Biochemical and Biophysical Research Communications 473 (2016) 462–470

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Myomir dysregulation and reactive oxygen species in aged human satellite cells

Ester Sara Di Filippo [a, b], Rosa Mancinelli [a, b], Tiziana Pietrangelo [a, b], Rita Maria Laura La Rovere [a, b], Mattia Quattrocelli [c], Maurilio Sampaolesi [c, d, *], Stefania Fulle [a, b, **]

[a] Interuniversity Institute of Myology (IIM), Department of Neuroscience Imaging and Clinical Sciences, University "G. d'Annunzio" Chieti-Pescara, Chieti, Italy
[b] Center for Excellence on Ageing (CeSI), "G. d'Annunzio" Foundation, Chieti, Italy
[c] Translational Cardiomyology Laboratory, Stem Cell Biology and Embryology Unit, Department of Development and Regeneration, KU Leuven, Leuven, Belgium
[d] Division of Human Anatomy, Department of Public Health, Experimental and Forensic Medicine, University of Pavia, Pavia, Italy

ARTICLE INFO

Article history:
Received 25 February 2016
Accepted 8 March 2016
Available online 11 March 2016

Keywords:
Aging
myomiRNAs
Human satellite cells
Sarcopenia
ROS

ABSTRACT

Satellite cells that reside on the myofibre surface are crucial for the muscle homeostasis and regeneration. Aging goes along with a less effective regeneration of skeletal muscle tissue mainly due to the decreased myogenic capability of satellite cells. This phenomenon impedes proper maintenance and contributes to the age-associated decline in muscle mass, known as sarcopenia. The myogenic potential impairment does not depend on a reduced myogenic cell number, but mainly on their difficulty to complete a differentiation program. The unbalanced production of reactive oxygen species in elderly people could be responsible for skeletal muscle impairments. microRNAs are conserved post-transcriptional regulators implicated in numerous biological processes including adult myogenesis. Here, we measure the ROS level and analyze myomiR (miR-1, miR-133b and miR-206) expression in human myogenic precursors obtained from *Vastus lateralis* of elderly and young subjects to provide the molecular signature responsible for the differentiation impairment of elderly activated satellite cells.

1. Introduction

During aging, skeletal muscle is subjected to progressive loss of muscle mass and strength, which is defined as sarcopenia. This is an age-related process known to be correlated to regenerative difficulties by satellite cells (SCs), adult muscle stem cells, which represent the main cell population involved in regeneration processes of adult skeletal muscle. They are in the quiescent state beneath the basal lamina, and upon activation they both proliferate and maintain undifferentiated phenotype or fuse each other and differentiate into multinucleated myotubes. However, in elderly subjects, the decreased muscle regenerative capability does not appear to be due to a reduced number of myogenic cells [1–3]. The mechanisms behind the impaired myogenic differentiation process of elderly people are not fully understood, although they appear to involve an imbalance between reactive oxygen species (ROS) production and antioxidant enzyme activity and alterations in the gene expression [2,4]. Among the ROS there are radicals as superoxide anion ($O_2^{\bullet -}$) that are normally produced by mitochondria at very low level in the cell, but $O_2^{\bullet -}$ production can increase producing high level of other ROS as H_2O_2 [5]. ROS can modulate the transcriptional profiles of many genes [6] and in suitable conditions provoke damage into the cells. Myogenic regulatory factors (MRFs) involved in differentiation programmes, including *MyoD1* (Myogenic Differentiation 1) and *Myog* (Myogenin) [7] can regulate, or be regulated by muscle-specific miRNAs, myomiRs (miR-1, miR-133, miR-206), small non-coding RNAs that binding to mRNA targets interfere with protein synthesis. Emerging evidence has

* Corresponding author. Department of Public Health, Experimental and Forensic Medicine, University of Pavia, Via Forlanini, 8, 27100 Pavia, Italy.
** Corresponding author. Department of Neuroscience Imaging and Clinical Sciences, University "G. d'Annunzio" of Chieti-Pescara, Via dei Vestini, 29, 66100 Chieti, Italy.
E-mail addresses: es.difilippo@unich.it (E.S. Di Filippo), r.mancinelli@unich.it (R. Mancinelli), tiziana@unich.it (T. Pietrangelo), ritalarovere@unich.it (R.M.L. La Rovere), mattia.quattrocelli@med.kuleuven.be (M. Quattrocelli), sampa@unipv.it, Maurilio.Sampaolesi@med.kuleuven.be (M. Sampaolesi), sfulle@unich.it (S. Fulle).

http://dx.doi.org/10.1016/j.bbrc.2016.03.030

demonstrated that these myomiRs are essential for myoblast differentiation, proliferation and apoptosis [8–10]. ROS can also modulate myomiRs since H_2O_2 exposure can induce concentration-dependent apoptosis mediated by increasing miR-1 expression [11].

We hypothesised that ROS-dependent MRF and myomiR can be altered and involved in the myogenic impairment of elderly SCs. To verify this we isolated SCs from skeletal muscle of young and aged men and measured: (1) levels of intracellular $O_2^{\bullet -}$ production; (2) myomiR expression (also in myotubes); (3) gene expression profile of MRF (also in myotubes) including *Pax3* (Paired Box 3 gene), *Pax7* (Paired Box 7 gene), *Myf5* (Myogenic Factor 5 gene), *MyoD1*, *Myog*, and proliferation, atrophy, ubiquitin-proteasome and inflammation pathways; (4) trans-membrane mitochondrial potential in living cells. The data from this study suggest that the alteration of myomiR expression and ROS production in old SCs could contribute to the impaired differentiation capacity of skeletal muscles in elderly subjects.

2. Materials and methods

2.1. Samples

Muscle samples were derived from *Vastus Lateralis* muscle biopsies using the percutaneous needle-biopsy [12]. Young (22.8 ± 4.0 years; n = 6) and old (76.6 ± 3.7 years; n = 8) healthy untrained male subjects underwent voluntary biopsies. These subjects all provided written informed consent before participating in the present study. This study was approved by the Ethics Committee for Biomedical Research, University of Chieti (PROT 1884 COET), and complied with the Declaration of Helsinky (as amended in 2000). The inclusion criteria were normal ECG and blood pressure, and absence of metabolic, cardiovascular, chronic bone/joint and muscular diseases.

2.2. Cell cultures

The muscle biopsies were processed according to the procedures of Fulle et al. [13]. The cells were cultured for growth and differentiation according to Pietrangelo et al. [2]. To assay the cell proliferative capability were calculated the PDL (population doubling level) at 20 days of culture. Pericytes were isolated according to Dellavalle et al. [14]. The alkaline phosphatase expression was detected by enzymatic activity using the NBT/BCIP kit (Roche).

2.3. Flow cytometric analysis

Human cell cultures were also evaluated by flow cytometric analysis for surface myogenic markers using CD56 (Mouse monoclonal antibody (Abcam, Cambridge, UK) and 5.1H11 policlonal antibody (Developmental Studies Hybridoma Bank (DSHB), Iowa City, Iowa, USA) [15]. The cells were detached with trypsin-EDTA 1X in PBS without phenol red calcium and magnesium (EuroClone, Milan, Italy) and washed with PBS. After washing, cells were incubated with CD56 and 5.1H11 Abs, following the manufacturer specifications and with the appropriate isotypes as internal controls, at FACS Canto (BD, Franklin Lakes, NJ, USA), then the data were displayed using the FlowJo software (Tree Star, Ashland, OR, USA). Data by FACS are mean ± SD of five independent experiments in triplicates and expressed as percentage.

