

## Proteomics-based investigation in C2C12 myoblast differentiation

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Skeletal muscle cell differentiation is a multistage process extensively studied over the years. Even if great improvements have been achieved in defining biological process underlying myogenesis, many molecular mechanisms need still to be clarified.

To further highlight this process, we studied cells at undifferentiated, intermediate and highly differentiated stages, and we analyzed, for each condition, morphological and proteomic changes. We also identified the proteins that showed statistical significant changes by a ESI-Q-TOF mass spectrometer. This work provides further evidence of the involvement of particular proteins in skeletal muscle development. Furthermore, the high level of expression of many heat shock proteins, suggests a relationship between differentiation and cellular stress. Intriguingly, the discovery of myogenesis-correlated proteins, known to play a role in apoptosis, suggests a link between differentiation and this type of cell death.

**Key words:** C2C12 cells, differentiation, proteome, TEM, 2-D electrophoresis, mass spectrometry.

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Skeletal muscle differentiation is a highly coordinated process, accompanied by deep changes in both cell morphology and gene expression patterns. In early myogenesis, specific transcription factors determine the commitment of mesodermal cells to myogenic progenitors and, then, myoblasts. Later, myoblasts exit from cell cycle and differentiate, becoming multinucleated myotubes (Muntoni *et al.*, 2002). However, a number of myoblasts persist in adult muscle as satellite cells, localised between basement membrane and muscle fiber sarcolemma (Cooper *et al.*, 1999). Although they are quiescent in adult muscle, satellite cells are responsible for muscle regeneration following denervation, physical exercise stress or muscle lesions. After damage, they migrate to the injured site and proliferate, fusing to pre-existing fibers or together, to form new myofibers (Hawke and Garry, 2001).

Molecular characterization of proteome is necessary for a complete understanding of biological systems (Giorgianni *et al.*, 2003) and previous studies have been also performed on gene expression profile during in vitro differentiation of C2C12 myoblast cell line (Delgado *et al.*, 2003; Tomczak *et al.*, 2004). A proteomic approach to myoblast differentiation was used by Tannu *et al.* (2004), who compared proteins in proliferating C2C12 cells and fully differentiated myotubes but not along myogenic differentiation. Also Kislinger *et al.* (2005) report an extensive LC-MS based shotgun profiling analysis of protein expression changes in differentiating C2C12 myoblasts.

Here we describe protein expression changes (by 2D-PAGE), correlated to morphological ones (transmission electron and confocal microscopy), at the undifferentiated condition (T0), like as 0 in T0 intermediate (T3-T5) and high (T7) stages of differentiation. We also identify, by electrospray

ionisation (ESI) (Griffiths *et al.*, 2001) tandem mass spectrometry, the proteins for each stage that show the major expression changes.

## Materials and Methods

### Cell cultures

C2C12 mouse adherent myoblasts were grown as described (Curci *et al.*, 2008). To induce myogenic differentiation, when about 80% cell confluence was attained, the medium containing 10% fetal calf serum was changed with a 1% new one. Cells have been observed at the undifferentiated stage and at 3, 5, 7 days of differentiation with a Nikon Eclipse TE 2000-S reverted microscope (RM) and photographed with a digital DN 100 Nikon system.

### Transmission electron microscopy

Cells, growing in flasks, were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 15 min, gently scraped and centrifuged at 520 g. Pellets were furtherly processed as reported (Burattini *et al.*, 2004).

### Immunofluorescence

Immunofluorescence (IF) was carried out in dishes containing cover slides, where cells had been seeded and cultured. Monolayers were fixed with 4% paraformaldehyde in PBS for 30 min, washed and permeabilized with 0.2% Triton X-100 in PBS for 10 min. For actin labelling, cells were treated with 5% normal horse serum (D.B.A.) and 2% bovine serum albumin (Sigma) in PBS for 30 min and then incubated with a mouse antibody against sarcomeric actin (1:100 in PBS, Sigma) overnight at 4°C. After rinsing, the monolayers were incubated with a FITC-conjugated horse anti-mouse secondary antibody (1:50 in PBS, D.B.A., Vector) for 75 min. Nuclei were counterstained with DAPI (1:20000 in PBS, Sigma) for 5 min. Cells were mounted with an antifading medium and analyzed by means a Zeiss LSM 510 Meta confocal microscope.

