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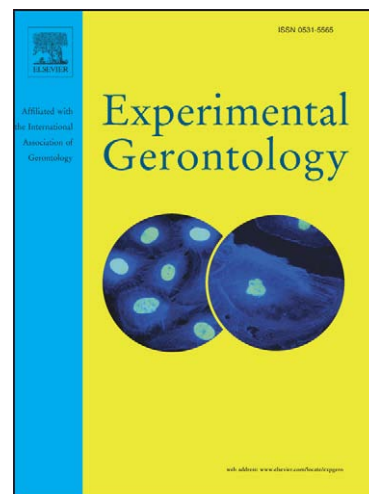
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**Molecular basis of the myogenic profile of aged human skeletal muscle satellite cells
during differentiation**

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Abstract

1
2 *Sarcopenia* is the age-related loss of muscle mass, strength and function. Human
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4 muscle proteins are synthesized at a slower rate in the elderly than in young adults, leading to
5
6 atrophy and muscle mass loss with a decline in the functional capability. Additionally, aging
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8 is accompanied by a decrease in the ability of muscle tissue to regenerate following injury or
9
10 overuse due to the impairment of intervening satellite cells, in which we previously reported
11
12 oxidative damage evidences. The aim of the present study was to determine the effects of
13
14 aging on myoblasts and myotubes obtained from human skeletal muscle, and characterize the
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16 transcriptional profile as molecular expression patterns in relation to age-dependent
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18 modifications in their regenerative capacity. Our data show that the failure to differentiate
19
20 does not depend on reduced myogenic cell number, but difficulty to complete the
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22 differentiation program. Data reported here suggested the following findings: i) oxidative
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24 damage accumulation in molecular substrates, probably due to impaired antioxidant activity
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26 and insufficient repair capability, ii) limited capability of elderly myoblasts to execute a
27
28 complete differentiation program; restricted fusion, possibly due to altered cytoskeleton
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30 turnover and extracellular matrix degradation, and iii) activation of atrophy mechanism by
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32 activation of a specific FOXO-dependent program.
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Introduction

Sarcopenia is defined as the age-related loss of muscle mass, strength and function (Vandervoort et al., 2001; Waters et al., 2000) associated with a decline in cell functional capability (Fulle et al., 2004). This process appears to begin in the fourth decade of life, and accelerates after approximately 75 years of age (Waters et al., 2000). Several factors of both intrinsic (metabolic pathway modifications, hormonal crisis, and cellular changes) and extrinsic (lifestyle and caloric administration) origin contribute to *sarcopenia*. However, certain factors appear more important than others. For instance, age-related changes in the neuromuscular system play an important role in the onset of *sarcopenia*. The numbers of spinal cord motor neurons and functioning motor units decline with age (Roth et al., 2000a; Roubenoff, 2001). This is a continuous, irreversible process throughout life (Waters et al., 2000). Another factor affecting *sarcopenia* is the rate of muscle protein synthesis. Accumulating evidence shows that the human muscle protein synthesis rate is lower in older adults, compared with young individuals (Nair, 1995; Proctor et al., 1998). These processes result in atrophy and loss of muscle mass. However, this theory was disputed by reports showing that essential amino acids (Drummond et al., 2008) or, however, high quality proteins sufficient ingestion, increase the muscle protein synthesis equally in both young and old subjects (Tang and Phillips, 2009). Aging is additionally related to the deregulation of Growth Hormone (GH) secretion and IGF-1 levels, testosterone (T) secretion in men, and ovarian steroid secretion in women (*menopause*). Interestingly, sex hormones are an important factor in maintaining muscle mass and strength in men, but not women. GH and IGF-1 play dominant roles in the regulation of protein metabolism. GH and T are required for protein maintenance, while IGF-1 levels are positively correlated with muscle protein synthesis rates, specifically, those of myofibrillar protein (actin and myosin filaments) and myosin heavy chains (part of the myosin-containing

1 cross-bridges) (Waters et al., 2000). A sustained decrease in these hormones is linked to a
2 reduction in muscle mass and increase in body fat (Janssen et al., 2000). In fact some reports
3 show that 6-month treatment with low-dose GH alone or with T in healthy elderly men
4 produces comparable increments in whole body protein turnover and protein synthesis
5 (Giannoulis et al., 2008)..

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11 Reactive oxygen species (ROS) additionally play a key role in triggering *sarcopenia* in
12 skeletal muscle during age progression (Hiona and Leeuwenburgh, 2008). Cells are exposed
13 to oxidative stress as a result of one or more of three factors: i) increase in oxidant generation,
14 ii) decrease in antioxidant protection, and iii) failure to repair oxidative damage. ROS cause
15 damage to lipids, nucleic acids and proteins (Berlett and Stadtman, 1997; Mecocci et al.,
16 1999), and lead to a functional deficit in the control of Ca^{2+} homeostasis in myofibers as a
17 consequence (Eu et al., 2000; Fulle et al., 2004; Fulle et al. 2005; Squier and Bigelow, 2000).
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29 The aging process is associated with a consistent decrease in the ability of muscle tissue to
30 regenerate following injury or overuse due to the impairment of intervening satellite cells
31 (Cooper et al., 2006). Satellite cells are undifferentiated quiescent mononucleated cells
32 present in muscle (Charge and Rudnicki, 2004; Mauro, 1961), which have properties of stem
33 cells and are activated as a consequence of strenuous muscle work and/or injury. In response
34 to muscle damage, satellite cells are activated to proliferate as myogenic precursor cells (mpc)
35 or myoblasts. Proliferating myoblasts migrate to the damaged region of the fiber, where they
36 differentiate and fuse to form myotubes via a similar process to that of myogenesis (*S. B. P.*
37 *Chargé and M. A. Rudnicki. Cellular and Molecular Regulation of Muscle Regeneration.*
38 *Physiol Rev* 84: 209–238, 2004). However, this capacity is reduced in the elderly, where
39 satellite cells are unable to execute the complete repair process (Hawke and Garry, 2001;
40 Seale and Rudnicki, 2000).
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58 Activation and proliferation stages of satellite cells are characterized by the expression of
59 myogenic regulatory factors (MRFs), and the phases of this process typically involve the
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1 sequential expression of proteins, including Pax3 (quiescent satellite cells), myoD
2 (proliferating mpcs), myogenin (differentiated myoblasts) and acetylcholine receptor clusters
3 (innervated fibers). In muscle from old rodents and humans, the regeneration process is
4 defective and contributes significantly to the loss of muscle mass (Conboy and Rando, 2005;
5 Renault et al., 2002b).

6
7 Despite several investigations, the precise role of satellite cells in the age-associated loss of
8 muscle mass and strength remains unclear. One of the initial hypotheses proposes a possible
9 age-dependent decrease in the satellite cell pool, which may explain the reduced efficiency of
10 muscle regeneration in older subjects (Renault et al., 2002a). However, this theory was
11 disputed by reports showing that satellite cell proportion and morphology are generally
12 conserved during the aging time-course, at least for the same muscle from healthy subjects
13 (Roth et al., 2000b).