2.4. Fluorescence-activated cell sorting

To enrich the cell cultures in myogenic precursor, at least 1,000,000 cells per sample were detached with trypsin-EDTA in PBS without phenol red, calcium and magnesium, and washed with PBS. After washing, the cells were incubated for 30 min at 4 °C with specific primary antibodies for human myoblasts that targeted a cell-surface protein, as CD56 and 5.1H11. After being washed with PBS, the cells were incubated with Alexa Fluor 488 donkey anti-mouse IgG (Molecular Probes, Life Technologies, Carlsbad, CA, USA) for 30 min at 4 °C, and then washed again in PBS. The human myogenic precursors (expressing both CD56 and 5.1H11) were isolated by fluorescence-activated cell sorting (FACS) at FACS Aria III (BD). These human $CD56^+/5.1H11^+$ cells were replaced in petri dishes and cultured in growth or differentiation medium for microRNA isolations and further RT-PCR analysis.

2.5. Immunocytochemistry and immunofluorescence analyses

To estimate the myogenic purity and the differentiative capacity of cell cultures immunocytochemistry assays were performed as described by Fulle et al. [13]. In addition, the myogenic differentiation was determined by fusion index as the ratio of the number of nuclei in differentiated myotubes (positive for MyHC) and the total number of nuclei. Immunofluorescence analysis was performed as described by Dellavalle et al. [14]. The following antibodies were used in this study: MF20 antibody at 1:5 dilution; anti smooth alpha actin (Sigma, 1:300 dilution) and anti-desmin (Sigma, 1:50 dilution); NG2 (Chemicon, 1:250 dilution); anti PDGF receptor beta (Cell Signaling Technologies, 1:500 dilution). After incubation, samples were washed three times with BSA/PBS and then incubated with the appropriate FITC or TRTC conjugated anti mouse or anti-rabbit IgG for 1 h at RT. After three final washes, the cover slips were mounted on glass slides and analysed under an inverted fluorescent microscope (Nikon). Immunostaining and fusion index data derived from at least 1000 cells counted in at least 10 different randomly chosen optical fields of each culture.

2.6. Quantitative real-time PCR for myogenic transcriptional factors

RNA was extracted with Purelink RNA Mini Kits (Invitrogen, Life Technologies) and genomic DNA contamination was removed using the TURBO DNA-Free kits (Ambion, Life Technologies). The RNA was quantified using a NanoDrop™ spectrophotometer. After 500 ng reverse transcribed by Superscript III First-Strand Synthesis SuperMix kit (Invitrogen, Life Technologies). Quantitative real-time PCR (qPCR) was performed on 1:5 diluted cDNA, via Platinum Sybr Green SuperMix-UDG (Invitrogen, Life Technologies), as 15 s at 95 °C, 45 s at 60 °C, for 40 cycles.

Primers: Pax3 FW TGTTCAGCTGGGAAATCCGAGACA, Pax3 REV GTCGATGCTGTGTTTGGCCTTCTT, Pax7 FW AAGCACTGTGCCCTCAGGTTTAGT, Pax7 REV GTCGATGCTGTGTTTGGCCTTCTT, MyoD1 FW TGCTCCGACGGCATGATGGACTA, MyoD1 REV TTGTAGTAGGCGCCTTCGTAGCAGTT, Myf5 FW TGAGAGAGCAGGTGGAGAACTACT, Myf5 REV AGACAGGACTGTTACATTCGGGCA, Myogenin FW TACAGATGCCCACAACCTGCACT, Myogenin REV TTTCATCTGGGAAGGCCACAGACA. Internal standard, GAPDH FW TGGTATCGTGGAAGGACTCATGAC, GAPDH REV ATGCCAGTGAGCTTCCCGTTCAGC.

The values represent the mean ± SD of three independent experiments performed in triplicate. GAPDH was used as the housekeeping gene, to normalise the mRNA levels, and the data are shown as ΔCt.

2.7. NBT assay

The spectrophotometric NBT (Nitro blue tetrazolium chloride, SIGMA-Aldrich, Milan, Italy) assay, based on the reduction of NBT in Nitro blue-formazan in the presence of $O_2^{\bullet -}$, was performed according to Sozio et al. [16]. Conventionally, NBT assay is used to

determine the production of $O_2^{\bullet -}$.

2.8. Measurement of ROS

Intracellular ROS were quantified by the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA Cat. No. D6883; Sigma) using a SPECTRAmax Gemini XS microplate fluorometer (Molecular Devices, Sunnyvale, CA, U.S.A.). The fluorescence intensity represents the mean of values registered during 5 min ± SEM [16].

2.9. Determination of mitochondrial membrane potential

The mitochondrial membrane potential was determined using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodide/chloride; Molecular Probes), which is a fluorescent cationic carbocyanine dye that accumulates in the mitochondria. The human myoblasts were plated in special-optics 96-well (Corning-Costar, SIGMA-Aldrich). The cells were loaded with JC-1 (10 μM, 100 μl/well) for 15 min at 37 °C. The fluorescence intensity of JC-1 was then measured at a constant temperature of 25 °C using a microplate reader (SpectraMAX Gemini XS; J-aggregates, excitation/emission 560/595 nm; JC-1 monomers, excitation/emission 488/522 nm). The fluorescence was recorded from each well every for 10 min, in imaging buffer. The cells were then stimulated with 100 nM H_2O_2 and all of the recordings were acquired on a computer using SoftMax Pro (version 5.0). The values of fluorescence are expressed as ratio between red and green fluorescence value emission means ± SEM at four time-points.

2.10. RT-PCR on custom TaqMan array cards

RT-PCR was performed on myoblasts, using TaqMan low density array (Applied Biosystems, Foster City, CA, USA) as described in Fulle et al. [17]. The relative quantification of target gene expression was evaluated with the data from the SDS software, using the arithmetical formula $2^{-\Delta\Delta Ct}$, according to the comparative Ct method, which represented the amount of the target normalised to the endogenous control (GAPDH).

2.11. miRNA isolation and quantification

Small RNA extractions from the young and old samples were performed using PureLink miRNA Isolation kits (Invitrogen, Life Technologies) following the manufacturer instructions. According to the procedure of La Rovere et al. [18]. The ubiquitously produced miR-16 was used as an internal control. miR-16 has been identified as a candidate control gene as showing the least variability in cell lines from Applied Biosystems (Application note TaqMan® MicroRNA Assays). In according to manufacturing indications, Ct values do not change in our samples between proliferation and differentiation condition. The relative quantification of the miRNA targets was carried out using the ΔCt formula ($Ct_{miRNA\ of\ interest} - Ct_{miR\text{-}16}$), according to the Ct method. Three independent experiments were performed in quintuplicate.