### Sample preparation for two-dimensional electrophoresis

Cells were detached from flasks by means of 0.25% trypsin for 10 min at 37°C, and washed with 0.1 M PBS, pH 7.4 added with a tablet of protease inhibitor cocktail (Roche diagnostic

Bmbh, Germany). After centrifugation at 1300 g, supernatant was removed and pellet was resuspended in 8 M urea, 4% CHAPS, 65 mM DTE, 40 mM Tris base and sonicated for 5 s on ice. After centrifugation at 21000 g, protein concentration was determined by Bradford assay (Bradford, 1976).

### Two-dimensional electrophoresis

Forty-five  $\mu\text{g}$  (analytical runs) or 500  $\mu\text{g}$  (semi-preparative runs) of total protein was used for each electrophoretic run. Two-dimensional electrophoresis (2-DE) was carried out as previously described (Sestili *et al.*, 2009). Briefly, isoelectric focusing was made on Immobiline strips providing a non linear pH 3-10 gradient (Amersham Biosciences) using a Multiphor II system (Amersham Biosciences). After IPG strip equilibration, the second dimension was carried out in a Laemmli system on 9-16% polyacrylamide linear gradient gels (18 cm x 20 cm x 1.5 mm) at 40 mA/gel constant current, until the dye front reached gel bottom.

### Protein visualization and image analysis

Analytical gels were stained with ammoniacal silver nitrate (Oakley *et al.*, 1980). Semi-preparative gels for mass spectrometry analysis were stained with Brilliant Blue G-Colloidal (Sigma-Aldrich, Saint Louis, USA) according to the manufacturer's procedure. Stained gels were scanned with a Scan-jet 4c (HP, Palo Alto, CA, USA) and the data were analysed with Melanie 3 computer software. Protein quantification values are calculated as relative volume (% Vol). Gel calibration was carried out using human plasma as internal standard (Bini *et al.*, 1996). To take into account experimental variation, 2-D gels were normalized by each value spot volume data by the total volume of all the matched spots in the 2-D gel image to obtain a normalized spot volume value. 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  $p < 0.05$ .

### In-gel digestion

Protein spots extracted from 2-D gels were rinsed with 100 mM ammonium bicarbonate and then dehydrated with acetonitrile. Reduction, alkylation and tryptic digestion were carried out according to the literature (Shevchenko *et al.*, 1996).

### Mass spectrometry

LC-ESI-MS/MS analysis was performed using a Q-TOF micro™ 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, MA, USA) as an analytical column.

For protein identification, MS/MS spectra were searched by MASCOT (Matrix science, www.matrixscience.com, U.K.) using 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.

### Results

When switched to differentiation medium, mitotic C2C12 myoblasts rapidly stop proliferating and initiate the differentiation program. The cells exhibit striking morphological changes along 0-10 days, finally fusing into mature multinucleated myotubes.