14
15 It is possible that the diversity between young and old individuals occurs due to different
16 capacities of activating the repair process, rather than variations in the cell number available
17 for damage repair. Thus, impaired activation, proliferation and/or differentiation of satellite
18 cells in older individuals may lead to deterioration of muscle regeneration (Cooper et al.,
19 2006). In addition, satellite cells from aged muscle display a tendency to accumulate fat with
20 reduced differentiation-dependent gene expression (Kirkland et al., 2002), which possibly
21 contributes to the deterioration of regeneration capability (Peterson, 1995; Taylor-Jones et al.,
22 1995).

23
24 Previously, we reported ROS damage in both mature muscle (Fulle et al., 2004) fibers and
25 muscle resident satellite cells in which some functional parameters are modified (Fulle et al.,
26 2005). In fact, our data revealed an age-dependent increase in lipid peroxidation in cultured
27 myotubes. In these substrates, the capacity of satellite cells to generate Ca^{2+} transients in
28 response to various physiological or pharmacological stimuli is significantly modified
29 (Beccafico et al., 2007).

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In the present study, we investigate the effects of aging on myoblasts and myotubes obtained from human skeletal muscle. In particular, we show here that the morpho-functional modifications found in myotubes derived from elderly skeletal muscles, that have a difficulty to complete a differentiation program, are related to alteration of expression of genes involved in several pathways as well as oxidative stress, cell adhesion/motility, protein balance and differentiation.

In conclusion, our results aid in defining specific functional mechanisms altered by aging and determining the molecular basis for altered gene expression in satellite cells.

Materials and Methods

1. Muscle samples

Biopsies from *vastus lateralis* (VL) or *glutaeus medius* (GM) muscles were obtained from healthy patients who underwent elective orthopedic surgery after informed consent. Each patient was a non-trained individual.

The following abbreviations were used for our samples: VL29M where VL is the typology of muscle, 29 represents the donor age, and M is donor sex.

Samples were derived from males and females of different ages, and categorized into two groups:

* *Adult individuals* (6 males, 38.0 ± 4.1 years of age: 28 (VL), 29 (VL), 34 (VL), 36 (VL), 48 (GM) and 53 (VL) years, respectively);

* *Aged individuals*, both males and females, (10 patients, 78.5 ± 7.7 years of age, including 5 males, 69 (GM), 71 (GM), 71 (VL), 76 (GM) and 81 (VL) years, 5 females, 73 (VL), 81 (VL), 84 (VL), 87 (VL) and 92 (VL) years, respectively).

Myoblasts from the autopsy of a 5-day-old infant (CHQ5B) were obtained from quadriceps muscle kindly provided by the Butler-Browne group of Faculté de Médecine Pitié-Salpêtrière de Paris.

1 Biopsies (muscle pieces 0.1-0.4g in weight) were performed using the procedure of Engel
2 (1994), and immediately treated to obtain explants, as described by Decary (1996). Each
3
4 biopsy sample was collected in Ham's F-10 medium (GIBCO, Invitrogen, Carlsbad,
5
6 California) supplemented with 50 µg/ml of gentamycin and stored at +4°C until processing,
7
8 which was generally performed within 24 h of surgery.
9

10 11 **2. Cell cultures**

12 Muscle biopsies were processed according to the procedure of Fulle et al. (2005) The first
13
14 mononucleated cells migrated out of the explants within 7 to 13 days from the beginning of
15
16 culture (independent of donor age). Cell cultures were considered *senescent* when they could
17
18 not reach one population doubling level (PDL) after three weeks of re-feeding (Decary et al.,
19
20 1997). The life span of our cultures was 55-65 PDL for newborn, 17-20 PDL for young adults
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22 and 15-20 PDL for the elderly. To induce the differentiation of mononucleated cells into
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24 multinucleated myotubes, cells were plated onto ECL-coated dishes. ECL is a cell attachment
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26 matrix gel (Upstate, Millipore Corporation, Billerica, MA, USA) containing entactin (E),
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28 collagen (C) and IV-laminin (L), which facilitates cell adhesion. The substrate of choice for
29
30 differentiation was plastic (bottom of cell-treated dishes, thermanox, optics polystyrene TC-
31
32 treated microplates), which was most favorable for satellite human cell growth and
33
34 differentiation.
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37 Myoblasts were plated at a confluence of 10.000 cells/cm² and maintained for two days in
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39 Growth Medium. At 2-3 days after plating, the medium was replaced with Differentiation
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41 Medium (DM) consisting of DMEM high Glucose (Euroclone, Pero, MI, Italy) supplemented
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43 with 5% Horse Serum (Euroclone), 50 µg/ml of Gentamycin (Euroclone), 10 µg/ml of Insulin
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45 (SIGMA Chemical Co.; St Louis, MO, USA) and 100 µg/ml of apo-Transferrin (SIGMA).
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48 We usually followed the differentiation process until day 12. The efficiency of differentiation
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50 was determined by counting the number of nuclei in 7 day-differentiated myotubes as a
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52 percentage of the total number of nuclei. The ratio of the two values was defined as Fusion
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Index (FI). At least 1500-2000 nuclei were counted in 25-30 different randomly selected fields.

Several experiments were performed on cells with PDL values of 15.3 ± 1.0 (newborn), 6.1 ± 0.4 (young adults) and 5.3 ± 0.5 (elderly) for undifferentiated cells or 19 ± 1.5 (newborn), 6.3 ± 0.6 (young adults), and 5.7 ± 0.3 (elderly) for differentiated cells.

3. Immunocytochemistry

Since cells obtained from explants are always contaminated with non-myogenic cells, it was important to estimate the myogenic purity of our cultures.

Myogenic purity was monitored by immunocytochemistry using desmin as a marker. Desmin is a cytoskeleton intermediate filament protein expressed early in myogenic populations (Behr et al., 1994; Kaufman and Foster, 1998).

Cells were fixed in 90% ethanol for 10 min at room temperature, and immunostaining performed as described by Fulle et al. (2005) D33 Monoclonal Mouse anti-Human (diluted 1:30) and Mouse Immunoglobulin Biotin Rabbit anti-Mouse (diluted 1:200) (DAKO, Dakocytomation, Glostrup, Denmark) were used as primary and secondary antibodies, respectively. Specific antibody binding was examined using the streptavidin biotinylated peroxidase visualization reagent (StreptABCComplex DAKO).

The percentage of desmin-positive myoblasts was calculated as the number of positive cells versus total cell number. At least 1000-2000 cells were counted in 25-30 different randomly selected fields.