2.12. Western blotting

The myoblasts cultured in growth medium, were lysed in RIPA buffer (Sigma–Aldrich) supplemented with 1:100 protease inhibitor cocktail (Sigma–Aldrich), and 1:100 phosphatase inhibitor cocktail (Sigma–Aldrich). Protein extract was quantified by Bio-Rad protein assay (Bio-Rad Laboratories, Milan, Italy). Total protein mixes (140 μg) were separated on a 4–12% Bis-Tris Plus Gels (Life Technologies) and transferred to a nitrocellulose membrane (iBlot 2 Dry Blotting System (Life Technologies). After blocking for 1 h at RT with 5% Skim-milk (Sigma–Aldrich) in TBS buffer supplemented with 0.1% Tween-20 (Sigma–Aldrich), the membrane was incubated overnight at 4 °C with primary antibodies, as follows: HSP60 (D6F1) XP Rabbit mAb (Cell Signalling Technology, Danvers, MA, USA) at 1:1000, HSP70 Antibody (Cell Signalling Technology) at 1:1000, GAPDH (Santa Cruz Biotechnology, INC) at 1:600. After incubation with anti-rabbit or anti-mouse IgG, HRP-linked Antibody (Cell Signalling Technology) at 1:2000 for 1 h at RT. Immunodetection was performed with the LiteAblot PLUS enhanced chemiluminiscent substrate (EuroClone). Images were taken at GelDoc machine (Bio-Rad). Western blots densitometric analysis was performed with ImageJ software, a Java-based image analysis package available free for download from the National Institutes for Health (NIH).

2.13. Statistical analysis

Unpaired *t*-test with Welch's correction was performed old *versus* young using Prism5 GraphPad software (Abacus Concepts, GraphPad Software, SanDiego, CA, USA). A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Myogenic characteristics of cultures

The percentage of two surface markers/$^{ve+}$ (CD56 and 5.1H11 [19]) cells plus desmin$^{/ve+}$ cells and fusion index were considered to depict the myogenic characteristics of our human myoblast populations derived from young and old subjects (Table 1). In young and old samples the percentage of desmin$^{/ve+}$ cells (69.7 ± 8.5 and 64.3 ± 6.1 respectively) and CD56$^{/ve+}$/5.1H11$^{/ve+}$ cells (75.1 ± 7.0 and 66.4 ± 5.3, respectively) were not significantly different, confirming a good myogenicity of our cultures. The FI was significantly different in young and old samples (52,1 ± 5,7 and 41,8 ± 8,1, respectively; $^*p < 0.05$); while the percentage of unfused desmin$^{/ve+}$ cells was higher in old samples than young ones (42.5 ± 8.0 and 32.5 ± 9.6, respectively, $^*p < 0.05$). Also the myoblasts proliferative capability after 20 days of culture was higher in young respect to old samples (9.1 ± 1.3 PDL and 6.7 ± 1.1 PDL, respectively, $^*p < 0.05$).

3.2. MRF expression profile of activated satellite cells and pericyte characterization from young and old muscles

The analysis of the MRFs, including *PAX-3*, *PAX-7*, *MYF5*, *MYOD* and *MYOG*, revealed that during proliferation state these genes were significantly down-regulated in the myoblasts from the elderly than those from the young subjects (Fig. 1a). In the old samples after 7 days in differentiation medium (Fig. 1a), the expression of earlier genes such as *Myf5* and *MyoD* was up-regulated, while *Myog* was down-regulated with respect to the young samples. This results in a reduced number of Myosin heavy chain (MyHC, Fig. 1b) positive myotubes in old samples compared to young ones despite the comparable amount of desmin positive cells (Desm, Fig. 1b). However, the number of pericytes and their typical markers, including alkaline phosphatase, PDGFRβ (Platelet-Derived Growth Factor Receptor Beta), NG2 (neural/glial antigen 2) and α-SMA (alpha Smooth Muscle Actin) were not affected by aging (Fig. 1c).

3.3. ROS measurement

Due to known implication of ROS in several cell signalling, including the above-mentioned correlation between ROS and

Table 1
Myogenicity, Fusion Index, Unfused Desm$^{/ve+}$ percentage and proliferation rate (PDL). The percentage of myogenic cells in our cultures was analysed by immunostaining for desmin and by FACS for two surface myogenic markers (CD56 and 5.1H11). Fusion index was determined by counting the number of nuclei in differentiated myotubes (positive for MyHC) after 7 days of culture in differentiation medium and reported as a percentage of the total number of nuclei; further they were counted the unfused desmin positive cells after 7 days differentiation. To assay the cell proliferative capability was calculated the PDL at 20 days of culture. Data by FACS are mean ± SD of five independent experiments in triplicates and expressed as percentage. Immunostaining and FI data derived from at least 1000 cells counted in at least 10 different randomly chosen optical fields of each culture. All data (n = 6 young; n = 8 old) are presented as mean ± SD $^{*}p < 0.05$.

Samples	Desmin$^{/ve+}$ cell %	CD56^{+}/5.1H11^{+} cell %	Fusion Index %	Unfused desmin$^{/ve+}$ cell %	PDL at 20 days of culture
Young	69.7 ± 8.5	75.1 ± 7.0	52.1 ± 5.7	32.5 ± 9.6	9.1 ± 1.3
Old	64.3 ± 6.1	66.4 ± 5.3	41.8 ± 8.1*	42.5 ± 8.0*	6.7 ± 1.1*