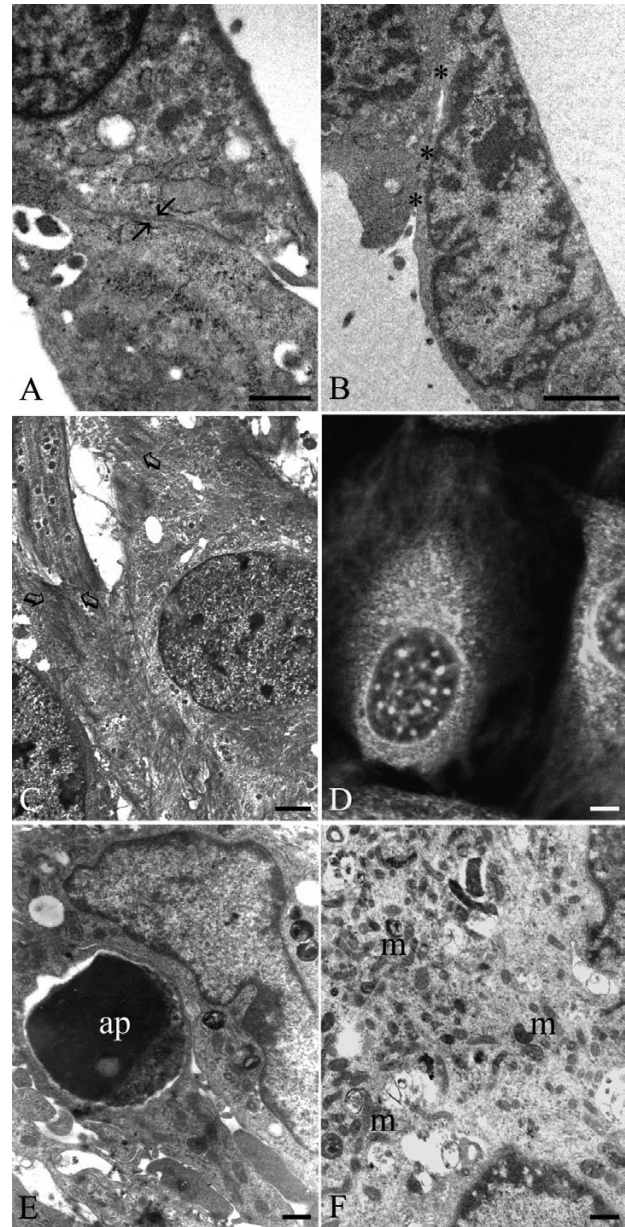
Figure 1 evidences some peculiarities appearing at TEM and/or confocal microscope observations, during C2C12 differentiation, which can be correlated, in our opinion, to proteome changes. Cell membrane contact areas are frequently present at early stage (Figure 1A) while focal cell fusions (Figure 1B) can be revealed at intermediate differentiation stage. In this condition stress fibers are clearly identifiable, at TEM, as peripheral aggregations of filaments (Figure 1C) and, at confocal microscopy, by  $\alpha$ -sarcomeric actin containing definite patches (Figure 1D). At late differentiation stage, occasional apoptotic cells appear: an apoptotic nucleus, with mostly condensed chromatin, is shown in Figure 1E. A large number of mitochondria also characterizes late C2C12 differentiation, as evidenced in 1F myotube.

In order to have a picture of the protein actually expressed during the different phases of differentiation, we performed a 2D-PAGE over a 10-day time span on differentiating C2C12 myoblasts.

Computer analysis of the different gels (n= 4 replicates), obtained from 2-DE analysis, revealed significant changes in 26 spots, shown in Figure 2.

The level of expression of many proteins appeared to increase in a linear way if compared to the differentiation grade, reaching higher values at T5 and T7: the average number of proteins in undifferentiated cells is in fact 934, at T3 1129; at T5 1252 and at T7 it is 1290.

Twenty-six spots were identified by ESI-Q-TOF



**Figure 1. C2C12 differentiation peculiarities, identified by TEM (A,B,C,E,F) and confocal microscope IF (D) at early (A), intermediate (B,C,D) and late (E,F) time. Cell membrane contacts (→) are followed by the fusion of focal membrane areas (\*).  $\alpha$  Sarcomeric actin can also be identified as stress fibers, well recognizable at TEM (⇔), and as strongly fluorescent cytoplasmic spots at IF (D), at the intermediate stage. Late differentiation is characterized by the appearance of apoptotic cells (ap) and by the significantly increased number of mitochondria (m). A-F, bar = 1  $\mu$ m**

MS. The application of tandem mass spectrometry (MS<sup>n</sup>) to peptide-derived ions using hybrid quadrupole-TOF instruments, provides both sequence and structural information, which increases the confidence in protein identification, since a single peptide sequence may be sufficient to identify a protein. Proteins and their quantitative changes are listed in Table 1. Their potential involvement in the myoblastic differentiation stage will be reported in the discussion.

