

Research Article

Morphofunctional and Biochemical Approaches for Studying Mitochondrial Changes during Myoblasts Differentiation

Elena Barbieri,¹ Michela Battistelli,² Lucia Casadei,¹ Luciana Vallorani,¹ Giovanni Piccoli,¹ Michele Guescini,¹ Anna Maria Gioacchini,¹ Emanuela Polidori,¹ Sabrina Zeppa,¹ Paola Ceccaroli,¹ Laura Stocchi,³ Vilberto Stocchi,¹ and Elisabetta Falcieri^{2,4}

¹ Department of Biomolecular Sciences, University of Urbino Carlo Bo, Via I Maggetti, 26, 61029 Urbino (PU), Italy

² DISUAN, University of Urbino Carlo Bo, 61029 Urbino, Italy

³ Department of Biopathology, Tor Vergata University of Rome, 00133 Rome, Italy

⁴ IGM, CNR, Orthopedic Rizzoli Institute, 40136 Bologna, Italy

Correspondence should be addressed to Elena Barbieri, elena.barbieri@uniurb.it

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This study describes mitochondrial behaviour during the C2C12 myoblast differentiation program and proposes a proteomic approach to mitochondria integrated with classical morphofunctional and biochemical analyses. Mitochondrial ultrastructure variations were determined by transmission electron microscopy; mitochondrial mass and membrane potential were analysed by Mitotracker Green and JC-1 stains and by epifluorescence microscope. Expression of *PGC1 α* , *NRF1 α* , and *Tfam* genes controlling mitochondrial biogenesis was studied by real-time PCR. The mitochondrial functionality was tested by cytochrome c oxidase activity and *COXII* expression. Mitochondrial proteomic profile was also performed. These assays showed that mitochondrial biogenesis and activity significantly increase in differentiating myotubes. The proteomic profile identifies 32 differentially expressed proteins, mostly involved in oxidative metabolism, typical of myotubes formation. Other notable proteins, such as superoxide dismutase (MnSOD), a cell protection molecule, and voltage-dependent anion-selective channel protein (VDAC1) involved in the mitochondria-mediated apoptosis, were found to be regulated by the myogenic process. The integration of these approaches represents a helpful tool for studying mitochondrial dynamics, biogenesis, and functionality in comparative surveys on mitochondrial pathogenic or senescent satellite cells.

1. Introduction

Skeletal muscle represents an important model for studying mitochondrial behaviour during cell growth and differentiation. Myoblasts cultured *in vitro*, if induced by cell confluence and serum deprivation, follow a myogenic program, which includes an active proliferation, withdrawal from the cell cycle, synthesis of muscle-specific proteins, and fusion into multinucleated myotubes [1, 2]. This event is accomplished by the activation of specific myogenic regulatory factors (MRFs) [3–5].

Recent studies suggest that mitochondria are involved in the regulation of the skeletal muscle physiology and play a critical role in cell growth, cell proliferation, cell

death, and cell differentiation [6–13]. In particular, mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression, the activity of myogenic factors, and by control of *c-Myc* expression [8, 14, 15]. Furthermore, differentiation appears to be a program which is dependent on both mitochondrial function and mitochondrial biogenesis, as indicated by the rapid increase in mitochondrial mass/volume, mtDNA copy number, mitochondrial enzyme activities, and mRNA levels within the first 48 hrs of myoblast differentiation [6, 7]. Mitochondrial DNA transcription and replication are key events in cellular differentiation, which requires interaction between the nucleus and the mitochondrion [16].

Several aging theories are associated with mitochondrial damage or with a decline in mitochondrial energy production in which links between mitochondria genome expression and senescence symptoms are not always recognized [17–19]. Our interest is particularly focused on the role that mitochondria may play in the proliferative and differentiation capacity of satellite stem cells. It is well documented that with aging, satellite stem cells lose both mitogenic and myogenesis abilities and may decrease in numbers in both mice and humans [20–23]. The C2C12 cell line *satellite* myoblasts could offer a suitable model for studying mitochondrial behaviour during the differentiation program.

In this study, we combined a morphological and biomolecular approach to analyze changes in mitochondrial phenotype, ultrastructure, biogenesis, and functional activity during C2C12 myoblast differentiation. Although the contribution of the proteomic profile of mitochondria during the myogenesis program is significant, it has not been described in the literature. In this paper we aim to better define the involvement of mitochondria in the regulation of muscle cell differentiation and discover new proteins potentially involved in the crosstalk between nuclei and mitochondria.

2. Material and Methods

2.1. Cell Line. Mouse C2C12 myoblasts were grown in flasks in the presence of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine at 37°C, and 5% CO₂. To induce myogenic differentiation, when 80%–90% confluence was obtained, the medium was changed to DMEM supplemented with 1% FBS. Cells were analyzed at the undifferentiated stage and at the early-, middle-, and late-differentiation stage. In order to eliminate divergences in the differentiation time points analyzed, we assessed several differentiation markers. The cells, grown in the presence of 10% fetal calf serum until 80% cell confluence, were considered undifferentiated cells, corresponding to day 0 of the differentiation process (T0). To induce differentiation, cells at T0 were switched to differentiation medium. They were analyzed in the early-differentiation stage, 24 h after serum removal (T1), in mid-differentiation, 3–5 days after serum removal, when myotubes containing one of two nuclei appeared (T3–5), and in the late-differentiation stage, that is, 7–10 days after serum removal, in the presence of long multinucleated myotubes (T7–10).

2.2. Estimation of Myoblast Fusion. Myoblasts and myotubes were methanol fixed and air dried under different experimental conditions. They were then stained with water 1:10 May Grunwald-Giemsa solution, washed, and mounted to evaluate cell fusion. Cells were considered fused if they contained two nuclei within one cytoplasmic continuity as reported by Ferri et al. [5]. The fusion percentage was evaluated as the number of nuclei in myotubes divided by the total number of nuclei in myoblasts and myotubes magnified by 100 (×40 objective) using a TE 2000-S Nikon reverted

microscope (RM) with a digital Nikon DN100 acquisition system. Twenty optical fields were randomly chosen. Data were expressed as means ± S.E.M.

2.3. Mitochondrial Ultrastructure. Undifferentiated and differentiated cell monolayers were washed and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 15 min, gently scraped, and centrifuged at 1200 rpm.

Cell pellets, as well as purified mitochondria, were further fixed by glutaraldehyde for 1 h. All specimens were OsO₄ postfixed, alcohol dehydrated, and embedded in araldite, as previously described [24]. Thin sections were stained with uranyl acetate and lead citrate and analysed with a Philips CM10 electron microscope. Mitochondrial density was calculated in 20 different areas of 10 × 15 cm at 28000 magnification. Mitochondrial sizes were evaluated at 28000 magnification using the Philips CM10 microscope and Megaview software system.

