenance of muscle mass. Nutr Metab Cardiovasc Dis 2013;23:S12-8.

31. Hawke TJ, Garry DJ. Myogenic satellite cells: Physiology to molecular biology. J Appl Physiol (1985) 2001;91:534-51. Erratum in: J Appl Physiol 2001;91:2414.

32. Chen W, Datzkiw D, Rudnicki MA. Satellite cells in ageing: Use it or lose it. Open Biol 2020;10:200048.

33. Alway SE, Myers MJ, Mohamed JS. Regulation of satellite cell function in sarcopenia. Front Aging Neurosci 2014;6:246.

34. Sousa-Victor P, García-Prat L, Muñoz-Cánoves P. Control of satellite cell function in muscle regeneration and its disruption in ageing. Nat Rev Mol Cell Biol 2022;23:204-26.
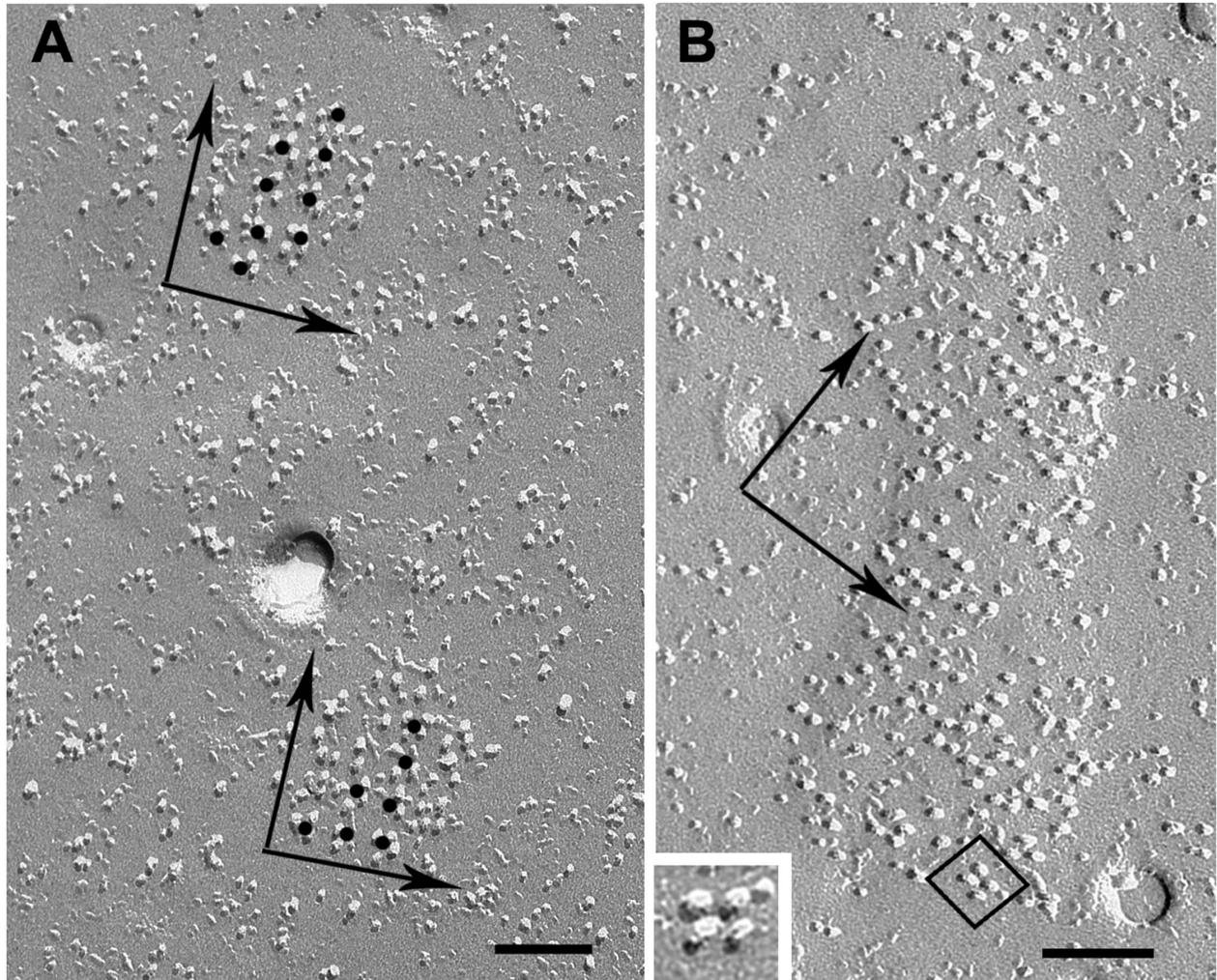
Figure 1. Arrays of DHPRs in the surface membrane of human myotubes. DHPR arrays are easily