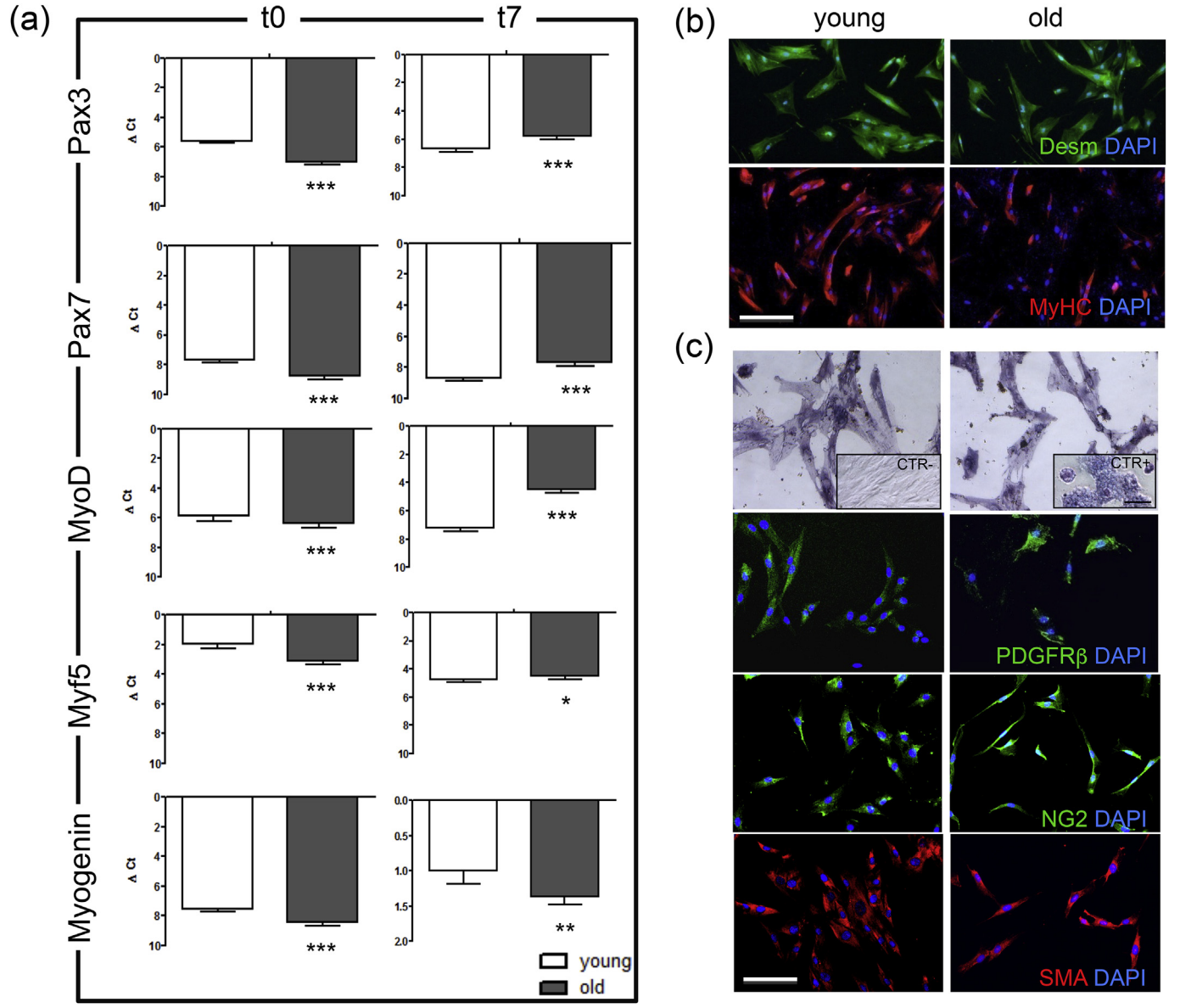

Fig. 1. Gene expression of MRFs in human myoblasts and myotubes of young and old subjects. (a) Levels of expression of myogenic genes (Pax3, Pax7, Myf5, MyoD1, Myogenin) under proliferation (t0) and after myogenic induction (t7). Three independent experiments were performed, each in triplicate. Data are means ± SD and were analysed using unpaired *t*-tests (old *vs* young). GAPDH was used as the housekeeping gene, to normalise the mRNA levels, and the data are shown as ΔCt ($Ct_{gene\ of\ interest} - Ct_{GAPDH}$). Smaller ΔCts indicate higher expression levels. $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$. (b) Immunofluorescence analysis for the localization of Desm (green) and MyHC (red) in satellite cells and myotubes respectively is shown. (c) Immunofluorescence analysis for the localization of PDGFR β (green), NG2 (green), and α-SMA (red) in pericytes isolated from young and old muscle biopsies; scale bar = 50 μm. The alkaline phosphatase staining in isolated pericytes is also reported (c, upper panels); human fibroblasts and human embryonic stem cells were used as negative (CTR-) and positive (CTR+) controls respectively; scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

apoptosis, we analysed ROS levels in young and elderly myoblasts by NBT assay and measurements of H_2DCF-DA fluorescence. The basal levels of intracellular ROS and more specific the $O_2^{\bullet -}$ level were significantly higher in elderly myoblasts than in young ones

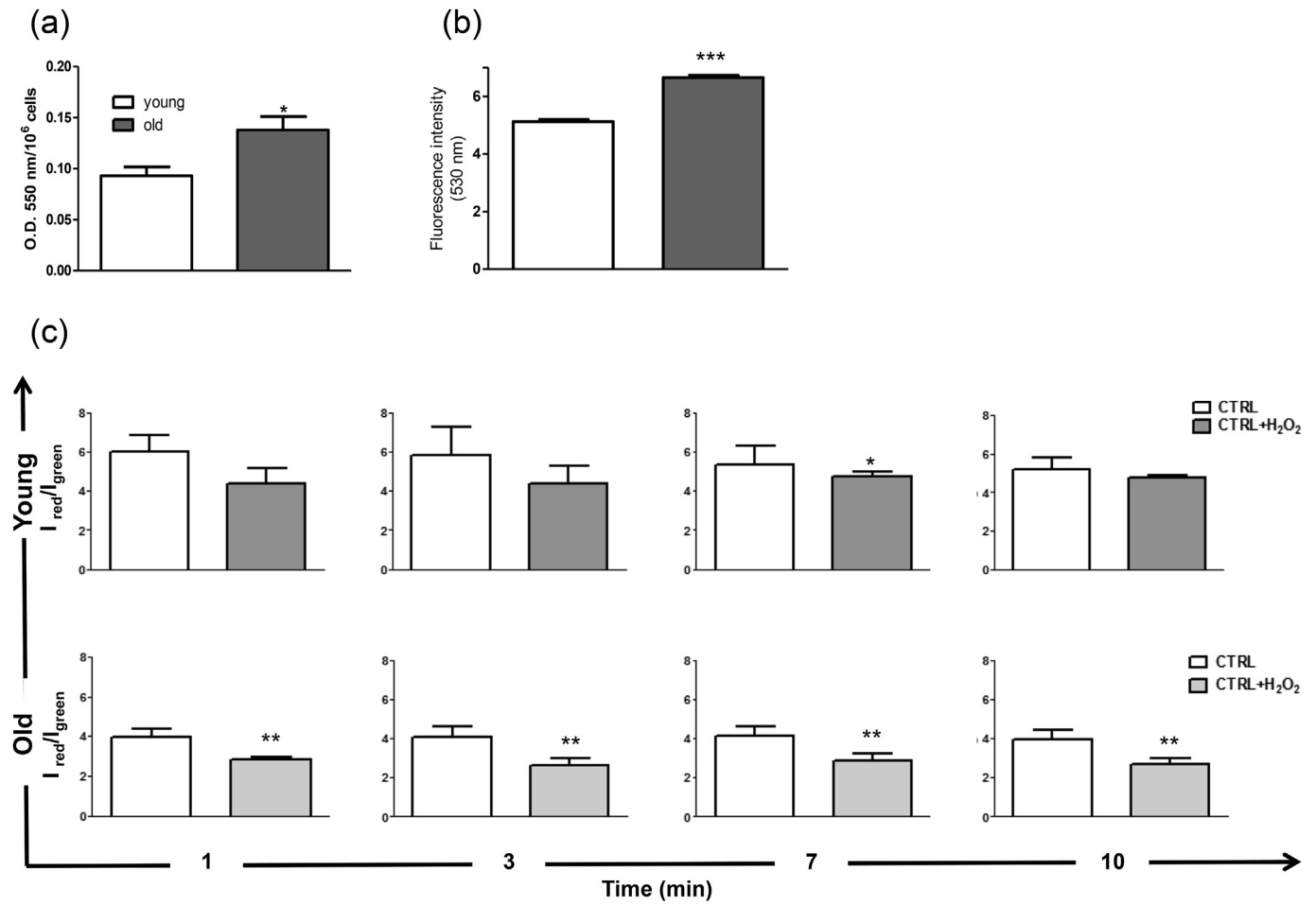

Fig. 2. ROS levels and time course of JC-1 fluorescence emission. Quantitative analyses in myoblasts from young and old subjects of $O_2^{\bullet -}$ (a) by NBT assay and intracellular ROS (b) levels by H_2DCF-DA fluorescence. Data are mean ± SEM of three independent experiments each performed in quintuplicate. *p < 0.05, ***p < 0.0001. Panel (c) shows time course of JC-1 fluorescence ratio between red and green emission at four time-points. The myoblasts were followed for 10 min without or with addition of 100 nM H_2O_2. Data are means ± SEM of three different experiments performed in quintuplicate. *p < 0.05, **p < 0.005.