2.4. Mitochondrial Mass and Membrane Potential. The fluorescent dye Mito Tracker Green FM (Molecular Probes), which covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues regardless of membrane potential (DWm) and JC-1 (Molecular Probes), a mitochondrial membrane potential sensor, were used to monitor mitochondrial mass and membrane potential respectively [25, 26]. The medium was removed from the culture dish and replaced with prewarmed growth medium containing 100 nM Mito Tracker Green or 2 µg/mL JC-1. After incubation for 20 min at 37°C, cells were immediately washed twice in cold PBS and analyzed using a Zeiss LSM 510 metaconfocal microscope. The variation of JC-1 signals was also analyzed by Zeiss LSM Image Examiner software.

2.5. Nucleic Acid Extraction and cDNA Synthesis. At each differentiation step, plates ($n = 3$) were washed with PBS, and nucleic acids were isolated. Total DNA and total RNA were extracted using QIAamp DNA kit (Qiagen, Chatsworth, Calif, USA) and RNeasy Mini Kit (Qiagen, Chatsworth, Calif, USA), respectively, following the manufacturer's instructions. Nucleic acid concentrations were estimated spectrophotometrically (DU-640; Beckman Instruments, Milan, Italy) at 260 nm. One microgram of DNase-treated total RNA was reverse transcribed using Omniscript RT (Qiagen, Chatsworth, Calif, USA) and random hexamers in a final volume of 20 µL as suggested in the manufacturer's protocol.

2.6. Construction of the Reference Plasmid pDGC. To construct the reference plasmid pDGC, a 98 bp amplicon of the mouse GAPDH, Acc. no. NM_008084, and a 100 bp sequence of mouse mtDNA located within the COXII, Acc. no. NP_904331.1, were inserted into the TA cloning and HindIII restriction sites, respectively, of the polylinker region of pDrive (Qiagen, Chatsworth, Calif, USA). The resultant dual-insert plasmid of 4048 bp, renamed pDGC, was purified by using DNA plasmid purification Kit (Qiagen, Chatsworth, Calif, USA) and was verified as having only one copy

of each insert by restriction enzyme digestion as well as DNA sequencing. Plasmid concentration was estimated spectrophotometrically (DU-640; Beckman Instruments, Milan, Italy) at 260 nm and was adjusted to give a stock solution of 1×10^9 molecule/ μ L. Further 10-fold serial dilutions down to a concentration of 1×10^1 molecule/ μ L were prepared.

2.7. Determination of mtDNA Content and mRNA Expression Levels by Quantitative Real-Time PCR. All quantitative real-time PCR reaction were carried out in a Bio-Rad iCycler iQ Multi-Color Real-Time PCR Detection System using 2x Quantitect SYBR Green PCR kit (Qiagen). The PCR conditions were set up as follows: hot start at 95°C for 10 min then 40 cycles of the two steps at 95°C for 30 sec and at 60°C for 30 sec. Reaction mix (25 μ L final volume) consisted of 12.5 μ L Mix Hot-Start (Qiagen), total DNA (50 ng) or cDNA (1 μ L) template, 2 μ L SYBR Green, and 0.3 μ M of each primer (Table 1). Threshold cycle (Ct) was determined on the linear phase of PCRs using the iCycler iQ Optical System software version 3 (BioRad, Milan, Italy). The specificity of the amplification products obtained was confirmed by examining thermal denaturation plots, by sample separation in a 3% DNA agarose gel and by sequencing. A precise determination of mitochondrial DNA (mtDNA) copy number was determined amplifying both COXII and GAPDH as mtDNA and nDNA targets, respectively. Quantification of mtDNA was performed by reference to a single recombinant plasmid (pDGC) containing a copy of each target DNA sequence (mitochondrial and nuclear). COXII and GAPDH gene copy number were determined by interpolating the threshold cycle (Ct) from standard curves that were obtained using serial dilution of the recombinant plasmid pDGC. The mtDNA/nDNA ratio was obtained, relating the mitochondrial and nuclear DNA quantities. The relative expression of *Tfam*, *PGC1- α* transcription factors, and COXII were quantified using 1 μ L of cDNA template and the PCR condition already described above. The amount of each target transcript was related to that of the reference gene (the ribosomal protein S16) using the method described by Pfaffl [28]. In fact, previous experiments have shown that S16 mRNA is stable during the differentiation process [5]. All oligonucleotide primers were designed using Primer Express version 1.0 (Perkin-Elmer Applied Biosystem) from the GenBank database and are listed in Table 1.

2.8. Preparation of Mitochondria for Enzymatic and Proteomic Analyses. About 3×10^7 cells were harvested and washed with $1 \times$ PBS buffer. The pellet was resuspended in 5 mL of an ice-cold solution containing 5 mM K^+ -Hepes, pH 7.4, 210 mM mannitol, 1 mM EGTA, 70 mM sucrose, and 55 μ g/mL digitonin and homogenized by 10 strokes in an ice-cold glass homogenizer. Nonlysed cells and nuclei were pelleted by centrifugation at 750 g for 20 min at 4°C, and the supernatant was centrifuged again at 8000 g for 15 min at 4°C. The resulting mitochondrial pellet was resuspended in 1 mL of 5 mM K^+ -Hepes, pH 7.4, 210 mM mannitol, and 70 mM sucrose at 37°C and treated for cytochrome oxidase activity and proteomic analysis as described below.

2.9. Enzymatic Activity of Cytochrome c Oxidase. Cytochrome c oxidase activity was determined spectrophotometrically using the Cytochrome c Oxidase Assay Kit (Sigma, MO, USA). Reactions were started by the addition of ferrocytochrome c. The difference in extinction coefficients ($\Delta\epsilon^{mM}$) between ferrocytochrome c and ferricytochrome c is 21.84 at 550 nm. One unit of enzyme will oxidize 1.0 μ mole of ferrocytochrome c per minute at pH 7.0 at 25°C. The proteins were determined according to the method of Lowry et al. [29] using bovine serum albumin as the standard.

2.10. Proteomic Analysis. Mitochondria were resuspended in urea lysis buffer (8 M urea, 4% CHAPS, 65 mM DTE, and 40 mM Tris base) and sonicated for 5 s on ice. Following centrifugation at 21000 g, protein concentration was determined by Bradford assay [30]. Aliquots were then stored at -80°C until use. Two dimensional electrophoresis (2-DE) was carried out as previously described [31]. Briefly, isoelectric focusing was made on Immobiline strips providing a nonlinear pH 3–10 gradient (GE Healthcare Italy, Milan, Italy) using an IPGphor system (GE Healthcare) and applying an increasing voltage from 200 V to 3500 V during the first 3 h, then stabilized at 5000 V for 20 h. After IPG strip equilibration, the second dimension was carried out in a Laemmli system on 9%–16% polyacrylamide linear gradient gels (18 cm \times 20 cm \times 1.5 mm) at 40 mA/gel constant current, until the dye front reached gel bottom. Forty-five μ g (analytical runs) or 500 μ g (semipreparative runs) of proteins were used for each electrophoretic run.