To verify the FI percentage measured in our cultures at day 7 of differentiation, we stained fast and slow myosin heavy chains (MHC) using the procedure described by Edom et al. (1994). The primary antibody used was monoclonal MF20 (diluted 1:50) (Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA, USA).

4. Myogenic purification

1 In some cultures, the myogenic cell number was too low to ensure efficient differentiation. In
2 these cases, we used a separation technique (MiniMACS separation system from Miltenyi
3 Biotec (Bergisch Gladbach, Germany)) to enrich cultures with myogenic cells and eliminate
4 fibroblasts and other non-myogenic cells.
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9 The MiniMACS separation system is equipped with 1 MiniMACS separation unit that is an
10 extremely powerful magnet, 1 MACS multistand, and MS MACS separation columns.
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12 Cell suspensions were separated and initially incubated with a specific primary antibody for
13 human myoblasts targeting a cell surface protein, 5.1H11 (Walsh and Ritter, 1981) (DSHB).
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15 Cells were washed carefully in phosphate buffer containing 0.5% BSA and 2 mM EDTA.
16

17 Subsequently, cells were labeled with the MACS Goat anti-mouse IgG MicroBeads secondary
18 antibody linked to a little magnetic bead. After magnetic labeling, cells were passed through
19 an MS column placed in the MiniMACS strong permanent magnet. Magnetically labeled cells
20 were retained in the column while unlabeled cells passed through, allowing effective
21 separation. After removal of the column from the magnetic field, the bound fraction
22 containing myogenic cells was eluted. Purified cells were counted and replaced in a Petri dish.
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36 **5. Transcriptome analysis**

37 **Microarray fabrication**

38 The human oligonucleotide set consists of 21,329 70mer sequences (Operon version 2.0)
39 designed on Human Unigene clusters, mainly in the 3'-terminal region. The individual genes
40 analysed via microArray were 10,664. Each oligonucleotide was spotted in two replicates on
41 a MICROMAX glass slide SuperChip I (PerkinElmer Life Sciences Inc., Massachusetts,
42 USA) using a Biorobotics Microgrid II robotic station fitted with split-pin (M-medical,
43 Milano, Italia). Details of the slide design and sequence selection are presented in the
44 manufacturer's website (<http://microcribi.cribi.unipd.it/e-index.htm>).
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58 **RNA isolation and amplification**

1 Total RNA from cultured human satellite cells (HSC) was isolated using the NucleoSpin®
2 RNA II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.
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4 The RNA 6000 LabChip kit was employed with the Agilent 2100 bioanalyzer (Agilent
5 Technologies, Palo Alto, California, USA) to determine the integrity of the RNA samples
6
7 obtained from HSC. On average, the ratio was 1.8.
8
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10 Aliquots of each RNA sample (2 µg) were amplified and labeled using an Amino Allyl
11
12 MessageAmp™ aRNA amplification and labeling kit (Ambion, Austin, USA).
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16 **Microarray prehybridization and hybridization**

17 For hybridization, labeled aaRNAs were combined with water and hybridization buffer (500
18 µl SSC 20X, 20 µl SDS 10%, 100 µl ssDNA 2 µg/µl, 500 µl formamide, 880 µl water) with
19
20 stirring at 65°C to a total volume of 125 µl.
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24 Microarray hybridization was performed on the hybridization station ArrayBooster™
25
26 (Advalitx, Brunthal, Germany) humidified with 250 µl of hybridization buffer.
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29 The hybridization solution was sandwiched between AdvaCard™ and the microarray.
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32 Hybridization proceeded overnight at 48°C. Two replicates of each experiment were analyzed
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34 using different microarray slides in which the sample and reference RNA labeled with either
35
36 Cy3 or Cy5 fluorochromes were crossed in both combinations.
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40 **Microarray washing**

41 Slides were washed in 1x SSC and 0.2% SDS solution for 4 min 0.1 x SSC and 0.2% SDS
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43 solution for 4 min, twice in 0.2 x SSC solution for 4 min, and finally, twice in 0.1 x SSC
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45 solution for 4 min.
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50 **Fluorescence scanning**

51 Digital images were generated using a GSI Lumonics LITE dual confocal laser scanner
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53 (ScanArray Microarray Analysis System - Perkin Elmer). All spot signal intensities were
54
55 collected manually using QuantArray Analysis Software (GSI Lumonics, Ottawa, Canada),
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57 corrected for local backgrounds, and arranged in an Excel file for further analysis.
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6. Statistical Analysis

Morphological and functional data

Data were presented as means \pm SEMs where possible and performed using GraphPad Prism 2.0 Ex.

Gene expression data

Normalized values were calculated for each spot, using MIDAS software (TIGR Microarray Data Analysis System) available at www.tigr.org. Values were converted to a logarithmic scale. Final values correspond to \log_2 ratio of the normalized intensities (e.g., $\log_2 I_{\text{elderly}}/I_{\text{young}}$) where positive numbers correspond to RNA overexpressed in the elderly.

Differentially expressed genes were detected with the SAM program available at <http://www-stat.stanford.edu/~tibs/SAM/index.html>.

Results

Consistent with the findings of Renault et al. (2000), the *in vitro* proliferative life span of our myoblasts is limited and related to donor age. The highest PDL from each cell culture decreases considerably between birth and about 20 years. Once the period of muscle growth ceases, the proliferative capacity of myoblasts remains stable at about 15-20 divisions.

Cell cultures were characterized as myogenic purity (% of desmin^{+ve} cells) and fusion index (%). Interestingly, the number of myogenic cells did not alter significantly during aging, but their ability to fuse (calculated in 7 day-differentiated myotubes) with each other decreased (from 80% to 20%) (Table 2). We additionally used the myogenic purification technique to enrich some cultures with myogenic cells (see Methods). For the VL34M and GM71M samples, we obtained an increase in the desmin-positive cell number (from 17% and 15% to 62% and 93% respectively), but not fusion index (FI), which does not increase, in the elderly sample, in proportion to myogenicity. Furthermore, the percentage of unfused desmin^{+ve} cells calculated in 7 day-differentiated cultures increased with age.

Moreover, the cultures display alterations from a morphologic viewpoint. As shown in Fig. 2, myotubes derived from newborn and young donors are large with several myonuclei, while those from elderly subjects are smaller in number and thinner, containing few myonuclei, confirming the decrease in FI. These characteristics become more pronounced with progression of donor age.

To ascertain whether these morphological alterations and previously reported functional changes (Beccafico et al., 2007; Fulle et al., 2005) in aged myotubes could be associated to gene expression changes, we performed a series of experiments to analyze the transcriptional profile during the early steps of the differentiation program (0, 4, 24 and 72 h). We assessed genes obtained from SAM analysis (see Experimental Procedures) according to Δ and false discovery rates reported in Table 1. The total number of differentially expressed genes was 225 and we did not insert genes which function is unknown. Several differentially expressed (up- and down-regulated) genes coding for proteins implicated in diverse pathways were observed at each differentiation time-point in the elderly respect to the young (Table 3).