(Fig. 2a, b).

3.4. Trans-membrane mitochondrial potential measurement

We wondered if the trans-membrane mitochondrial potential ($\Delta\Psi_m$) could be affected by age impairing the homeostasis of these organelles. The living myoblasts from the old muscle samples, in the presence of JC-1 probe, showed a $\Delta\Psi_m$ significantly lower than for the young muscle samples. Moreover, the treatment with the oxidant agent 100 nM H_2O_2, consistently decreased the $\Delta\Psi_m$ of elderly myoblasts demonstrating specific difficulty to recover and maintain the $\Delta\Psi_m$ to control values (Fig. 2c).

3.5. miRNA expression

The MRF expression can affect myomiRs regulation, in particular miR-1, miR-133b and miR-206, which at the same time modulate the MRF family members affecting myogenesis. Furthermore, it is demonstrated that high levels of ROS may increase the expression of miRNAs, in particular miR-1. For these reasons we analysed the expression of muscle-specific miRNA. During the proliferation state (Fig. 3a), in old muscle samples miR-1was significantly up-regulated, miR-133b was slightly down-regulated and miR-206 did not significantly change with respect to young muscle samples. During differentiation (Fig. 3b), both miR-1 and miR-133b were significantly up-regulated in elderly muscle respect to young muscle samples, while miR-206 expression did not change. All these miRNAs were up-regulated in differentiation than proliferation conditions. The same analysis in the enriched cultures of $CD56^{/ve+}/5.1H11^{/ve+}$ myogenic precursors obtained by sorting young and old myoblast populations, confirmed the myomiR expression results (data not shown).

3.6. Heat shock protein expression

Since miR-1 can inhibit Hsp60 and Hsp70, normal inhibitors of apoptotic mitochondrial pathway [11] we assessed the Heat shock proteins (HSPs) expression, finding them significantly less expressed in aged myoblasts than in young ones (Fig. 4e, f).

3.7. Specific pathway gene expression

In this scenario, specific transcriptome signature of selected genes of proliferation, atrophy, ubiquitin-proteasome and inflammation pathways related to regeneration and sarcopenia were studied in satellite cells (Fig. 4a, b, c, d). The analysis of key cell-cycle and proliferation genes showed significant down-regulation of cyclin dependent kinases (*CDK1*, *CDK2*, *CDK4* and *CDK7*), *CCNB1*(human cyclin B1), *CCNE2* (human cyclin E2) (Fig. 4a). CyclinB1 and CDK1 interact with each other and are involved in

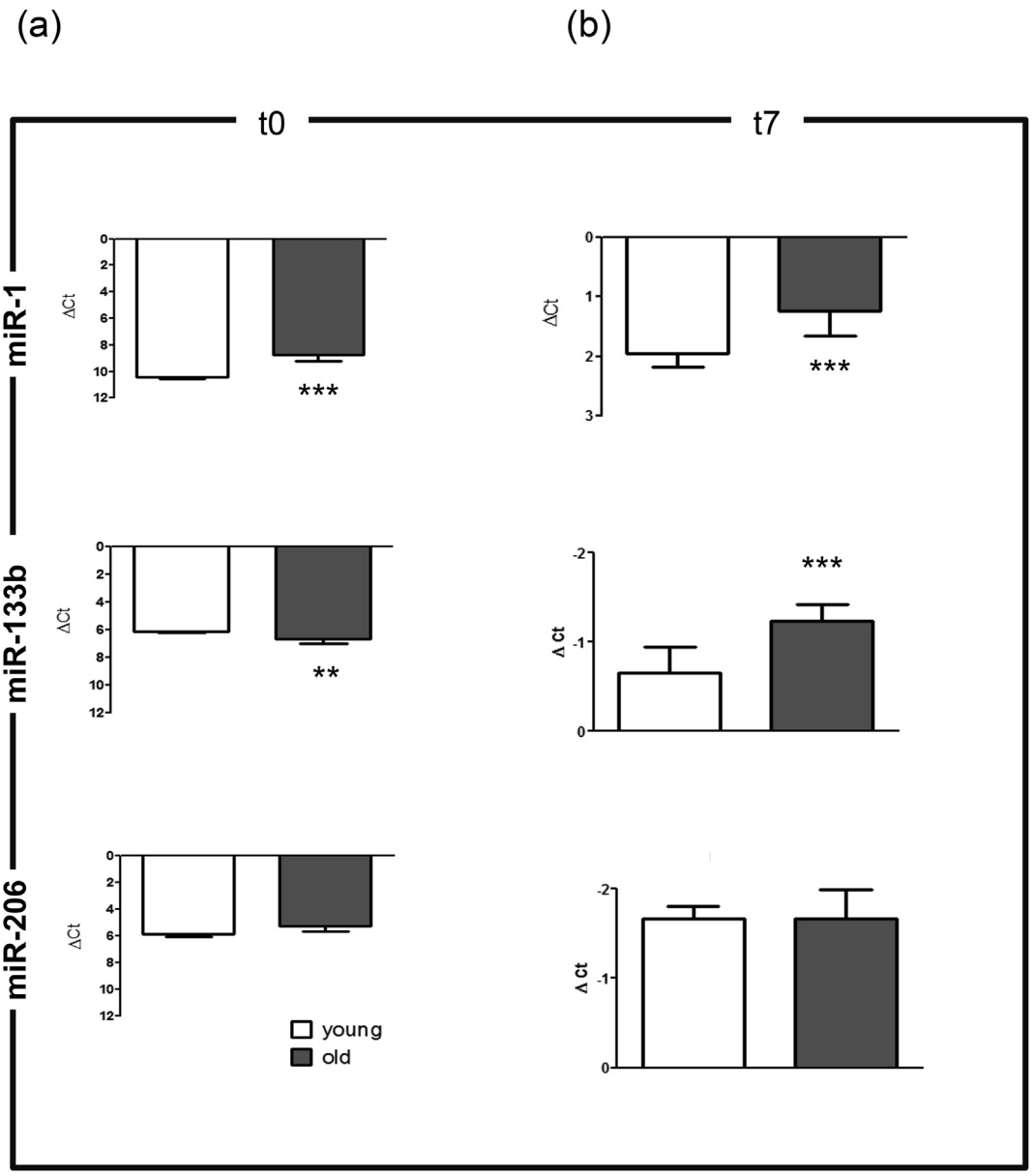

Fig. 3. myomiR analysis in SCs isolated from young and old subjects. The expression levels of miR-1, miR-133, miR-206 were analysed during proliferation (a) and after 7 days from myogenic induction (b). The ubiquitous miR-16 was used as housekeeping gene to normalize miRNA levels. Three independent experiments were performed in quintuplicates and results are shown as ΔCt ($Ct_{miRNA\ of\ interest} - Ct_{miR\text{-}16}$). Data are mean ± SEM; $^{**}p < 0.005$, $^{***}p < 0.0001$.

cell-cycle control for the regulation of entry into mitosis [20]. Cyclin E2 forms a complex with, and functions as a regulatory subunit of, CDK2 and it is responsible for cell-cycle transition from G1 to S phase. Instead *DMTF1* and *MSTN* resulted up-regulated. Of the atrophic pathway, *FOXO1* was up-regulated while *AKT1*, *PIK3CA*, *MTOR* genes, part of the PI3K-AKT-mTOR pathway that is involved in protein synthesis as a positive regulator, and *MAPK1* were down-regulated (Fig. 4b). Looking at the ubiquitin-proteasome system, our selected genes (*MUL1*, *PSMC6*, *UBA1* and *UBE2A*) were up-regulated (Fig. 4c). As ROS related-apoptosis can be triggered by alterations in the inflammatory system, the expression of some cytokines were also analysed. The genes *IL1R1*, *IL18*, *IL15* were up-regulated while *IL6* and *LIF* were down-regulated (Fig. 4d).