Analytical gels were stained with silver nitrate [32], while semipreparative gels for mass spectrometry analysis were stained with Brilliant Blue G-Colloidal (Sigma-Aldrich, Saint Louis, USA) according to the manufacturer's procedure. Gel images were acquired by Fluor-S MAX multi-imaging system (BioRad Laboratories Italy, Segrate, Italy), and the data were analysed with ImageMaster 2D Platinum software. To test the significant differences in the relative protein levels for each spot, a paired Student's *t*-test statistic was applied at a significant level of $P < .05$.

The gel digestion procedure was adapted from Shevchenko et al. [33] as previously described [34].

LC-ESI-MS/MS analysis was performed using a Q-TOF microTM mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray nanoflow electrospray ion source and a CapLC system. The sample was analyzed using a Symmetry C18 nano column (Waters, Milford, Mass, USA) as an analytical column. For protein identification, MS/MS spectra were searched by MASCOT (Matrix science, www.matrixscience.com, UK) using the database of NCBI nr. For unmatched peptides, however, good quality MS/MS spectra were manually sequenced using de novo sequencing process (carried out by PepSeq of the Masslynx 4.0 software, Micromass), and the obtained sequence was subsequently used in ExPASy TagIdent.

2.11. Statistical Analysis. Unless noted otherwise, the results were expressed as mean values \pm S.E.M. for the indicated number of measurements. Results from PCR real-time

TABLE 1: List of primer pairs.

Genes	Primers (forward)	Primers (reverse)	References
<i>Mouse COXII</i>	5'-CATCTGAAGACGTCTCCACTCAT-3'	5'-TCGGTTTGATGTTACTGTTGCTTGAT-3'	this study
<i>Mouse TfamA</i>	5'-GGGAGCTACCAGAAGCAGAA-3'	5'-CTTTGTATGCTTTCCACTCAGC-3'	this study
<i>Mouse PGC1-α</i>	5'-CGGAAATCATATCCAACCAG-3'	5'-TGAGGACCGCTAGCAAGTTTG-3'	[27]
<i>Mouse S16</i>	5'-TGAAGGGTGGTGGACATGTG-3'	5'-AATAAGCTACCAGGGCCTTTGA-3'	[5]
<i>Mouse GAPDH</i>	5'-TGACGTGCCGCCTGGAGAAA-3'	5'-AGTGTAGCCCAAGATGCCCTTCAG-3'	[27]

TABLE 2: Mitochondrial area and number variability during differentiation by means of ultrastructural observations of resin-embedded sections.

Differentiation day	Δ cell mitochondria area/ 10×15 cm total surface	Δ isolated mitochondria area/ 10×15 cm total surface	Number of mitochondria/ 10×15 cm total area
$T = 0$	$3.30E-02 \pm 0.005$	$6.90E-02 \pm 0.009$	6 ± 0.89
$T = 1$	$9.30E-02 \pm 0.008$	$9.80E-02 \pm 0.008$	10 ± 1.14
$T = 4$	$8.20E-02 \pm 0.004$	$8.10E-02 \pm 0.004$	13 ± 0.91
$T = 7$	$3.40E-02 \pm 0.008$	$5.40E-02 \pm 0.005$	15 ± 0.86

analysis were compared with the ANOVA test, followed by a post hoc test using Tukey's multiple comparison test. The threshold of significance for the ANOVA and the Tukey's test was fixed at $P \leq .05$.

3. Results

3.1. Cell Differentiation. The monolayer organization, as directly analysed at RM and by means of Giemsa staining, deeply changes from undifferentiated myoblasts to myotubes. In the undifferentiated condition (Figures 1(a), 1(b), and 1(c)), myoblasts appear as fusiform or star-shaped cells, mostly flattened and closely adherent to the substrate. At the initial differentiation stage (Figures 1(d), 1(e), and 1(f)), intercellular spaces disappear, cells progressively align, and, occasionally, elongate. Four days after differentiation induction (Figures 1(g), 1(h), and 1(i)), early myotubes, with 2 or more centrally located nuclei, appear ($T = 4$, fusion index = $38 \pm 3.4\%$). The late differentiation condition (7 days) is characterized by the presence of highly structured myotubes (Figures 1(j), 1(k), and 1(l)). These are 100–600 μm syncytia and contain even more than 20 nuclei, mainly centrally located or, occasionally, aligned in parallel rows ($T = 7$, fusion index $84.6 \pm 6\%$).

3.2. Morphofunctional Changes in Mitochondrial Content. Changes in mitochondrial ultrastructure were determined by transmission electron microscopy (TEM). Figure 1 shows the progression of C2C12 cell differentiation and the related mitochondrial behaviour. Their number per area significantly increases from the undifferentiated condition (c), through the initial (f) and the intermediate (i) differentiation stages, to the final phase, characterized by myotubes, which show the maximal mitochondrial content (l). Conversely, the size of single mitochondria, appears to change throughout differentiation. It increases in the undifferentiated stage (c) reaching maximal values at initial differentiation condition

(f). It then steadily decreases (f, i), showing minimal values in the late differentiation stage (l). TEM of isolated mitochondria further highlights mitochondrial changes. Table 2 represents mitochondrial number and area variability during differentiation. They undergo a progressive rounding from 0 (Figure 1, inset c) to 7 day (Figure 1, inset l) after differentiation induction.

Analysis of mitochondria suggests a numerical increase of mitochondrial cristae from the undifferentiated to differentiated condition (Figure 1, insets: c, f, i, and l) probably correlated with the reported increase in enzymatic activities [6].

Figure 2 describes mitochondrial characteristics during differentiation, analysed by confocal microscopy, after Mito Tracker green (a–d) and JC-1 (e–h) staining, both specific mitochondrial dyes. The first covalently binds to mitochondrial proteins and is generally considered an available indicator of mitochondrial mass. The second undergoes characteristic fluorescence changes according to the mitochondrial membrane $\Delta\Psi$, thus revealing functional mitochondrial alterations. In myoblasts (a, b, c, and d), both fluorescent probes show a perinuclear mitochondrial distribution. Indeed, at initial differentiation stages, numerous mitochondria can be identified as clearly distinguishable single organelles. Moreover, after differentiation induction, mitochondrial mass increased appearing uniform in myotubes (e and f). Mitochondrial membrane potential also increased, highlighted by JC-1 main red staining (g), still more evident in late differentiation condition shown in (h). Graphs of lower panel show the increasing level of red fluorescence JC-1 intensity from myoblasts (i) to late myotubes (j).

3.3. mtDNA Content. To ensure accurate quantification of mtDNA, we applied a PCR-based assay using a dual-insert reference plasmid, containing both mtDNA and nuclear DNA targets [35]. In this work, pDrive plasmid was used to

TABLE 3: Identification of mitochondrial protein differentially expressed during myogenesis.