detectable and occupy patches of membrane of different area size. The patch of membrane on

which DHPR arrays reside is slightly raised (better visible in panel B). Alignment of tetrads

components in the arrays is particularly well visible (more easily viewable by obtaining a grazing

view in the direction of the arrows) since the members of the group, even if somewhat incomplete,

can be overlaid by an approximately tetragonal grid of dots marking the centre of each tetrad (panel

A). Inset: higher magnification of a tetrad (four particles) marked by the box in panel B. *Scale bar:*

0.1 μm.

Table 1. Quantitative freeze-fracture electron microscopy analysis of DHPR clusters in myotubes from a young subject (28 years) at 9 days of differentiation, and in myotubes from an old subject (71 years) at 9 and 12 days of differentiation.

	A	**B**	**C**	**D**	**E**
	No. of DHPR clusters /100 μm²	**Average area of DHPR clusters, μm²**	**Area of clusters/ total area (%)**	**% of tetrads /DHPRs cluster**	**Tetrads /1 μm² of cluster area**
YM9d	6.4 ± 3.3 (25)	0.078 ± 0.095 (346)	4.0 ± 3.1 (25)	49.7 ± 1.8 (191)	119.1 ± 40.0 (191)
OM9d	4.4 ± 1.6* (25)	0.048 ± 0.022* (143)	1.3 ± 0.5* (25)	27.7 ± 3.8* (220)	93.7 ± 29.7* (220)
OM12d	7.2 ± 5.7[#] (39)	0.074 ± 0.057 (365)	2.8 ± 1.7 (39)	40.4 ± 6.3 (336)	99.4 ± 44.7 (336)