## Discussion

### Undifferentiated stage

The presence of high quantity of vimentin during the starting phase of differentiation and its decrease during development progression confirm the phase-specific role of this protein. Runembert *et al.* (2002) reported that vimentin does not affect cell proliferation and differentiation directly, but participates in preserving proper transport

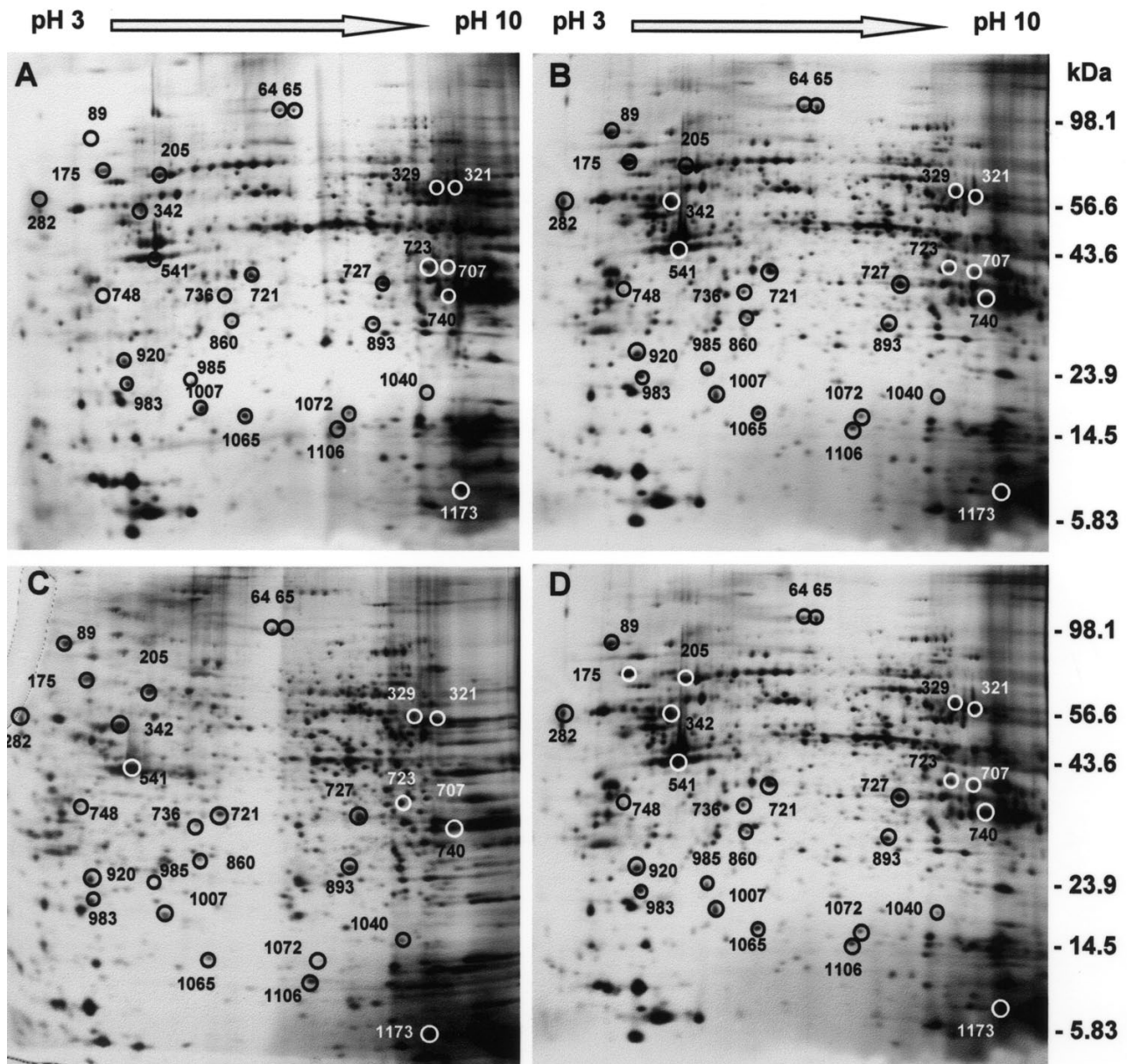
functions, possibly by maintaining membrane physical state.