No.	Protein	Score	NCBI nr	Peptides	MW	PI	Localization
1	Malate dehydrogenase, mitochondrial (MDH2)	162	DEMSMM	IFGVTTLDIVR, VDFPQDQLATLTGR, IQEAGTEVVK	35589	8.93	Mitochondrial matrix
2	Malate dehydrogenase precursor (MDH2)	345	DEMSMM	VAVLGASGGIGQPLSLLLK, IFGVTTLDIVRANTFVAELK, VDFPQDQLATLTGRIQEAGTEVVK, MIAEAIPELK	35589	8.93	Mitochondrial matrix
3	Aldehyde dehydrogenase 2, mitochondrial (Aldh2); putative uncharacterized protein	143	Q3TVM2_MOUSE	TIPIDGDFFSYTR, VAEQPTLTALYVANLIK, EAGFPFGVNVNIPGFGPTAGAAIASHEGVDK	56560	7.03	Mitochondrial matrix
4	Aldehyde dehydrogenase precursor, mitochondrial	411	I48966	TIPIDGDFFSYTR, LGPALATGNVVVMK, TFVQENVYDEFEVER, TEQGPQVDETFQK, GYFIQPTVFGDVK, TIEEVVGR, YGLAAAVFTK	56502	7.53	Mitochondrial matrix
5	Voltage-dependent anion channel 1 (VDAC1)	161	VDAC1_MOUSE	LTFDSSSPNTGK, VTQSNFAVGYK, LTLSALLDGK	32331	8.55	Mitochondrial outer membrane
6	Voltage-dependent anion channel 1 (VDAC1)	344	VDAC1_MOUSE	GYGFGLIK, WTEYGLTFEK, LTFDSSSPNTGK, VTQSNFAVGYK, VNNSSLIGLYTQTLKPGIK, LTLSALLDGK	32331	8.55	Mitochondrial outer membrane
7	Pyruvate dehydrogenase (lipoamide) beta. (Pdhb protein)	288	Q99LW9_MOUSE	TYYMSAGLPVPVVER, DFLIPIGK, IMEGPAFNFLDAPAVR, VTGADVPMPIYAK, VLEDNSVPQVK	34814	5.63	Mitochondrial matrix

TABLE 3: Continued.

No.	Protein	Score	NCBI nr	Peptides	MW	PI	Localization
8	Prohibitin	396	A39682	DLQNVNITLR,	29802	5.57	Mitochondrial intermembrane space
				ILFRPVASQLPR,			
				IYTSIGEDYDER,			
				VLPSITTEILK,			
				FDAGELITQR,			
9	ATP synthase D chain, mitochondrial (ATP5H)	131	ATP5H_MOUSE	AAIISAEGDSK,	18607	5.52	Mitochondrial inner membrane
				AAELIANSLATAGDGLIELR,			
				NITYLPAGQSVLLQLPQ			
				ANVAKPGLVDDFEK,			
				YTALVDQEEKEDVK			
10	Ubiquinol-cytochrome c reductase core protein 1	135	Q3THM1_MOUSE	TDLTDYLNK, IQEVDQAQMLR	52806	5.89	Mitochondrial inner membrane
11	Fumarate hydratase precursor, mitochondrial (FH)	170	UFRT	AAAEVQNQYGLDPK, AIEMLGGLGSK, VAALTGLPFVTPNPK	54429	9.06	Mitochondrial
12	Superoxide dismutase precursor	72	DSRTN	GDVTTQVALQPALK	24659	8.96	Mitochondrial matrix
13	Aconitase 2, mitochondrial (ACO2)	340	Q3UDK9_MOUSE	DINQEVYNFLATAGAK, SQFTITPGSEQIR, NTIVT'SYNR, FNPETDFLTGK, NAV'TQEFQVPD'TAR, WVVIGDENYGE'GSSR	85376	8.08	Mitochondrial matrix
14	Dihydrolipoamide dehydrogenase (DLDH)	139	Q99LD3_MOUSE	ADGSTQVIDTK, EANLAAAF'GHPINF	54238	7.99	Mitochondrial matrix

TABLE 3: Continued.

No.	Protein	Score	NCBI nr	Peptides	MW	PI	Localization
15	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit (ATP5B)	998	Q3TFD7_MOUSE	LVLEVAQHLGESTVR,	56207	5.25	Mitochondrial inner membrane
				TIAMDGTEGIVR,			
				VLDSGAPIK,			
				IPVGPETLGR,			
				IMNVIGEPIDER,			
				VVDLLAPYAK,			
				IGLFGGAGVGK,			
				TVLIMELINNVAK,			
				EGNDLYHEMIESGVNLK,			
				VALVYQGMNEPPGAR,			
				VALTGLTVAEYFR,			
				FTQAGSEV/SALLGR,			
				AIAELGIYPADVPLDSTSR,			
				IMDPNIVGNEHYDVAR,			
				ILQDYK,			
16	Protein disulfide isomerase A3 (Pdia3)	94	PDIA3_MOUSE	FLSQPFQVAEVFTGHMGK	56472	5.88	Endoplasmic reticulum, also present in mitochondria (see discussion).
				DASVVGFPR,			
				GFPTIYFSPANK,			
				ELNDFISYLQR			