4. Discussion

The impairment of myogenic differentiation in human elderly myoblasts has been confirmed in the present work by the reduced fusion index values of human myoblasts, despite similar percentage of desmin$^{/ve+}$ and CD56$^{/ve+}$/5.1H11$^{/ve+}$ cells between young and old myoblasts. In injured muscle or during growth, the SCs become activated, and they proliferate (*Pax3* and *Pax7* highly expressed) and activate *Myf5*, necessary for initial expansion of SCs [21], and *MyoD1*, with the down-regulation of *Pax3* and *Pax7*, followed by *Myog* expression increase and terminal differentiation. SC self-renewal is assured by a minority of SCs that maintain high *Pax7* levels [22]. Indeed we observed this typical MRF modulation during differentiation of our human myoblast populations derived from young subjects, whereas in elderly subjects all MRF genes were down-regulated.

Recent studies show that pericytes may have a profound impact in skeletal muscle regeneration, fibrosis and fat accumulation [23]. Our data clearly showed that pericytes are present in the elderly muscles in a sufficient number, and they express typical pericytic

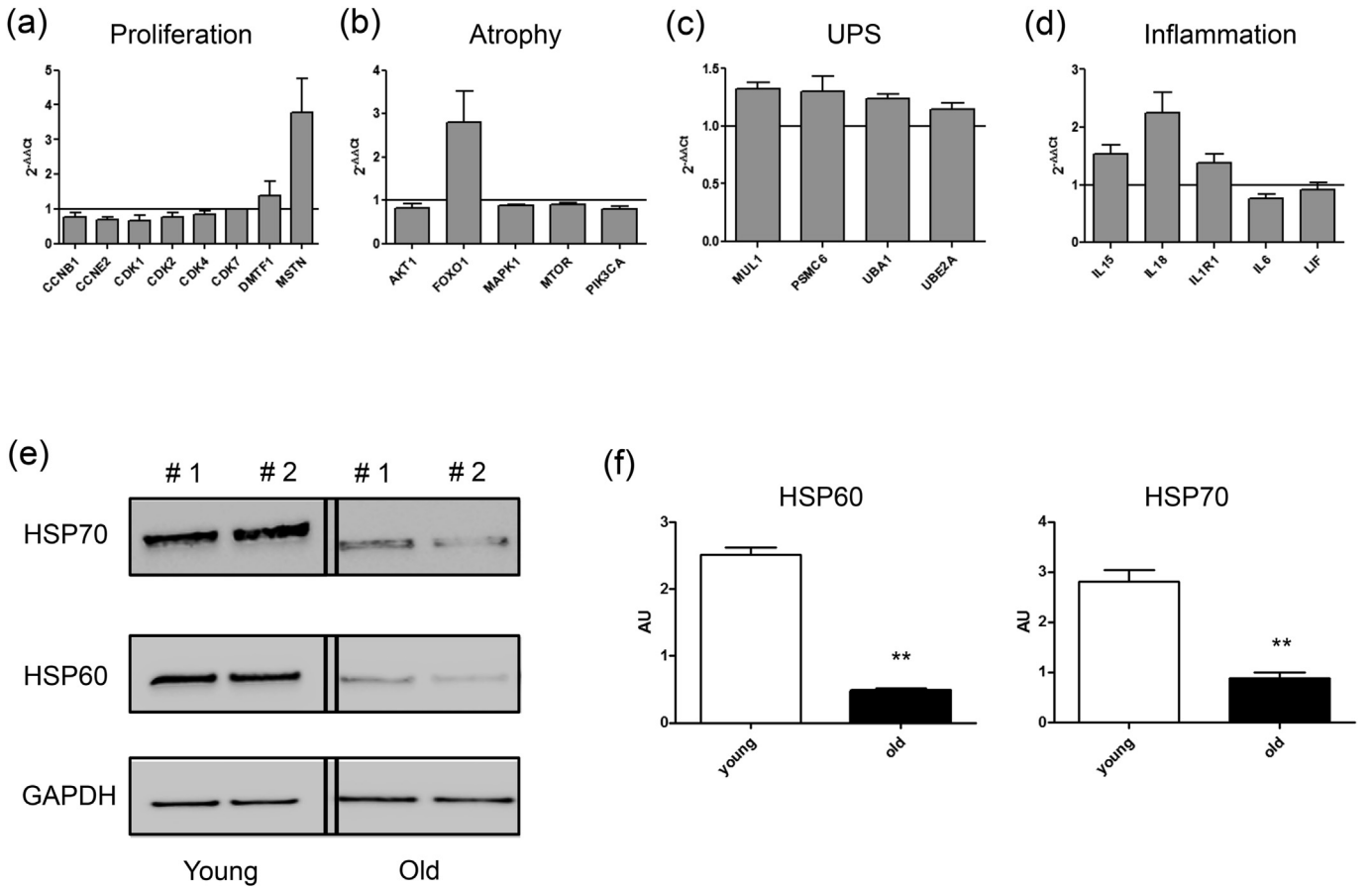

Fig. 4. Specific signalling pathways and expression of Hsp60 and Hsp70 during proliferation in myoblasts derived from young and old subjects. Expression levels of genes involved in proliferation (a), atrophy (b), ubiquitin-proteasome system (UPS) (c) and inflammation (d) pathways. Gene expression are analysed using RT-PCR with TaqMan low density arrays. Data are means ± SEM of three independent experimental sets (n = 4 each) and were analysed using unpaired t-tests (old *vs* young). e) Representative patterns of Hsp60, Hsp70 and GAPDH (as loading control) expression in myoblasts deriving from two young and two old samples. f) Densitometric analysis expressed as AU (arbitrary units) of all performed Western blots. $^{**}p < 0.005$.

markers similarly to their young counterparts, although we cannot exclude an impairment in their functions.

We have recently established that in the elderly skeletal muscle a high percentage of the myogenic precursors are subjected to spontaneous apoptosis triggered by caspase-9 [17]. It is knows that the ROS accumulation, that is typical during ageing, through the perturbation of pro- and anti-apoptotic rheostat, could trigger the mitochondria-dependent apoptosis. The ROS could be produced by different biochemical pathways, and in particular by mitochondrial activity, which produces the superoxide anion ($O_2^{\bullet -}$) as potentially one of the most dangerous ROS [5]. We measured a general oxidant level, and in particular $O_2^{\bullet -}$ production significantly increased in elderly myoblasts compare to young ones.