1. Oxidative management

Genes up-regulated in undifferentiated cells derived from the elderly included *Glutathione Peroxidase 4 (phospholipid hydroperoxidase)* (GPX4) that codes for a unique protein involved in the detoxification of oxidative damage to membrane lipids and *SHC (Src homology 2 domain containing) transforming protein 1* (SHC1) encoding a DNA repair enzyme involved in the aging process, a signaling pathway inducing elevation of extracellular oxidant levels, cytochrome c release and apoptosis, and the oxidative stress response (Abdelwaheda et al., 2007).

Moreover, at the undifferentiated level (0 h), *polymerase (DNA directed) kappa* (POLK) encoding a low-fidelity DNA polymerase that duplicates DNA, leading to accumulating damage and genetic instability, was up-regulated (Bavoux et al., 2005). However, at 4, 24 and

72 h of differentiation, *Glutathione S-transferase theta-2* (GSTT2) coding for a detoxification enzyme that preserves the intracellular glutathione pool was down-regulated.

2. Cell adhesion and motility

Another signaling pathway that is significantly modified with aging contains genes involved in the regulation of cytoskeletal actin and focal adhesion. In undifferentiated cells, increased expression of two genes involved in actin assembly and motility in several cell processes, was observed, specifically, *Actin-related protein 2/3 complex* (ARPC1B) coding for protein phosphorylating the ARP 2/3 complex subunit 1-B and *ARP2 actin-related protein 2 homolog* (ACTR2) (Galletta et al., 2008). Additionally, we observed enhanced expression of *RAP1*, *GTP-GDP dissociation stimulator 1* (RAP1GDS1), and at 4 h of differentiation, *GTP binding protein 1* (DRG1) encoding two different GTPases, the first involved in integrin-mediated cell adhesion and the second in cytoskeleton regulation (de Bruyn et al., 2003). Furthermore, at the undifferentiated stage, the *Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)* (ITGAV) gene that codes for receptors for extracellular matrix (ECM)-mediated cell adhesion and cytoskeletal organization was up-regulated.

Expression patterns of genes of the extracellular matrix organization pathway were additionally altered. At each differentiation time-point, *Matrix metalloproteinase 1* (MMP1) gene coding for an enzyme that digests specific extracellular matrix components was down-regulated (Saffarian et al., 2004). At 4 h of differentiation, the following genes were up-regulated: (i) *Alpha-2-macroglobulin* (A2M) encoding a protein that inhibits numerous proteases, including collagenases, and *Fibroblast growth factor receptor 2* (FGFR2) involved in cell division, regulation of cell growth and maturation, and embryonic development, (ii) *Laminin gamma2* (LAMC2) implicated in a wide variety of biological processes, including cell adhesion, differentiation, migration, signaling and neurite outgrowth, and (iii) *Protocadherin beta 5* (PCDHB5; also up-regulated at 72 h of differentiation), which codes for neural cadherin-like cell adhesion proteins. Expression of *Asporin (LRR class 1)* (ASPN)

1 encoding an extracellular cartilage matrix protein was enhanced at each time-point except at
2 72 h, and at 24 of differentiation, *Leptin receptor gene-related protein* (HSOBRGRP)
3
4 implicated in fibrogenesis, at least in liver tissue, was up-regulated (Cayón et al., 2006). We
5
6 observed down-regulation of different genes for membrane proteins, including *Collagen, type*
7
8 *XV, alpha 1* (COL15A1) at 24 and 72 h, *Proteoglycan 4* (PRG4) at 4 and 24 h, and
9
10 *Hyaluronan synthase 1* (HAS1) at 24 h of differentiation.
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13 3. Differentiative trophism

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15 Prior to the start of the myogenic pathway (0 h), *Fasciculation and elongation protein zeta 2*
16
17 (*zygin II*) (FEZ2) and *Axotrophin* (AXOT), two key genes in axonal elongation, were up-
18
19 regulated. After 4 h of differentiation, it was also up-regulated *Tumor necrosis factor receptor*
20
21 *superfamily, member 12* (TWEAK receptor), a translocating chain-association membrane
22
23 protein (also designated Fn14) that contributes to nerve regeneration via a Rac1 GTPase-
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25 dependent-mechanism (Tanabe et al., 2003) and the regulation of myogenic differentiation.
26
27 However, the specific muscle protein Myosin, heavy polypeptide 3, skeletal muscle,
28
29 embryonic (MYH3), was up-regulated at 72 h of differentiation. This is a developmental form
30
31 of essential MLC (MLC1a-emb) that is diagnostic of muscle regeneration, but normally
32
33 absent in adult muscle.
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37 In addition, we observed up-regulation of *Protocadherin alpha subfamily C, 2* (PCDHAC2) at
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39 24 and 72 h, and *Protocadherin beta5* (PCDHB5 playing a role in adhesion with neuronal
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41 cells), which belongs to a family of proteins important in axonal development, at 4 and 72 h
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43 of differentiation (Tissir et al., 2005).
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47 Finally, we have found the gene *Netrin 1* (NTN1) down-regulated at 72 h of differentiation.
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51 Netrins comprise a small family of secreted proteins related to laminins that associate with
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53 cell membranes and ECM and are very important to promote myotube formation (Kang et al.,
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55 2004).
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58 4. Protein balance

1 Sarcopenic muscle presents atrophy, which depends, at least in part, on a decrease or
2 imbalance in protein metabolism. In our experiments, several genes linked to protein
3 metabolism were differentially expressed during *in vitro* myogenesis. In particular, at 0 h, we
4 observed up-regulation of the metalloendoprotease gene, *Metalloprotease 1 (pitrilysin family)*
5 (MP1), that plays a broad role in general cellular regulation, and the subunit α of 20S
6 proteasome, *Proteasome (prosome, macropain) subunit, alpha type 5* (PSMA5). Proteasomes
7 cleave peptides via an ATP/ubiquitin-dependent process in a non-lysosomal pathway.
8
9 Notably, expression of *Proprotein convertase subtilisin/kexin type 1* (PCSK1N) encoding
10 convertase that processes latent precursor proteins into their biologically active products was
11 reduced after 24 h of differentiation, and *Protease, serine, 23* (SPUVE) that encodes a
12 specific protease was increased. Moreover at 4 h of differentiation, *Carboxypeptidase N,*
13 *polypeptide 1, 50 kD* (CPN1) was up-regulated, while at 72 h, significantly reduced
14 expression of mRNA encoding mitochondrial ribosomal proteins such as *ADP-ribosylation*
15 *factor domain protein 1, 64kD* (ARFD1) was observed.
16