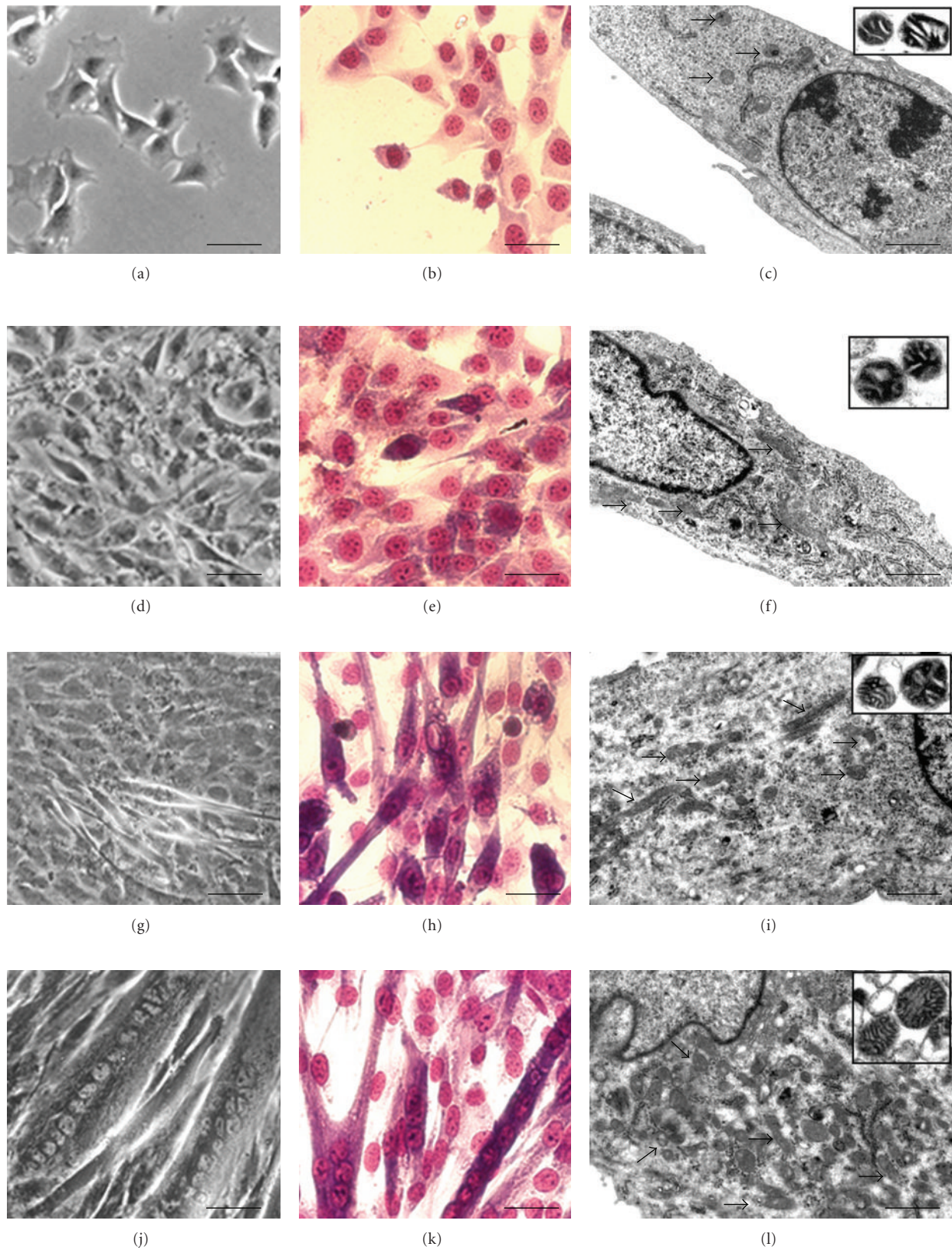


FIGURE 1: Undifferentiated (a, b, c), early differentiation (d, e, f), intermediate differentiation (g, h, i) and late differentiation stages (j, k, l), are indicated by RM (a, d, g, j), Giemsa staining (b, e, h, k), and TEM (c, f, i, l). Mitochondrial morphology is further detailed by the correspondent insets, showing TEM analysis of isolated mitochondria. C2C12 cell differentiation morphological progression is evident, as well as mitochondrial behaviour in the various stages. (a, b, d, e, g, h, j, k): Bar = 20 μm ; (c, f, i, l): Bar = 0.5 μm ; insets, Bar = 0.1 μm .

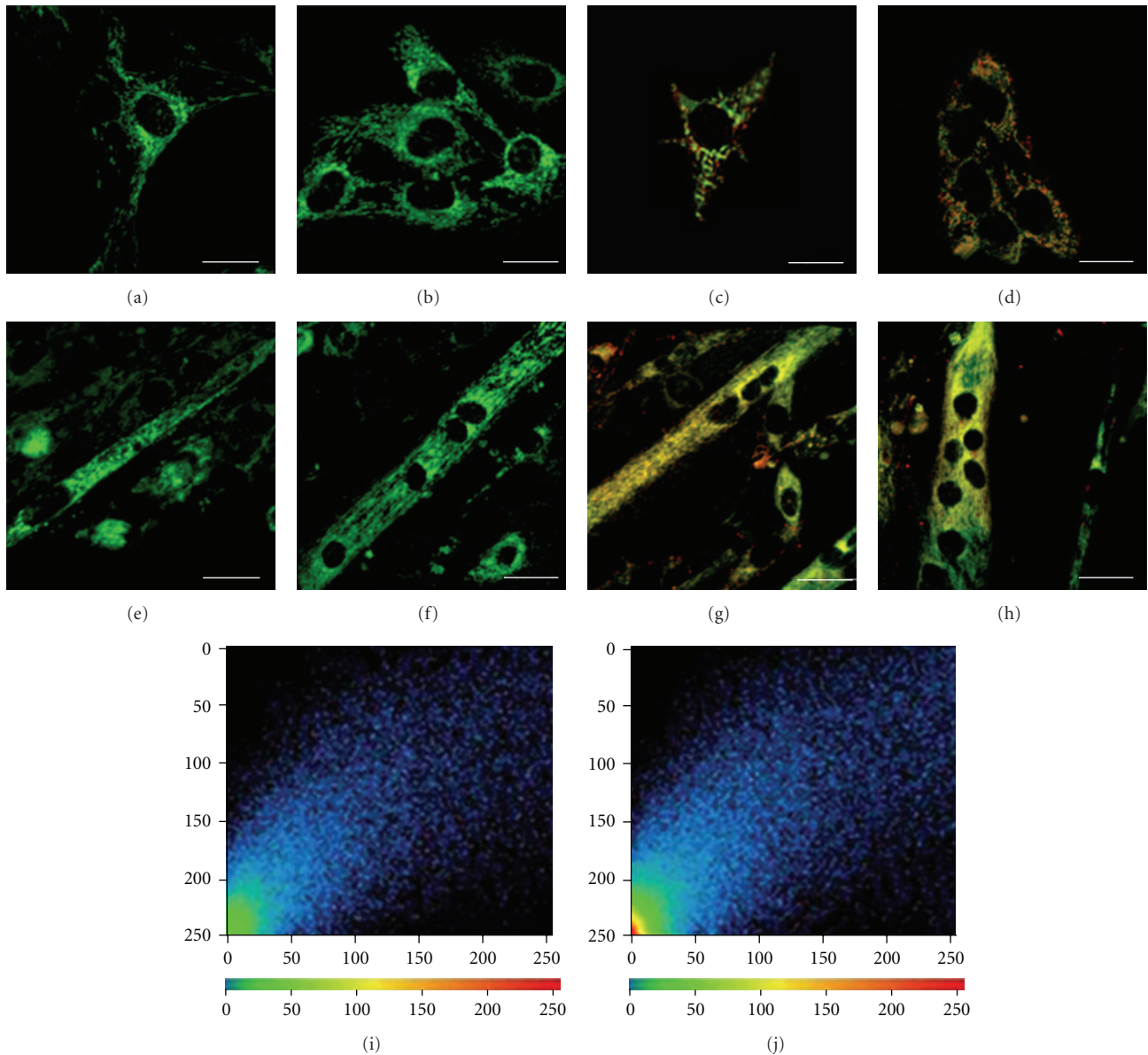


FIGURE 2: Confocal microscopy of C2C12 myoblasts (a–d) and late myotubes (e–h), after Mito Tracker (a, b, e, f) and JC-1 (c, d, g, h) staining. Graphs of lower panel show the different fluorescence JC-1 intensity in myoblasts (i) and late myotubes (j). (a–h): Bar = 20 μ m.

construct the reference plasmid pDGC, containing a single copy of *COXII* and *GAPDH* segments, the mitochondrial and nuclear target genes, respectively.

As shown in Figure 3(a), twenty-four hours after differentiation induction, the relative amount of mtDNA undergoes a 2-fold increment at the intermediate period of differentiation ($T = 3$) reaching a plateau level at the final stage of maturation ($T = 7$).