In parenthesis, columns A and C number of micrographs analysed; columns B, D and E number of DHPR clusters analysed. *$p < 0.05$ vs YM9d and [#]$p < 0.05$ vs OM9d.
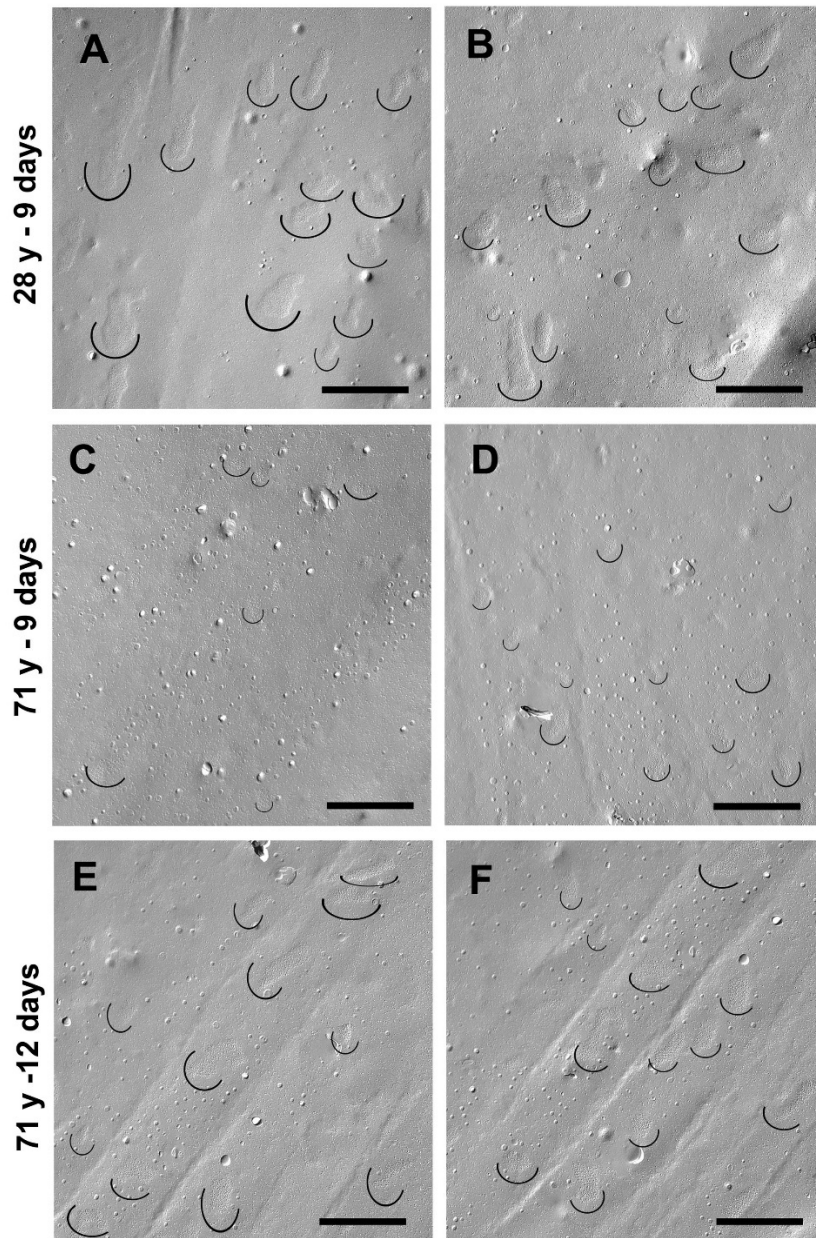
Figure 2. Representative freeze-fracture replicas images of young myotubes at 9 days and of old myotubes at 9 and 12 days of differentiation. In each electron micrograph, DHPR clusters have been appropriately marked by drawing a black line that partly follows the patch profile to facilitate visualization and comparison between arrays of particles of young and old myotubes. *Scale bar: 0.5 μm.*
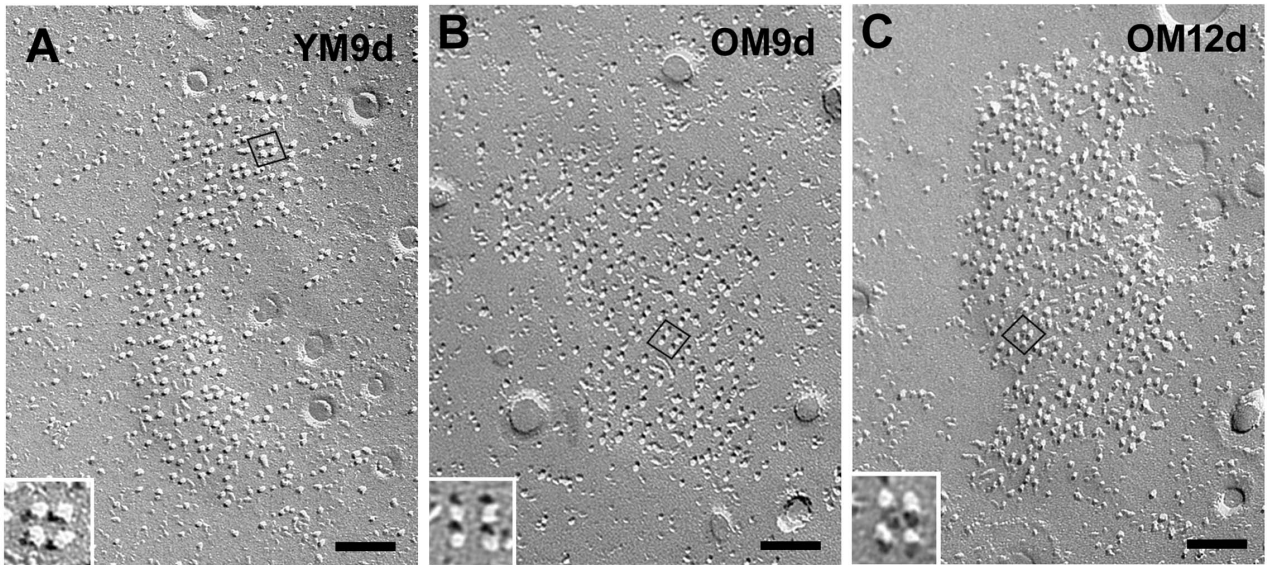
Figure 3. Representative freeze-fracture replicas images of clusters of tetrads. EM images of tetrads from the young subject at 9 days (A) and from old subject at 9 (B) and 12 days (C) of differentiation. Insets: examples of complete tetrads. *Scale bar: 0.1 μm.*