The intrinsic apoptotic process involves ROS-dependent transmembrane potential ($\Delta\Psi_m$) alteration of mitochondria [24]. We found $\Delta\Psi_m$ significantly decreased in elderly myoblasts. Generally the $\Delta\Psi_m$ decrease is linked to a complex mechanism that also involves $[Ca^{2+}]_i$ increase and in turn leads to mitochondrial Ca^{2+} uptake provoking $O_2^{\bullet -}$ production via mitochondria uncoupling [25]. The $\Delta\Psi_m$ decrease is demonstrated also after the exposure to H_2O_2, when the $\Delta\Psi_m$ collapsed. We also found that the ROS, and specifically $O_2^{\bullet -}$ production, and miR-1 levels in elderly myoblasts were significantly higher than those in young ones. Therefore, unbalanced mitochondrial activity and the presence of oxidative stress alter critical cellular processes that in turn negatively affect elderly myogenesis. miR-206 is normally up-regulated by Pax7/MyoD1 cascade and Myogenin. The lack of miR-206 modulation in the elderly with respect to the young samples is consistent with literature since miR-206 expression in skeletal muscle tissue was found not different across time or between young and old samples [26].

The specific MRF and miRNA modulation could be responsible for impaired fusion index since myomiRs regulate metabolic pathways and they are also altered in the aged male skeletal muscles [26]. Here we show for the first time that in old male satellite cells miR-1 is up-regulated, miR-133b is down-regulated during proliferation, while miR-206 is unaltered.

Data reported in the literature demonstrated that the modulation of miR-1 and miR-133b has been linked to the activation of apoptosis [25,27]. Moreover, it has been demonstrated in other muscle cells that miR-1 participated in the oxidant-dependent apoptotic insult [27] and that HSP70 [28] and HSP60 [29] are miR-1 targets. Interestingly, we observed the up-regulation of miR-1 only in proliferating elderly myoblasts and consequently the protein content of Hsp60 and Hsp70 was significantly reduced compared to young ones.

Nevertheless, we found miR-1 and miR-133b up-regulated in myotubes similarly to what observed in male myofibers by Nielsen et al. [26]. This suggests a specific role of elderly myoblasts in determining the detrimental changes occurring in skeletal muscle during aging. Both miR-1 and miR-133 are induced by SRF and play important roles in myogenic proliferation and differentiation. In

general, miR-1 pushes myoblasts toward terminal myogenic differentiation [25].

The role of miR-133 during differentiation process is quite controversial in literature [30–33]. Chen et al. showed that miR-133 hinders myoblast differentiation and promotes myoblast proliferation [30] while other groups showed that miR-133 promotes myogenic differentiation [31–33]. This discrepancy might be due to different experimental conditions and the different effects on cardiac and skeletal myocytes. Thus it remains controversial whether miR-133 promotes or inhibits muscle cell proliferation. Here, we reported in elderly myotubes an impairment of differentiation along with both the *Myogenin* down-regulation and miR-133b up-regulation, accordingly with Chen et al. [30], who showed that miR-133 overexpression repressed *Myogenin* in C2C12 cells.

Considering the impairment of elderly myoblasts in ROS management, we investigated some other pathways possibly involved in oxidative stress defective state. Genes representative of cell cycle, atrophy, ubiquitin-proteasome system (UPS) and inflammation were dysregulated in elderly myoblasts. The genes involved in cell cycle progression (*CCNB1*, *CCNE2*, *CDK1*, *CDK2*, *CDK4* and *CDK7*) were all down-regulated in old samples. This could explain the lower proliferation rate at 20 days of cultures of elderly myoblasts with respect to young ones. The highest percentage, after 7 differentiation days, of unfused Desmin^{ve+} cell in our culture of elderly samples, further confirms the inability to progress to a terminal differentiation. *DMTF1* accordingly with the literature, we found *MSTN Myostatin* overexpression along with *MyoD1* and *Pax3* expression down-regulation in elderly myoblasts [34]. Furthermore, also *FOXO1* up-regulation, modulated by IGF-1/PI3K/AKT/mTOR pathway [35], is consistent with the low levels of AKT-P we previously found in aged myoblasts and myotubes [3]. Moreover, p53 overexpression [36] could depend on *DMTF1*, thus promoting apoptosis [37]. It is worth mentioning, we previously showed apoptotic commitment in elderly myoblasts [17]. The down-regulation of *PI3K* and *LIF* along with the up-regulation of *IL-18* in elderly myoblasts and it is involved in apoptosis and tissue destruction [38,39]. This situation seems further exacerbated by ubiquitin proteasome system pathway. *MUL1* that results up-regulated in elderly samples, promotes mitochondrial fragmentation, and inhibits cell growth.

In conclusion we showed that during aging activated SCs display a complex picture of impaired regenerative potential, linked to altered ROS production and miR-1 and miR-133 dysregulation. The findings of this study suggest that myomiR modulation and ROS inhibitors should be further explored for combined therapy in sarcopenic muscles.

Conflict of interests

The authors declare that there is no conflict of interests.

Acknowledgements

We thank Dr. Vittore Verratti for his valuable skill in the needle-biopsies execution and Christofer Berrie for his manuscript generous editing. Sampaolesi is supported by Opening The Future Campaign (EJJ-OPTFUT-02010), CARE-MI FP7 (#QPG-394601), AFM-Téléthon (#18073), Programmafinanciering KUL PF/10/00300, CARIPLO (2015-0634), FWO (#G088715N, #G060612N, #G0A8813N), GOA (EJJ-C2161-GOA/11/012), IUAP-VII/07 (EJJ-C4851-17/07-P) and OT#09-053 (EJJ-C0420-OT/09/053) grants. Mancinelli, Pietrangelo and Fulle are supported by University "G. d'Annunzio" of Chieti-Pescara local research grants and by MIUR research grants [FIRB 2012 (RBFR12BUMH_005 Mancinelli), PRIN 2012 (2012N8YJC3_001 Pietrangelo) and PRIN 2010-2011 (2010R8JK2X_007 Fulle)].

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.03.030.