Stathmin expression is important in cell proliferation. Cell-cell contacts (Figure 1 A), probably mediated by adhesion molecules such as cadherins, are responsible for the high density-induced expression of stathmin, which might then be involved in the control of myoblast proliferation (Balogh *et al.*, 1996). In our case stathmin expression is higher during the starting phase (cell proliferation), and decreases during cell differentiation.

Gelsolin-like capping protein plays a role in organizing actin filaments during sarcomere formation. Hug *et al.* (1995) monitored its levels in Dictyostelium cells and found changes in resting and chemoattractant-induced actin assembly, consistent with the *in vitro* properties of capping protein. Cell movement is activated with capping protein increase and decreased with their down regulation. So capG overexpression in our model may

**Table 1. Identification of proteins differentially expressed during myoblast differentiation.**

| Spot (ID) | Protein   | Time (high expression)         | Score | Sequence coverage (%) /no. of peptides matched | NCBI ID      | pI   | Nominal mass |
|-----------|---|--------------------------------|-------|--|--------------|------|--------------|
| 342       | Vimentin  | T <sub>0</sub>                 | 1654  | 62/27  | Vime-mouse   | 5.06 | 53524        |
| 1065      | Stathmin  | T <sub>0</sub>                 | 253   | 29/4   | STMN1_mouse  | 5.77 | 17133        |
| 721       | Acidic ribosomal phosphoprotein P0                          | T <sub>0</sub>                 | 61    | 3/1  | Q5PWB6_mouse | 5.91 | 34165        |
| 1173      | 10 kDa heat shock protein, mitochondrial (Hsp10)            | T <sub>0</sub>                 | 277   | 53/6   | CH10_MOUSE   | 8.18 | 10825        |
| 723       | Capping protein   | T <sub>0</sub>                 | 470   | 26/8   | Q99LB4_mouse | 6.47 | 38745        |
| 282       | Carteculin  | T <sub>0</sub>                 | 230   | 12/6   | Q3TVD2_MOUSE | 4.34 | 47992        |
| 920       | Rho GDP-dissociation inhibitor 1                            | T <sub>0</sub>                 | 258   | 28/5   | GDIR_MOUSE   | 5.12 | 23262        |
| 541       | Actin alpha, skeletal muscle                                | T <sub>3</sub> -T <sub>5</sub> | 246   | 10/3   | A24904       | 5.23 | 42024        |
| 329       | IMP dehydrogenase   | T <sub>3</sub> -T <sub>5</sub> | 198   | 6/3  | JT0565       | 6.84 | 55750        |
| 175       | Heat shock 70 kDa protein 5 (grp78)                         | T <sub>3</sub> -T <sub>5</sub> | 328   | 9/4  | Q9DC41       | 5.01 | 72378        |
| 89        | Endoplasmic precursor (Hsp90kDa beta member 1)              | T <sub>3</sub> -T <sub>5</sub> | 110   | 2/1  | A29317       | 4.74 | 92418        |
| 1106      | Superoxide dismutase  | T <sub>3</sub> -T <sub>5</sub> | 277   | 32/4   | SODC_MOUSE   | 6.03 | 15802        |
| 1040      | Interferon-activable protein (p205)                         | T <sub>3</sub> -T <sub>5</sub> | 7     | 14/1   | Q8C4X3_mouse | 5.17 | 16510        |
| 736       | Docking protein 4   | T <sub>3</sub> -T <sub>5</sub> | 10    | 3/1  | BAC27829     | 8.18 | 37017        |
| 205       | Heat shock protein, 70 kDa 8                                | T <sub>3</sub> -T <sub>5</sub> | 827   | 31/15  | Q6NZD0_MOUSE | 5.28 | 70828        |
| 727       | Annexin I   | T <sub>3</sub> -T <sub>5</sub> | 607   | 32/9   | LUMS1        | 6.97 | 38710        |
| 1072      | 4733401I105 Rik protein                                     | T <sub>3</sub> -T <sub>5</sub> | 114   | 8/1  | Q9D643       | 6.96 | 13237        |
| 64        | Gelsolin cytosolic  | T <sub>7</sub>                 | 141   | 1/1  | A32621       | 5.53 | 80827        |
| 65        | Gelsolin cytosolic  | T <sub>7</sub>                 | 141   | 1/1  | A32621       | 5.53 | 80827        |
| 1007      | ATP synthase D chain, mitochondrial                         | T <sub>7</sub>                 | 237   | 24/4   | ATPD_MOUSE   | 5.52 | 18607        |
| 748       | Annexin A5  | T <sub>7</sub>                 | 159   | 4/1  | Q99LA1       | 4.83 | 35716        |
| 740       | Glyceraldehyde-3-phosphate dehydrogenase                    | T <sub>7</sub>                 | 189   | 21/5   | DEMSG        | 8.44 | 35787        |
| 707       | Alcohol dehydrogenase                                       | T <sub>7</sub>                 | 361   | 32/9   | AK1A1_MOUSE  | 6.87 | 36433        |
| 860       | Glucocorticoid modulatory element binding protein 1 (GMEB1) | T <sub>7</sub>                 | 9     | 3/1  | Q80Y88       | 4.73 | 61020        |
| 321       | Choline transport-like protein 3 (CTL3)                     | T <sub>7</sub>                 | 30    | 1/1  | Q921V7       | 7.70 | 67259        |
| 893       | Hypothetical protein  | T <sub>7</sub>                 | 54    | 8/1  | Q9D643       | 6.96 | 13237        |