17 The reduced anabolic processes, together with increased catabolic processes observed in
18 elderly myoblasts and myotubes possibly attribute to atrophy. In fact, during the early
19 differentiation phases (4 h), increased expression of *Forkhead box O1A (rhabdomyosarcoma)*
20 (FOXO1A) involved in atrogin-MAFbx pathways was evident.
21

22 **Discussion**

23 The possibility of a regenerative process in muscle that occurs using the quiescent satellite
24 cell pool is closely correlated with their ability to successfully complete myogenesis. The
25 ability of muscle to regenerate successfully following damage diminishes with age (Brooks
26 and Faulkner, 1990) and this is a main contributor to the development of muscle weakness
27 and physical frailty (Faulkner et al., 2007). Under our experimental conditions, differentiation
28 is not executed precisely due to morphological and functional modifications in myoblasts and
29

1 myotubes derived from old subjects, consequently leading to delayed and incomplete
2 myogenic differentiation. This finding is further confirmed by the inability of elderly
3 myoblasts to fuse and achieve a complete differentiative program, also upon enrichment of
4 the culture with myogenic cells, after purification procedure (see Methods). In particular, the
5 difficulty to fuse and differentiate into myotubes may be derived, at least in part, from
6 alterations in the actin cytoskeleton regulation that, in turn, modify the shape/morphology and
7 motility of activated cells. Cellular adhesion, migration and invasion are fundamental
8 properties of most cell types during development and normal tissue/cellular function. For
9 these cells, cell-cell and cell-substrate adhesion is essential to the maintenance of polarity,
10 differentiation and tissue architecture. During development some cells, including myoblasts,
11 migrate large distances. In order to migrate these cells need to overcome the physical and
12 biochemical barrier of the extracellular matrix. Up-regulated genes that we found, such as
13 ARP2 (encoding the major protein of the ARP 2/3 complex), PAK1 and Cdc42, are involved
14 in cytoskeleton regulation. In fact, the ARP 2/3 complex located on the cell surface is
15 essential for cell shape and motility through lamellipodial actin assembly and protrusion
16 (Vadlamudi et al., 2004). These genes, together with RAP1 (that codes for a GTPase
17 regulating integrin-mediated cell adhesion), are involved in focal adhesion processes, and
18 thus, extracellular matrix regulation (de Bruyn et al., 2003). The observed alterations in genes
19 involved in the last pathway (MMP1, A2M, FGF receptor 2) support the assumption of
20 abnormal deposition of the extracellular matrix that may give rise to a pseudofibrotic state,
21 characteristic of aging. In addition, MMP1 is involved in specific protein substrate digestion
22 of the extracellular matrix, and is fundamental in facilitating cellular migration when
23 myocytes fuse and form myotubes (Saffarian et al., 2004). These results are consistent with
24 the increase in membrane rigidity in myotubes of the elderly (Fulle et al., 2005) that may be
25 attributed to enhanced lipid peroxidation (Beccafico et al., 2007), increasing the difficulty of
26 migration and fusion. Therefore, the lower motility of senescent myoblasts and myotubes,
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1 also observed by other investigators (Renault et al., 2000), may be attributed to increase in
2 extracellular matrix deposition. This event is confirmed by the up- or down-regulation of
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4 various proteoglycans, collagens and laminins (see Table 3) at several differentiation time-
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6 points, in addition to the deregulation of cytokines and interferon α . Moreover, hyaluronic
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8 acid, collagen IV, TIMP-1, α -2-macroglobulin, and TGF β 1 are correlated with a moderate
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10 serious fibrotic state (Lu et al., 2003). In addition, the leptin receptor gene possibly involved
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12 in fibrogenesis and hepatic inflammation is up-regulated (Bethanis and Theocharis, 2006).
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14 The expression patterns of these genes collectively suggest that a fibrotic state develops
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16 during the differentiation program, which may be responsible, at least in part, for
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18 differentiation problems. However, other factors may contribute to failed myogenesis. For
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20 instance, the TWEAK receptor, a direct regulator of skeletal muscle regeneration that inhibits
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22 terminal differentiation in both the established C2C12 cell line and primary mouse muscle
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24 myoblasts, already was up-regulated in early stage of differentiation (Girgenrath et al., 2006).
25
26 Moreover also the down-regulation of NTN1, a gene that normally develops an activity that
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28 contributes to the promyogenic effects of cell-cell contact, can affect the adhesive function
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30 and impair the fusion process (Srinivasan et al., 2003).
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32 Furthermore, the difficulty to complete differentiation is confirmed by the up-regulation of
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34 MYH3. In fact, both embryonic and perinatal MHC genes coding for "transitional" isoforms
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36 (MHC emb and MHC neo) are expressed during fetal and postnatal development, and
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38 subsequently substituted with adult isoforms. In the adult, these transitional isoforms are
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40 expressed only during regeneration, and based on the differentiation of satellite cells (Weiss et
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42 al., 1999).
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52 We previously demonstrated that muscle tissue derived from aged subjects display a
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54 significant increase in DNA and lipid damage (Mecocci et al., 1999) as a consequence of
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56 ROS accumulation (Fanò et al., 2001) stemming from the decreased activity of a number of
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58 antioxidant enzymes. A similar situation described in myoblasts and myotubes derived from
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1 the elderly (Fulle et al., 2005), is also confirmed in the present study in which, during the
2 proliferation stage, polymerase (DNA directed) kappa that duplicates DNA leading to
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4 accumulating damage and genetic instability (Bavoux et al., 2005) and SHC1 involved in the
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6 aging process and in the oxidative stress response (Abdelwaheda et al., 2007), are up-
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8 regulated.
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10 The increase in membrane lipid peroxidation (Beccafico et al., 2007) related to alterations in
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12 antioxidant enzyme activities (GPX and GST) (Fanò et al., 2001), reported previously by our
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14 group, was confirmed by the transcriptional profile reported in this study showing alterations
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16 in the expression patterns of genes encoding antioxidant enzymes. This condition leads to
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18 increased membrane rigidity (Fulle et al., 2005), and together with other factors, may be
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20 responsible for insufficient ability to fuse into myotubes noted during *in vitro* myogenesis of
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22 elderly satellite cells. The oxidative stress-induced state may also underlie the inability of
23
24 elderly cells to align calcium channels (calcium release units, CRU) correctly during
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26 myogenesis, and thus uncouple the E-C mechanism in a way that prevents the correct
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28 development of stimulus-response coupling, as reported previously (Beccafico et al., 2007).
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30 We have already demonstrated that in adult muscle fibers of elderly there are alterations of
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32 CRU (Boncompagni et al., 2006).
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41 Sarcopenia is characterized by progressive denervation with loss of motor units (McNeil et
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43 al., 2005). Since successful muscle regeneration is strictly dependent on the appropriate cross-
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45 talk between newborn fibers and motoneurons connected to immature regenerating muscle,
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47 we analyzed whether the neuronal recruitment pathway was involved in satellite cells derived
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49 from the elderly. At the undifferentiated state, FEZ2 and Axotrophin, two key genes in axonal
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51 elongation, were up-regulated (Table 3), in agreement with previous reports (Welle et al.,
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53 2003). Furthermore, protocadherin genes were up-regulated at 24 and 72 h of differentiation.
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57 These up-regulated patterns may represent a cellular attempt to activate a reinnervation
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59 pathway, which is ultimately unsuccessful.
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In the scenario of differentiating myotubes particular emphasis is placed on the genes that codify for protein that regulate protein balance: proteins that are involved in myofibrillogenesis or in activation/inactivation or modulation of specific pathway. The protein balance derives from anabolic (synthesis) and catabolic (degradation) processes. If the anabolic processes predominate, the muscle can go towards hypertrophy, if those catabolic predominate can become atrophic. During the degradation process the proteins are tagged by a small protein called ubiquitin and degraded by proteasome by proteolysis. The proteasomal degradation pathway is essential for many cellular processes, including the cell cycle, the regulation of gene expression, and responses to oxidative stress. But also for sarcopenia (where it verifies an increase of atrophy) one of the main causes is altered muscle protein synthesis (Morley et al., 2001; Welle and Thornton, 1998). In fact, during aging, deregulation of autocrine/paracrine (as well as mIGF-1 and myostatin) and endocrine (cortisol and insulin) factors that control skeletal muscle ability to perform specific protein synthesis is observed (Harridge, 2007). To confirmation of that it has been demonstrated that a short-term blockade of myostatin, through stage-specific administration of a myostatin antagonist or prolonged absence utilizing myostatin-null mice (Siriatt et al., 2006), significantly enhanced muscle regeneration in aged mice after injury and during sarcopenia. Furthermore, antagonism of myostatin led to satellite cell activation, increased Pax7 and MyoD protein levels, and greater myoblast and macrophage cell migration, resulting in enhanced muscle regeneration after notexin injury in aged mice (Siriatt et al. 2007).