3.4. mRNA Expression Level of Mitochondrial Biogenesis “Master” Genes. An increase in mitochondrial biogenesis reflects an enhanced expression of nuclear and mitochondrial genes [36–38]. Two master genes involved in the mitochondrial biogenesis, the nuclear transcriptional coactivator peroxisome proliferative activated receptor, gamma,

coactivator 1 alpha (*PGC1 α*), and the mitochondrial transcription factor A (*Tfam*) were quantified using RT real-time PCR during differentiation. *PGC1 α* induces mitochondrial biogenesis by interacting with several nuclear transcription factors [36–39], and *Tfam* is involved in the mitochondrial genome transcription [40, 41], replication [42], and it is also crucial for maintaining mitochondrial DNA [43].

As shown in Figure 3(b), *PGC-1 α* expression does not change during the first 24 h from the induction of differentiation while progressively increasing up to 9.2-fold in differentiated myotubes on the 7th day compared to the myoblasts at time T_0 .

The *Tfam* expression level during the myoblasts differentiation is slightly shifted compared to the *PGC-1 α*

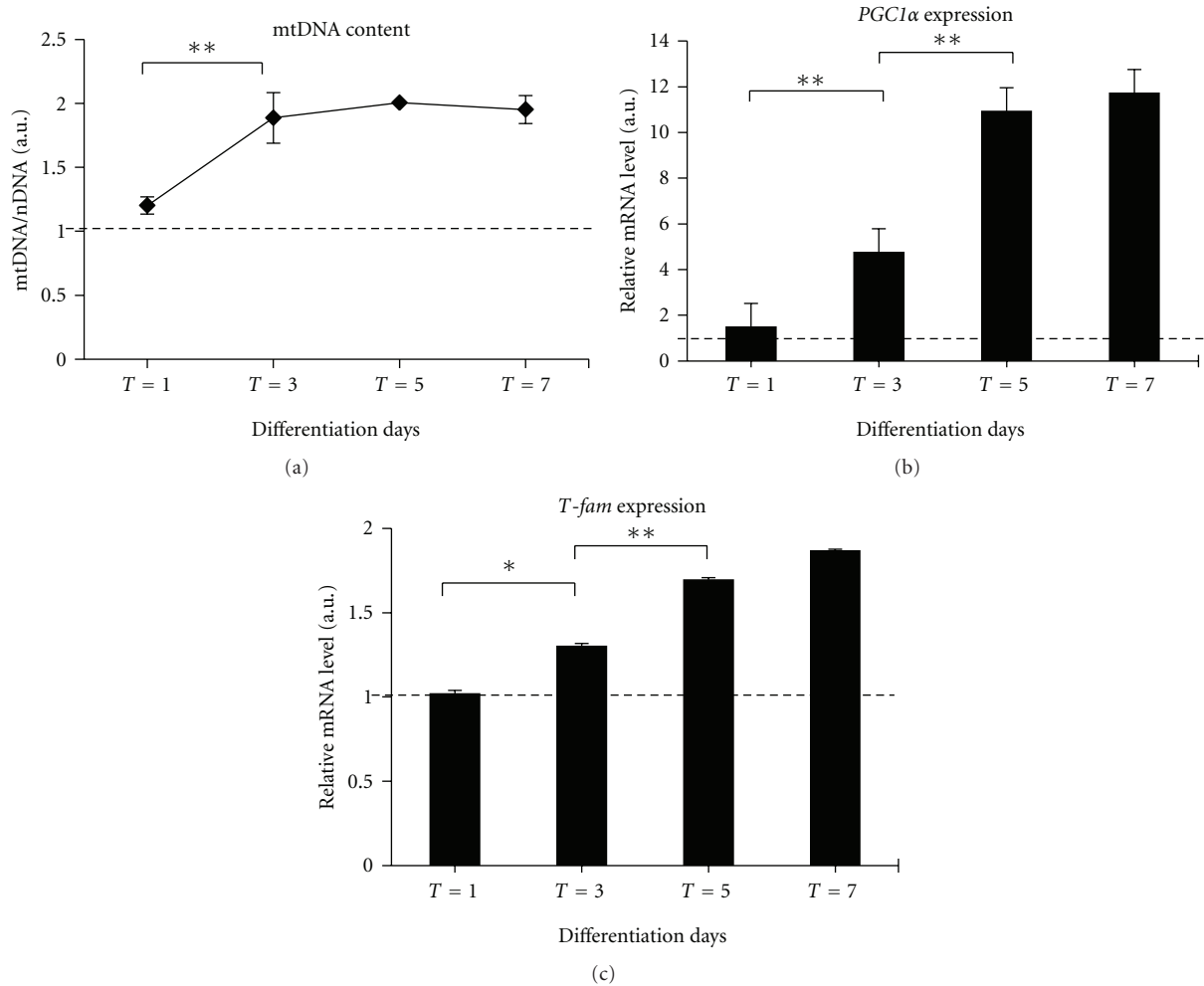


FIGURE 3: Evaluation of mitochondrial biogenesis during myoblast differentiation. In (a), determination by real-time PCR of mtDNA content expressed as mtDNA/nDNA ratio (*COXII*/*GAPDH*), as described in Section 2. In (b), quantitative analyses of *PGC-1α* and *T-fam* by real-time PCR. The amount of each target transcript was related to that of the reference gene (the ribosomal protein S16). Data are expressed as the mean \pm SEM of three experiments; all samples were analyzed in triplicate. Results from PCR real-time analysis were compared with the ANOVA test, followed by a post hoc test using Tukey's multiple comparison test. The threshold of significance for the ANOVA and the Tukey's test was fixed at $*P \leq .05$; $**P \leq .01$.

expression; in fact, it increased significantly between days 3–7 (Figure 3(c)).

3.5. Cytochrome c Oxidase Activity and *COXII* Expression Level. The mitochondrial enzymatic activities of cytochrome oxidase reflecting the respiratory chain activities were significantly higher in myoblasts able to differentiate (Figure 4(a)).

In addition, we evaluated the expression level of the corresponding gene coding for the subunit II of mitochondrial cytochrome c oxidase (*COXII*), which represents a target gene for mitochondrial transcriptional activity [27, 44, 45]. On days 3–7, the mitochondrial *COXII* transcript levels were significantly higher than in proliferating myoblasts (Figure 4(b)).

3.6. Changes in Mitochondrial Proteomic Profile. To highlight significant changes in mitochondrial proteome during

differentiation, we performed a 2D page on mitochondria isolated from C2C12 myoblasts over a 7-day time span differentiation. A total of 994 spots (mean) could be resolved on a silver-stained large 2DE gel, where we loaded 45 μ g of total protein. A larger amount of protein *per spot* was necessary for protein identification, thus we used preparative gels stained with Brilliant Blue G-Colloidal. To evaluate the possible presence of cellular contaminants, we compared the mitochondrial map with that of the whole cellular lysate in which we had previously identified several cytosolic and membrane proteins [46]. The comparison of 2D maps of mitochondria and whole cell lysate allowed us to state that the preparation of mitochondria contained little or no cellular contaminants.