**Figure 2.** 2-DE of 45  $\mu$ g of C2C12 myoblast during differentiation. (A) Undifferentiated stage (time 0); (B) Early intermediate differentiation stage (3 days); (C) Late intermediate differentiation stage (5 days); (D) High differentiation phase (7 days). Gels were stained with silver nitrate.

be correlated to cell motility requirement.

Acidic ribosomal protein (P0) is a neutral protein, related to acidic ribosomal phosphoproteins P1 and P2 family (Rich and Steitz, 1987). Zaho *et al.* (2003) found a decrease of P0 during skeletal muscle development by analyzing gene expression changes, by microarray. The early increased expression of P0 could so confirm its specific role in cell proliferation.

Rho family protein role in muscle differentiation was shown in C2C12, in which it regulates myogenin and MEF2 gene expression (Charrasse *et al.*, 2006). Proliferating myoblasts show high levels of RhoA which are markedly lower in differentiating cells (Iwasaki *et al.*, 2008). Here we report an high expression of rho GDP-dissociation inhibitor 1 during early differentiation, when a proliferative state must be maintained.

Mitochondria contain several members of the major chaperone families, crucial for maintaining mitochondrial function: hsp10 is a eukaryotic cpn10 homologue and, therefore, together with cpn60 is essential for mitochondrial protein biogenesis (Hartman *et al.*, 1992).

### **Intermediate differentiation stage**

Interferon activable protein (p205) is involved in both lymphoid and myeloid differentiation (Asefa *et al.*, 2006). Our results suggest that p205 is involved in withdrawal from the cell cycle. Major expression level of p205, can be correlated with cell proliferation arrest and cell differentiation start. In fact, we found a high p205 expression at the intermediate differentiation stage.

During myoblast differentiation, hsp90 balances the phosphorylation state of akt by modulating its dephosphorylation by PP2Ac. Akt is an hsp90-dependent serine-threonine kinase that plays critical role in muscle differentiation too (Yun and Matts, 2005). In this work we found a major expression of hsp90 in the central phase of differentiation.

Glucose-regulated proteins (GRPs) are localized in the endoplasmic reticulum (ER) (Hirano *et al.*, 1995) and up-regulated in response to ER stress. Our findings suggest a role for GRPs correlated to myoblast differentiation.

Hsp70 is involved not only in protein folding, but also in differentiation and cell-cycle progression. Lee *et al.* (1998) suggested that it may inhibit the differentiation of early stage T cells. Furthermore, expression of hspa 8 was markedly down-regulated upon differentiation also in human embryonic stem cells (Son *et al.*, 2005).