Obviously the up-regulation at 0 h of the only 20S proteasome subunit alpha gene does not prove that proteolysis was increased, but this event associated with a down-regulated expression, at 72 h of differentiation, of genes involved in anabolic processes, as well as those encoding mitochondrial ribosomal proteins, it suggests an unbalance of the protein synthesis rate with activation of degradation processes. This theory is in agreement with the findings of

1 Welle and Thornton (1998) showing down-regulation of transcripts relevant to mitochondrial
2 ribosomal proteins in skeletal muscle, suggesting higher levels of protein degradation in older
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4 regenerating fibers. The imbalance in these mechanisms may be responsible of the activation
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6 of an atrophy process. This hypothesis is supported by the observation that during early
7
8 differentiation (4 h), expression of the *FOXO1A* gene is increased. The *FOXO1A* gene is very
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10 important for the induction of atrophy via the atrogen-MAFbx pathways (Sandri et al., 2004).
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14 **Conclusions**

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16 In previous studies, we reported modifications in morphological, biochemical and functional
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18 characteristics of pool satellite cells in the elderly that may be responsible for insufficient
19
20 regeneration capability (Beccafico et al., 2007; Fulle et al., 2005). Here, we show that this
21
22 failure does not depend on a reduced number of myogenic cells but difficulty to term the
23
24 differentiation program, affecting the regeneration process, due to several factors as a result of
25
26 the alteration in the expression patterns of several genes. The main involved points are:
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32 • accumulation of oxidative damage in molecular substrates, probably due to impaired
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34 antioxidant activity (upregulation of polymerase κ and SHC1);
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38 • myoblasts show limited capability to execute a complete differentiation program with
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40 insufficient repair capability; scarce fusion, probably due to altered turnover of
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42 cytoskeleton and extracellular matrix degradation;
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46 • possible atrophic process induction due to specific proteolytic systems (unbalance
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48 protein synthesis) also sustained by the activation of a specific FOXO-dependent
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50 program (up-regulation FOXO1A).
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Legends to figures and tables