The study of quantitative changes of individual proteins in a purified mitochondrial fraction showed that 32 mitochondrial proteins increased significantly in abundance

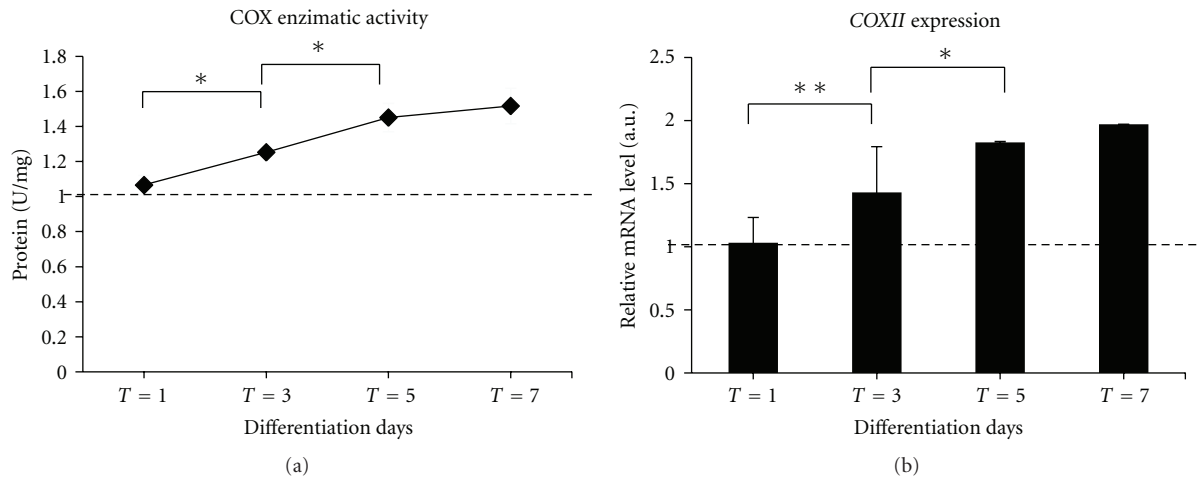


FIGURE 4: Time course change of cytochrome oxidase (COX) enzymatic activity and transcription level of *cytochrome oxidase subunit II (COXII)* gene at progressive differentiation stages. (a) Quantitative analysis enzymatic activity. (b) The expression level of *COXII* is related to *S16* mRNA gene level. Results from PCR real-time analysis were compared with the ANOVA test, followed by a post hoc test using Tukey's multiple comparison test. The threshold of significance for the ANOVA and the Tukey's test was fixed at $*P \leq .05$; $**P \leq .01$.

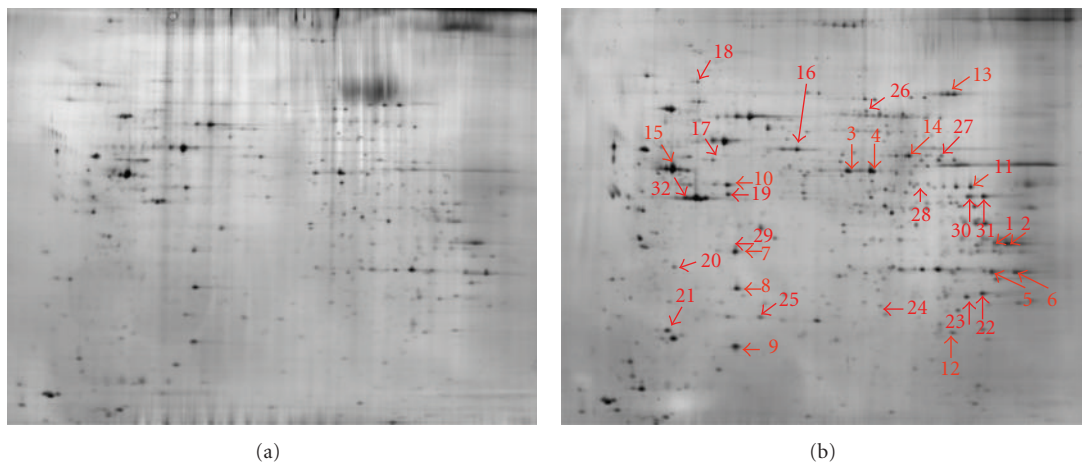


FIGURE 5: Image of a silver-stained 2-DE gel of 45 μ g purified mitochondrial proteins from C2C12 myoblasts at 0 (a) and 7 (b) days of differentiation time. Differentially expressed spots are indicated by arrows and numbered according to Table 3.

(Figure 5). The proteins showing the greatest expression changes were also characterized by electrospray ionisation (ESI) tandem mass spectrometry. In particular, the major changes occurred between T1 and T4 time of differentiation, while fewer differences were shown between T0-T1 and T4-T7 (Table 3).

The main mitochondrial proteins which could be detected in fully differentiated syncytia were involved in the citric acid cycle (malate dehydrogenase: MDH2, fumarate hydratase: FH, and aconitase 2: ACO2) or belong to the pyruvate dehydrogenase complex (pyruvate dehydrogenase, lipoamide beta: PDHB, dihydrolipoamide dehydrogenase), complex III (ubiquinol-cytochrome c reductase core protein 1: UQCRC1) and complex V (ATP synthase, H⁺ transporting mitochondrial F1 complex, beta subunit: ATP5B, and ATP synthase d chain: ATP5H) of the respiratory chain.

This was also interesting for the superoxide dismutase (MnSOD), a voltage-dependent anion-selective channel protein 1 (VDAC1), and the protein disulfide-isomerase A3 (Pdia3) that were differentially expressed during differentiation.

4. Discussion

In this study, we described temporal mitochondrial changes during the myogenic program of C2C12 myoblasts by analyzing complementary key parameters for mitochondrial dynamics, biogenesis, and functionality. Of particular interest is the contribution of the proteomic approach to better define the pattern of mitochondrial protein expression accompanying differentiation in myotubes and potentially involved in the crosstalk between nuclei and mitochondria.

Morphological analysis performed by fluorescence microscopy with markers of mitochondrial mass/volume and $\Delta\Psi$, as well as ultrastructural analysis, allowed us to acquire more information regarding the mitochondrial organization and dynamics in C2C12 myoblast differentiation.

Mitochondrial organization in myoblasts was perinuclear, and it was possible to discriminate individual mitochondrion by both MitoTracker Green and JC1 staining. This type of mitochondrial distribution is described in the literature for other cell types including fibroblasts [47], pancreatic acinar cells [48, 49], astrocytes, and neurons [50].

On the contrary, in myotubes, morphological observation by epifluorescence did not allow us to discriminate individual mitochondrion, showing homogeneous staining, representative of the mitochondrial network, well described in skeletal muscle tissue [51, 52]. TEM analysis showed a mitochondrial remodeling during differentiation and alignment of organelles along the myotubes.

At this level, we cannot show the formation of a network equal to that which is found in skeletal muscle fibers, where mitochondria are arranged in crystal structures closely related to the sarcoplasm [51, 52]. Indeed, the sarcomeres of myotubes are only sketched [53], but they may support the development of a mitochondrial network during myotube maturation.