References

[1] S. Beccafico, C. Puglielli, T. Pietrangelo, R. Bellomo, G. Fanò, S. Fulle, Age-dependent effects on functional aspects in human satellite cells, Ann. N. Y. Acad. Sci. 1100 (2007) 345–352.
[2] T. Pietrangelo, C. Puglielli, R. Mancinelli, S. Beccafico, G. Fanò, S. Fulle, Molecular basis of the myogenic profile of aged human skeletal muscle SCs during differentiation, Exp. Gerontol. 44 (2009) 523–531.
[3] S. Beccafico, F. Riuzzi, C. Puglielli, et al., Human muscle satellite cells show age-related differential expression of S100B protein and RAGE, Age Dordr. 33 (2011) 523–541.
[4] G. Fanò, P. Mecocci, J. Vecchiet, et al., Age and sex influence on oxidative damage and functional status in human skeletal muscle, J. Muscle Res. Cell Motil. 22 (2001) 345–351.
[5] C.L. Quinlan, I.V. Perevoshchikova, M. Hey-Mogensen, A.L. Orr, M.D. Brand, Sites of reactive oxygen species generation by mitochondria oxidizing different substrates, Redox Biol. 1 (2013) 304–312.
[6] K. Gundersen, Excitation-transcription coupling in skeletal muscle: the molecular pathways of exercise, Biol. Rev. Camb. Philos. Soc. 86 (2011) 564–600.
[7] P.K. Rao, R.M. Kumar, M. Farkhondeh, S. Baskerville, H.F. Lodish, Myogenic factors that regulate expression of muscle-specific microRNAs, PNAS 103 (2006) 8721–8726.
[8] Z.P. Huang, R. Espinoza-Lewis, D.Z. Wang, Determination of miRNA targets in skeletal muscle cells, Methods Mol. Biol. 798 (2012) 475–490.
[9] M.J. Drummond, J.J. McCarthy, M. Sinha, et al., Aging and microRNA expression in human skeletal muscle: a microarray and bioinformatics analysis, Physiol. Genomics 43 (2011) 595–603.
[10] M. Sharma, P.K. Juvvuna, H. Kukreti, C. McFarlane, Mega roles of microRNAs in regulation of skeletal muscle health and disease, Front. Physiol. 5 (2014) 239.
[11] J.M. Shookhoff, G.I. Gallicano, The emerging role of microRNAs in adult stem cells, in: D.G. Phinney (Ed.), Adult Stem Cells – Biology and Methods of Analysis, Springer New York Dordrecht Heidelberg, Humana Press, London, 2011, pp. 54–97.
[12] T. Pietrangelo, L. D'Amelio, C. Doria, R. Mancinelli, S. Fulle, G. Fanò, Tiny percutaneous needle biopsy: An efficient method for studying cellular and molecular aspects of skeletal muscle in humans, Int. J. Mol. Med. 27 (2011) 361–367.
[13] S. Fulle, S. Di Donna, C. Puglielli, et al., Age-dependent imbalance of the antioxidative system in human satellite cells, Exp. Gerontol. 40 (2005) 189–197.
[14] A. Dellavalle, M. Sampaolesi, R. Tonlorenzi, et al., Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells, Nat. Cell Biol. 9 (2007) 255–267.
[15] F. Trimarchi, A. Favaloro, S. Fulle, L. Magaudda, C. Puglielli, D. Di Mauro, Culture of human skeletal muscle myoblasts: timing appearance and localization of dystrophin-glycoprotein complex and vinculin-talin-integrin complex, Cells Tissues Organs 183 (2006) 87–98.
[16] P. Sozio, L.S. Cerasa, S. Laserra, et al., Memantine-sulfur containing antioxidant conjugates as potential prodrugs to improve the treatment of Alzheimer's disease, Eur. J. Pharm. Sci. 49 (2013) 187–198.
[17] S. Fulle, S. Sancilio, R. Mancinelli, V. Gatta, R. Di Pietro, Dual role of caspase enzymes in satellite cells from aged and young subjects, Cell Death Dis. (2013) e955.
[18] R.M. La Rovere, M. Quattrocelli, T. Pietrangelo, et al., Myogenic potential of canine craniofacial satellite cells, Front. Aging Neurosci. 6 (2014).
[19] F.S. Walsh, M.A. Ritter, Surface antigen differentiation during human myogenesis in culture, Nature 289 (1981) 60–64.
[20] Y. Li, Y.L. Chen, Y.T. Xie, et al., Association study of germline variants in CCNB1 and CDK1 with breast cancer susceptibility, progression, and survival among Chinese Han women, PLoS One (2013) e84489.
[21] S. Ustanina, J. Carvajal, P. Rigby, T. Braun, The myogenic factor Myf5 supports efficient skeletal muscle regeneration by enabling transient myoblast amplification, Stem Cells (2007) 2006–2016.
[22] M. Buckingham, F. Relaix, The role of Pax genes in the development of tissues and organs: Pax3 and Pax7 regulate muscle progenitor cell functions, Annu. Rev. Cell Dev. Biol. 23 (2007) 645–673.
[23] D. Costamagna, E. Berardi, G. Ceccarelli, M. Sampaolesi, Adult stem cells and skeletal muscle regeneration, Curr. Gene Ther. 15 (2015) 348–363.
[24] S. Fulle, L. Centurione, R. Mancinelli, et al., Stem cell ageing and apoptosis, Curr. Pharm. Des. 18 (2012) 1694–1717.
[25] T. Pietrangelo, E.S. Di Filippo, R. Mancinelli, et al., Low intensity exercise training improves skeletal muscle regeneration in women by managing the oxidative status, Front. Physiol. 6 (2015) 399.
[26] S. Nielsen, T. Hvid, M. Kelly, et al., Muscle specific miRNAs are induced by testosterone and independently upregulated by age, Front. Physiol. 4 (2014)

394.
[27] W.H. Davin Townley-Tilson, T.E. Callis, Da-Zhi Wang, MicroRNAs 1, 133, and 206: critical factors of skeletal and cardiac muscle development, function, and disease, Int. J. Biochem. Cell Biol. 42 (2010) 1252–1255.
[28] L. Yong-Eng, H. Chung-Ye, L. Yi-Ling, C. Ruei-Ming, MicroRNA-1 participates in nitric oxide-induced apoptotic insults to MC3T3-E1 cells by targeting heat-shock protein-70, Int. J. Biol. Sci. 11 (2015) 246–255.
[29] Z.X. Shan, Q.X. Lin, C.Y. Deng, et al., miR-1/miR-206 regulate Hsp60 expression contributing to glucose-mediated apoptosis in cardiomyocytes, FEBS Lett. 584 (2010) 3592–3600.
[30] J.F. Chen, E.M. Mandel, J.M. Thomson, et al., The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation, Nat. Genet. 38 (2006) 228–233.
[31] N. Liu, S. Bezprozvannaya, A.H. Williams, et al., microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart, Genes Dev. 22 (2008) 3242–3254.
[32] A. Carè, D. Catalucci, F. Felicetti, et al., MicroRNA-133 controls cardiac hypertrophy, Nat. Med. 13 (2007) 613–618.
[33] B. He, J. Xiao, A.J. Ren, et al., Role of miR-1 and miR-133a in myocardial ischemic postconditioning, J. Biomed. Sci. 18 (2011) 18–22.
[34] Y. Elkina, S. von Haehling, S.D. Anker, J. Springer, The role of myostatin in muscle wasting: an overview, J. Cachexia Sarcopenia Muscle 2 (2011) 143–151.
[35] M. Sandri, L. Barberi, A.Y. Bijlsma, et al., Signalling pathways regulating muscle mass in ageing skeletal muscle: the role of the IGF1-Akt-mTOR-FoxO pathway, Biogerontology 14 (2013) 303–323.
[36] L. Barberi, B.M. Scicchitano, M. De Rossi, et al., Age-dependent alteration in muscle regeneration: the critical role of tissue niche, Biogerontology 14 (2013) 273–392.
[37] D.P. Frazier, R.D. Kendig, F. Kai, et al., Dmp1 physically interacts with p53 and positively regulates p53's stability, nuclear localization, and function, Cancer Res. 72 (2012) 1740–1750.
[38] L.C. Hunt, E.M. Tudor, J.D. White, Leukemia inhibitory factor-dependent increase in myoblast cell number is associated with phosphotidylinositol 3-kinase-mediated inhibition of apoptosis and not mitosis, Exp. Cell Res. 316 (2010) 1002–1009.
[39] L. Austin, J. Bower, J. Kurek, N. Vakakis, Effects of leukemia inhibitory factor and other cytokines on murine and human myoblast proliferation, J. Neurol. Sci. 112 (1992) 185–191.