1
2 Fig. 1. Proliferative life span of human satellite cells. Examples of three cell populations
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4 isolated from donors of different ages serially passed and cultured until division ceases. At
5
6 each passage, the Population Doubling Level was calculated by counting the cell number.
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10 Fig. 2. MF20 immunostaining to identify myotubes (7 days of differentiation) derived from
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12 several donors of different ages (5 day-old newborn, 36, 53 and 92 years old) expressing
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14 Myosin Heavy Chain. Bar = 5 μ m.
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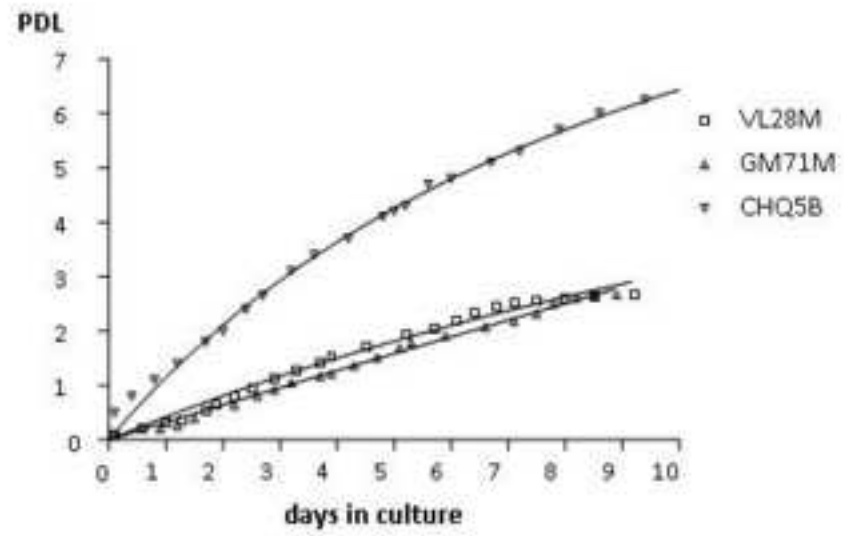
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19 Table 1: Statistical parameters utilized for SAM analysis
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23 Table 2. Outline of samples utilized to determine the percentage of myogenicity, fusion index
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25 and desmin-^{+ve} unfused cells. Information on muscle type, age and sex of samples is provided
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27 in the first column.
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31 Table 3. Descriptive table of differentially expressed genes.
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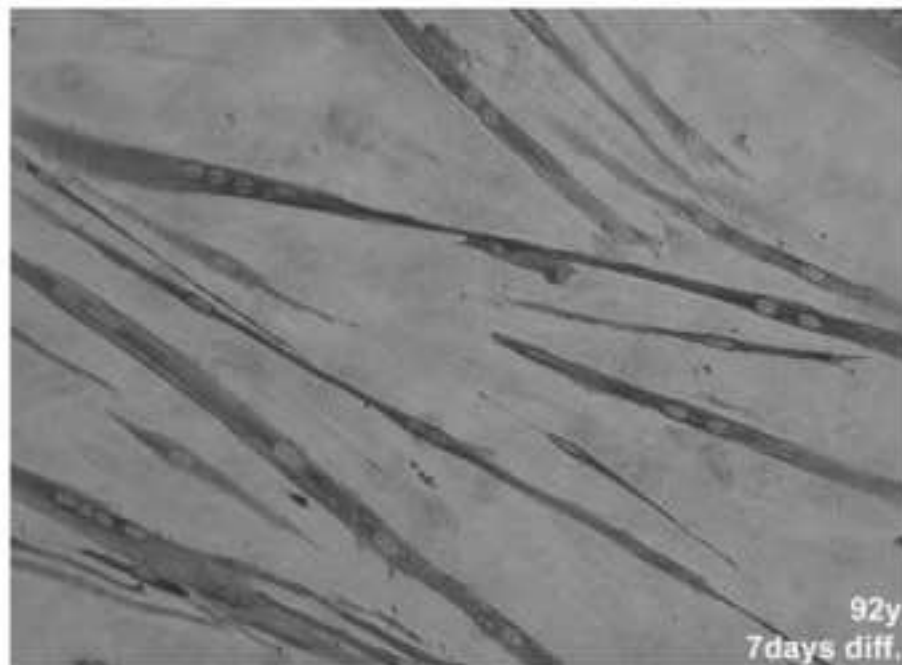
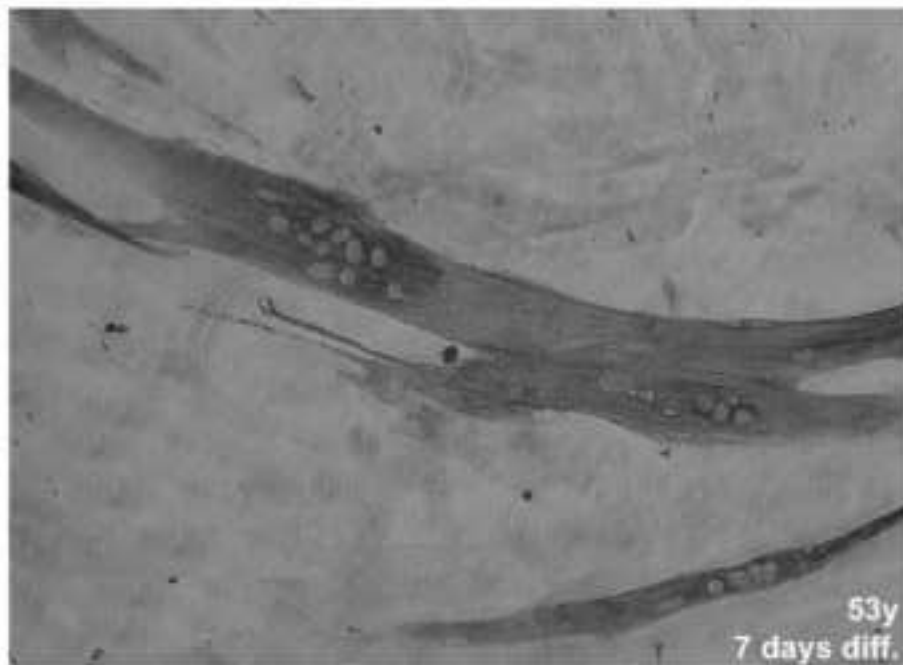
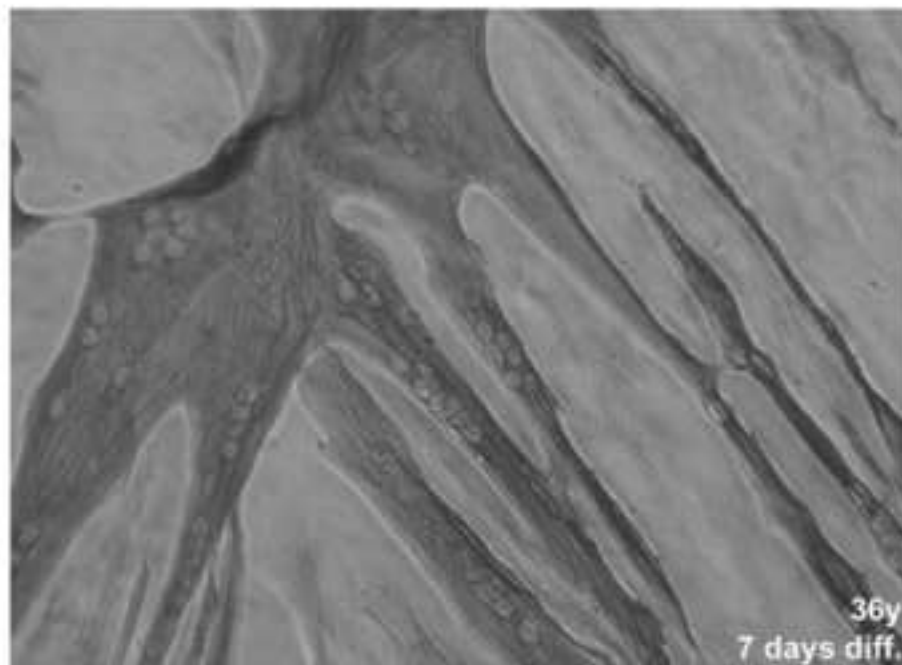
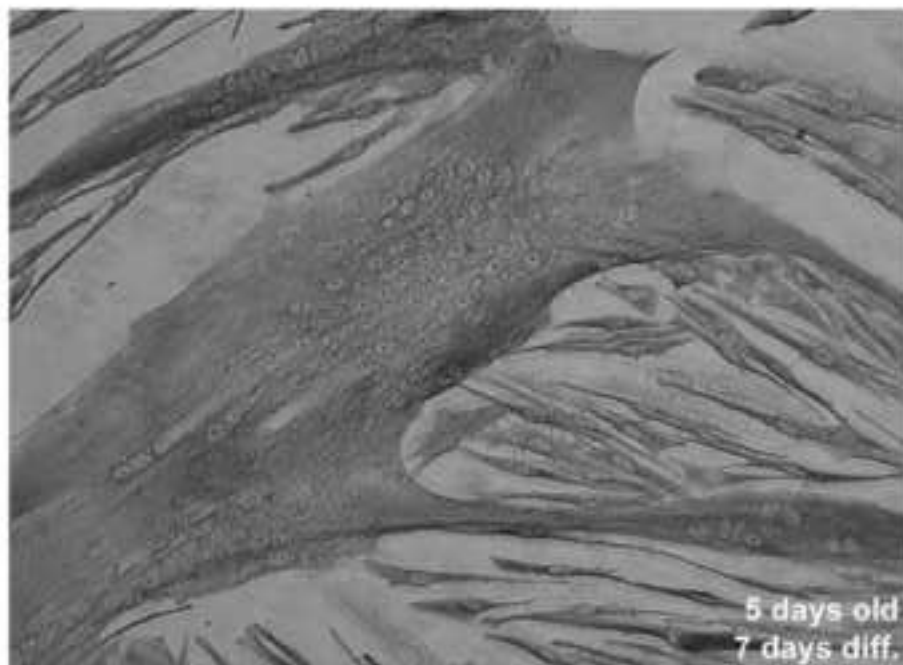
| Statistical Parameters | 0 h | 4 h | 24 h | 72 h |
|------------------------|-------------|-----|------|------|
| Δ | 3.6 | 1.7 | 2.5 | 1.3 |
| Fdr median | \emptyset | 1.9 | 0.9 | 1.0 |