Annexin I (A1) is one of the 20 known members of the annexin family of calcium and phospholipid-binding proteins. It is known mainly for its ability to promote aggregation and fusion of phospholipid vesicles (Blackwood and Ernst, 1990). So the major expression level of A1 in intermediate differentiation stage could be correlated to the myoblast fusion process (Figure 1B). We show that docking protein 4 (dok4) is involved in skeletal muscle development; in particular, its major expression is linked to differentiation. Furthermore, dok4 plays a positive role in activation of the MAP kinase pathway (Grimm *et al.*, 2001), confirming the presence of common pathways between apoptosis and differentiation

(Figure 1E) and in agreement with A1 increase. In both cases it brings, indeed, to an increase of MAPK.  $\alpha$ -Actin is associated to contractile function and this explains the finding of a strong actin expression at intermediate differentiation stage (Figure 1 C,D).

Concerning the increment of superoxide dismutase expression, we suppose that it is reliable to regulation of antioxidant defenses in response to cellular differentiation (Franco *et al.*, 1999).

### **High differentiation stage**

We found a high level of expression for annexin A5 in the final stage of differentiation. The literature reports that A5 is located in myotubes, where it has been described to appear around day 8 of embryonic development, possibly implicated in fusion process (Arcuri *et al.*, 2002).

Gelsolin is best known for its involvement in actin cytoskeleton dynamics. Its role in myogenesis was reported by Scholz and Hinssen (1995), who show that gelsolin increased steadily from the myoblast to terminally differentiated myotubes and hypothesize a functional role of gelsolin in myofibrillar assembly.

ATP synthase is a known mitochondrial enzyme, needed for mitochondrial oxidative phosphorylation. The increase of one of its components during the final phase can be due to the increase of ATP requirement in mature myotube, which progressively acquires contractile capacity, as well as mitochondrial functionality (Figure 1 F).

GMEBs were originally examined for their role in modulating the properties of glucocorticoid receptor-mediated transactivation. Members of the muscle-specific RING finger proteins (MURF-1, 2, and 3) act as cytoskeletal adaptors and signalling molecules by associating with myofibril components, microtubules and/or nuclear factors. GMEB1 may be involved in myofibril signal pathway and muscle gene expression, in agreement with its identification during late myogenesis, when the expression of muscle specific genes is induced.

To conclude, during early differentiation we find proteins able to stimulate myoblast proliferation and to inhibit their differentiation, proteins involved in the maintenance of cellular and nuclear shape, as well as in cell motility, migration and actin filament organization.

At the intermediate differentiation stage, our

study reveals proteins capable of inducing cell cycle arrest and promoting maturation, thus allowing progenitor cells to differentiate. Furthermore, in this stage myoblasts start fusing into multinucleated myotubes. Annexin 1, could be one of the proteins responsible for this event. We also found that dock 4 plays a positive role in the activation of the MAP kinase, essential for myogenesis (Keren *et al.*, 2006) and probably involved in this particular stage of differentiation program.

At this stage we also identified an increased expression of  $\alpha$ -actin, involved in the progression of contractile activity.

The main proteins which can be detected in fully differentiated syncytia are: annexin A5, CTL3, gelsolin and ATP synthase, potentially correlated to myotube formation (annexin 5), synthesis of the membrane (CTL3), myofibrillar assembly (gelsolin) and mitochondrial functionality (ATP synthase).

Intriguingly, in every stage of myogenic differentiation program we found proteins known to be involved in apoptotic process: this suggests a link between mechanisms regulating apoptosis and those controlling differentiation (Fernando *et al.*, 2002). Moreover, a number of highly expressed heat shock proteins (hsp70 kDa protein 5, hsp 70 kDa protein 8, hsp10, hsp90) suggests a close relationship between differentiation and cellular stress (Tarricone *et al.*, 2006).

Therefore, our proteomic data can be viewed as a resource for target follow-up studies centred on one or more biochemical pathways of particular interest.

As a conclusion, this work provides a description of myogenic differentiation program from the morphological and proteomic point of view, addressed to the hypothetical role of each identified protein expressed in a stage-specific manner. It furtherly highlights the mechanisms governing myoblast differentiation.

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