The mitochondrial counting *per* area of cell surface, obtained by TEM, showed that the number of mitochondria increased from undifferentiated to differentiated conditions. Mature myotubes contained approximately 2-fold more mitochondria than myoblasts. However, in the first 24 hours after induction of differentiation, the mitochondria increased in size up to 3-fold gradually decreasing in size only after the intermediate phases to reach the same size observed in myoblasts at $T = 0$, in mature myotubes ($T = 7$). This observation suggests that mitochondria first undergo fusion and then fission, which allows their distribution during syncytia formation as previously reported [54]. Nevertheless, the mitochondria in myotubes showed a greater extension of mitochondrial cristae than mitochondria in myoblasts. Marked stimulation of the biosynthesis of the phospholipid cardiolipin during the differentiation phases has been observed in previous studies on L6E9 myoblasts and other cells [54, 55]. It is probably necessary to supply the proper amount of functional mitochondrial inner membrane for the respiratory chain proteins involved in oxidative metabolism [7].

All the parameters observed through morphological analysis confirm a linear increase in mitochondrial biogenesis during differentiation. The morphological analysis corroborates the progression of the myogenic process and the increase in biochemical markers such as the transcription factors of mitochondrial biogenesis *PGC-1 α* and *Tfam*.

Of particular interest was the timing of mtDNA replication compared to mitochondrial biogenesis. Although mitochondrial biogenesis increased linearly during differentiation, mtDNA content increased significantly from the early days of differentiation already reaching a plateau at the intermediate stage. Hence, our investigation shows a slight difference in timing between DNA replication and

mitochondrial biogenesis. This shift could be explained by the biological cycle of mitochondria [56]. Mitochondrial fission is preceded by an extension of the organelles and the mtDNA replication phase. Although there is a slight shifting, the correlation between the number of copies of mtDNA and mitochondrial biogenesis is positive ($r^2 = 0.85$, data not shown).

In several studies, the measure of mtDNA copy number has been considered proportional to the number of mitochondria, a golden star for mitochondrial density [57–60]. However, changes in mitochondrial abundance regardless of the mtDNA copy number may occur, especially in peculiar conditions such as during alterations in the rates of intracellular ROS generation [61]. Franko et al., investigating C2F3 mouse myoblasts, showed that an increment in mtDNA does not always correlate with the proliferation of mitochondria or with their activity [62]. In this investigation, the mtDNA copy number of C2C12 myoblasts significantly increased during the early-intermediate differentiation phases ($T = 1$ and $T3$) up to 2-fold remaining constant during the myotube maturation. Likewise, over the course of myoblast differentiation in rat cell line L6, a small but significant increase in mitochondrial DNA copy number was observed by [27]. Furthermore, in a recent study on the regulation of mitochondrial biogenesis during myogenesis, mtDNA copy number was determined as a marker for mitochondrial density using QPCR, and the mtDNA copy number was 4-fold higher in fully differentiated myotubes than it was in myoblasts [60].

Interestingly, during differentiation, an increased mtDNA transcriptional activity and oxidative metabolism correspond to an enhanced mitochondrial biogenesis, as highlighted by the upregulation of *COXII* mRNA levels and cytochrome c oxidase activity ($r^2 = 0.83$ and $r^2 = 0.97$, resp., data not shown).

In our investigation, we integrated the mitochondrial changes observed by multiple key determinants with proteomic analysis.

In the literature, mitochondrial proteomic maps of differentiating myoblasts are not available; hence, this work presents the first proteomic profile of mitochondria during the myogenesis program. Previously, proteome-based investigations have been carried out to provide a description of the myogenic differentiation program [46, 63, 64]. We employed a proteomic approach using two dimensional electrophoresis, particularly helpful for investigating the subset of cellular proteins, such as organellar proteins, due to the reduced complexity of the protein sample [65].

In particular, analyzing the differentially expressed proteins in the mitochondrial proteome map during the myogenic process, we observed that also the enzymes involved in cellular respiration, such as pyruvate dehydrogenase, MDH2, FH, ACO2, and more markedly HB and 5B ATP synthase subunits, representative of oxidative phosphorylation, increase linearly with the mitochondrial biogenesis showing a positive correlation ($r^2 = 0.915$, data not shown). These findings are consistent with the differentiating cells' greater reliance on aerobic metabolism compared to the glycolytic

metabolism that characterizes the undifferentiated myoblasts [7].

Moreover, our observations are in agreement with Moyes and coworkers who demonstrated an mRNA increment for pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase, cytochrome c oxidase, and NADH dehydrogenase [6]. The increment of Krebs cycle and respiratory chain proteins supports the augmented mitochondrial functionality also confirmed by the COX enzymatic activity during myoblast differentiation [6, 7].

The data obtained using the proteomic approach are consistent with the increase in mitochondrial function and membrane depolarization highlighted by JC-1 and in agreement with the increase of mitochondrial cristae observed by TEM. These data support the evidence described by Sauvanet (2010) assuming that mitochondrial bioenergetics and dynamics are linked and that mitochondrial morphology reflects their functional status [66].

Proteomic analysis revealed other notable proteins involved in the crosstalk between nuclei and mitochondria, such as MnSOD, a recently described cell protection molecule whose role is the maintenance of myoblast mitochondrial function and the preservation of the potential of myoblast stem cell differentiation [67]. In our model, the increment of MnSOD expression is highlighted during myotube formation ($T = 4$ – $T = 7$), as recently documented by Kislinger and collaborators [64].

Another interesting protein associated with the progressive myoblast differentiation is the protein transport voltage-dependent anion-selective channel protein 1. It has been reported that its gene expression changes during myoblast differentiation [68], and it has been recognized as a key protein in mitochondria-mediated apoptosis, since it is a target for the pro- and antiapoptotic Bcl2-family of proteins, and for its function in releasing apoptotic proteins located in the intermembrane space [69]. Apoptosis is considered to be essential for normal skeletal muscle development by eliminating cells with defects or undergoing damage during differentiation [70–73]. The detection of mitochondrial myogenesis-correlated proteins, known to play a role in apoptosis, supports the link between differentiation and this type of cell death [46, 74].

Another protein induced during differentiation belongs to the protein disulfide isomerase (PDI) family: disulfide-isomerase A3 (Pdia3). These molecules are best known for their role as chaperones in protein-folding reactions in the endoplasmic reticulum [75]. However, mitochondrial localization has been documented in the outer membrane of rat liver mitochondria [76–78], bovine liver mitochondria [79], in a mitochondrial fraction of *Arabidopsis* [80] and a chloroplast isoform in *Chlamydomonas* [81]. PDI may act in mitochondria in several ways: enhancing protein folding of newly synthesized proteins, reducing disulphides required to activate proteins, controlling mitochondrial membrane permeability, and playing a role in the assembly and function of some enzymatic systems [76, 77].

In conclusion, this investigation demonstrates that the proteomic approach, integrated with the classical morpho-functional and biochemical analyses, provides a complete

scenario of mitochondrial dynamics, biogenesis and functionality useful in comparative surveys of mitochondrial pathogenic or senescent satellite cells.

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