Table 1



| Cell culture | Myogenicity % (desmin ^{+ve}) | Fusion Index (%) | Desmin ^{+ve} unfused (%) |
|---------------------------|---|---------------------|-----------------------------------|
| CHQ5B (newborn) | 85 | 80 | ---- |
| VL28M | 49 | 48 | ---- |
| VL29M | 71 | 73 | 20 |
| VL34M | 17 | 15 | ---- |
| <i>after purification</i> | 62 | 54 | ---- |
| VL36M | 69 | 54.3 | 37.9 |
| GM48M | 53 | 43 | 48.8 |
| VL53M | 44 | 46 | 36.6 |
| GM69M | 60 | 45 | ---- |
| GM71M | 15 | 17 | ---- |
| <i>after purification</i> | 93 | 27 | ---- |
| VL71M | 65 | 55 | ---- |
| VL73F | 53 | 40 | ---- |
| GM76M | 47 | 48 | ---- |
| VL81M | 71.5 | 40 | 66 |
| VL81F | 76 | 22 | ---- |
| VL84F | 36.3 | 37 | 75.5 |
| VL87F | 60 | 23 | 58.3 |
| VL92F | 32.6 | 24 | 66.3 |

Table 2



| Gene Symbol | Description | Mean Log2 Ie/Iy \pm SD 0 h differentiation | Mean Log2 Ie/Iy \pm SD 4 h differentiation | Mean Log2 Ie/Iy \pm SD 24 h differentiation | Mean Log2 Ie/Iy \pm SD 72 h differentiation |
|----------------------------|--|---|---|--|--|
| Oxidation Management | | | | | |
| GPX4 | <i>Glutathione Peroxidase 4 (phospholipid hydroperoxidase)</i> | 2.56 \pm 0.38 | | | |
| (SHC1) | <i>SHC (Src homology 2 domain containing) transforming protein 1</i> | 1.99 \pm 0.02 | | | |
| POLK | <i>Polymerase (DNA directed) kappa</i> | 2.46 \pm 0.1 | | | |
| GSTT2 | <i>Glutathione S-transferase theta-2</i> | | -1.26 \pm 0.13 | -1.27 \pm 0.13 | -1.38 \pm 0.25 |
| Cell adhesion and motility | | | | | |
| ARPC1B | <i>Actin-related protein 2/3 complex</i> | 2.67 \pm 1.4 | | | |
| ACTR2 | <i>ARP2 actin-related protein 2 homolog</i> | 2.20 \pm 0.07 | | | |
| RAP1GDS1 | <i>RAP1, GTP-GDP dissociation stimulator 1</i> | 3.16 \pm 0.18 | | | |
| DRG1 | <i>GTP binding protein 1</i> | | 1.13 \pm 0.21 | | |
| ITGAV | <i>Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)</i> | 3.67 \pm 0.35 | | | |
| MMP1 | <i>Matrix metalloproteinase 1</i> | -3.18 \pm 0.08 | -2.14 \pm 0.32 | -3.60 \pm 0.28 | -1,23 \pm 0.14 |
| A2M | <i>Alpha-2-macroglobulin</i> | | 1.93 \pm 0.31 | | |
| FGFR2 | <i>Fibroblast growth factor receptor 2</i> | | 1.25 \pm 0.14 | | |
| LAMC2 | <i>Laminin gamma2</i> | | 1.29 \pm 0.50 | | |
| PCDHB5 | <i>Protocadherin beta 5</i> | | 1.16 \pm 0.16 | | 1.28 \pm 0.2 |
| ASPEN | <i>Asporin(LRR class 1)</i> | 2.60 \pm 0.12 | 2.03 \pm 0.32 | 1.53 \pm 0.16 | |
| HSOBRGRP | <i>Leptin receptor gene-related protein</i> | | | 1.67 \pm 0.26 | |
| COL15A1 | <i>Collagen, type XV,</i> | | | -1,88 \pm 0.03 | -2,15 \pm 0.19 |

| | | | | | |
|--------------------------|--|-----------|------------|------------|------------|
| | <i>alpha 1</i> | | | | |
| PRG4 | <i>Proteoglycan 4</i> | | -1.19±0.22 | -1.43±0.27 | |
| HAS1 | <i>Hyaluronan synthase 1</i> | | | -1.21±0.14 | |
| Differentiative trophism | | | | | |
| FEZ2 | <i>Fasciculation and elongation protein zeta 2 (zygin II)</i> | 2.15±0.06 | | | |
| AXOT | <i>Axotrophin</i> | 2.94±0.18 | | | |
| TWEAK | <i>Tumor necrosis factor receptor superfamily, member 12</i> | | 0.67±0.07 | | |
| MYH3 | <i>Myosin, heavy polypeptide 3, skeletal muscle, embryonic</i> | | | | 1.09±0.17 |
| PCDHAC2 | <i>Protocadherin alpha subfamily C, 2</i> | | | 1.50±0.08 | |
| NTN1 | <i>Netrin 1</i> | | | | -2,63 |
| Protein balance | | | | | |
| MP1 | <i>Metalloprotease 1 (pitrilysin family)</i> | 3.44±0.42 | | | |
| PSMA5 | <i>Proteasome (prosome, macropain) subunit, alpha type, 5</i> | 2.62±0.10 | | | |
| PCSK1N | <i>Proprotein convertase subtilisin/kexin type 1</i> | | | -1.79±0.26 | |
| SPUVE | <i>Protease, serine, 23</i> | | | 1.27±0.04 | |
| CPN1 | <i>Carboxypeptidase N, polypeptide 1, 50 kD</i> | | 1.39±0.08 | | |
| ARFD1 | <i>ADP-ribosylation factor domain protein 1, 64 kD</i> | | | | -1.12±0.24 |
| FOXO1A | <i>Forkhead box O1A (rhabdomyosarcoma)</i> | | 1.12± 0.17 | | |

Table 3