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THE EFFECT OF SERUM WITHDRAWAL ON THE PROTEIN PROFILE OF QUIESCENT HUMAN DERMAL FIBROBLASTS IN PRIMARY CELL CULTURE

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Key words: proteome, dermal fibroblasts, primary cell culture, ROS production, serum withdrawal

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ABSTRACT

The effect of serum deprivation on proliferating cells is well known, by contrast it is not always clear its role on confluent cells in primary cell culture. In order to investigate the response of quiescent cells to serum deprivation, normal human dermal fibroblasts were grown, for 48h after confluence, in the presence or absence of 10% FBS. Cell viability, cell morphology and ROS production were evaluated by light microscopy and FACS analysis. Moreover, proteome was investigated by 2D-GE and differentially expressed proteins were identified by mass spectrometry. Serum withdrawal caused cell shrinkage, without significantly modify the total cell number. ROS production, as evaluated by the DH2 probe, was markedly increased after serum deprivation. By proteome analysis, 41 proteins appeared to change their expression, the great majority of protein changes were related to the glycolytic pathway, the cytoskeleton and the stress response. Interestingly, in our experimental conditions, annexin 2 exhibited the most dramatic changes, being down-regulated more than ten folds in the absence of serum. Data indicate that human dermal fibroblasts in primary cell culture can adapt to environmental changes, even though serum withdrawal represents a stress condition capable for instance to increase ROS production and to influence cell metabolism.

INTRODUCTION

Serum factors represent a fundamental source of nutrients, growth factors and adhesive molecules necessary for in vitro cell growth and maintenance. Serum deprivation is in fact a commonly used method to synchronize cell lines in the G0 phase of the cell cycle, since the absence of mitotic signal, as through serum deprivation in G1 phase, leads to a rapid exit from the cell cycle into a non dividing state (G0) [1, 2].

In quiescent cultures, serum growth factors, even at very low concentrations, maintain cell viability [3, 4], consistently with the observation that complete serum starvation causes cell death [5, 6] and favours the synthesis of extracellular matrix proteins in the surviving cells [7]. In addition, there are several reports in the literature concerning the effect of serum withdrawal on the expression of single proteins in different cell types [8, 9, 10], but a comprehensive analysis of changes occurring in the protein profile upon serum starvation has been never approached. It has to be underlined that there are conditions such as tissue damage and repair where fibroblasts do not only experience oxygen deficiency but also restricted supply of nutrients [6] and a similar condition may also occur in fibrotic process or during aging, suggesting that growth factors abundance influences matrix composition [6] and turnover [11] as well as cell metabolism and behaviour [1].

Aim of the present study was to investigate the influence of serum depletion on quiescent human dermal fibroblasts in primary cell culture. Cell viability was assessed by cell morphology and cell count; changes in the protein profile were investigated by proteome analysis, whereas serum starvation-induced stress response was evaluated by measurements of reactive oxygen species.

MATERIALS AND METHODS

Cells and treatments

Human dermal fibroblasts were taken during surgery after informed consent from 3 clinically healthy females (45 ± 7 years), which did not exhibit any sign of genetic, metabolic or connective tissue disorders. The adopted procedure was in accordance with the guidelines of the ethical committee of the Modena University Faculty of Medicine. Biopsies were taken from the upper thigh. Fibroblasts were obtained from small skin fragments placed in DMEM plus 50% FBS (fetal bovine serum) until a consistent number of cells appeared to grow around tissue explants. Trypsinized cells were subcultured one or two times, stored in liquid nitrogen and used between 4rd and 6th passages. Unless otherwise specified, cells were routinely cultured in 75cm^2 flasks (Nunc, Roskilde, Denmark) with 18 ml of DMEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 ug/ml streptomycin.

Cell viability

Dermal fibroblasts were plated at a density of 2,5 x 10^4 cells into 25 cm^2 flasks (Nunc) in 5 ml DMEM until confluence, thereafter cells were grown for 48 hours in the presence or in the absence of 10% FBS. During treatment, cells were observed at the inverted microscope (Leica DM-IL) in order to evaluate their morphology. Digital images were taken on cells at confluence and after 3, 6, 24 and 48 hours of treatment. For cell counting, cells were trypsinized, washed with PBS and stained with the trypan blue dye (Sigma). The number of dye-excluding cells was counted by mean of the Neubauer chamber.

After serum depletion for 48h, fibroblasts were trypsinized, centrifuged and resuspended in PBS with propidium iodide at the final concentration of 1 mg/ml. After staining with propidium iodide for 5 min., cells were analyzed on an EPICS XL flow cytometer (Coulter, USA).

For all conditions and cell lines, viability assays were performed in duplicate.

F-actin staining

Cells cultured in 25 cm^2 flask until confluence were cultivated for further 48h in the presence and in the absence of 10% FBS. Trypsinized cells were centrifuged for 10 min at 1000g, washed in PBS and suspended in 1ml of 3% paraformaldehyde in PBS for 10 min at 4°C and centrifuged again for 5 min. Samples were permeabilized by addition of Triton X-100 for 10min at 4°C. After a rapid centrifugation, cells were incubated with phalloidin-FITC labeled for 30 min at 4°C. Washed cells were suspended in 500 µl of PBS and analyzed on an EPICS XL flow cytometer (Coulter, USA).

ROS production

Intracellular levels of the reactive oxygen species (ROS) were estimated by flow cytometry using the dihydroethidium probe (DH2) (Molecular Probes, Eugene, OR) [12]. Confluent fibroblasts cultured up to 48 hours in the presence or in the absence of 10% FBS, were treated with 1 µM DH2 for 60 minutes at 37 °C, trypsinized and collected in 500 µl of PBS, transferred to polystyrene tubes and analyzed for red fluorescence using a 610 nm long pass filter in a EPICS XL flow cytometer (Coulter, USA).

Proteome analysis

Sample preparation

Human dermal fibroblasts at confluence were grown for 48h in DMEM plus or minus 10% FBS. Afterwards, cells were detached from flasks by incubation in 0.25% Trypsin in PBS for 10min at

37°C. After washes in DMEM plus FBS and proteinase inhibitors (1mM EDTA, 10μM ε̃-aminocaproic acid, 50mM benzamidine) cells were centrifuged at 1000g for 10min. After supernatant removal, pellet was resuspended in PBS plus proteinase inhibitors, centrifuged at 1000g for 10min, and immediately resuspended in lysis buffer (8 M urea, 2% CHAPS, 65 mM dithioerythritol, 2% Pharmalyte pH 3–10 and trace amount of bromophenol blue). Cells from each subject were kept separate during experiments. Protein concentration was determined before addition of the lysis buffer, according to Bradford [13]. 2D-GE was performed essentially as described by Bjellqvist et al. [14].

Two-dimensional gel electrophoresis

Samples containing 60 μg (analytical gels) or 1 mg (preparative gels) of protein underwent two-dimensional gel electrophoresis using the Immobiline/polyacrylamide system [14]. Each sample was run in duplicate. Isoelectric focusing was performed on IPGphor system (GE- Healthcare) at 16°C using two different protocols. For analytical gels, passive rehydratation was done for 16h (500 V for 1h, 500-2000V for 1h, 3500V for 3h, 5000V for 30 min and 8000 V for 12h). For preparative gels a preliminary step at 200V constant for 12h was added. Thereafter, immobilized pH gradient strips were reduced (2% dithioerythritol) and alkylated (2.5% iodoacetamide) in equilibration buffer (6M urea, 50mM Tris-HCl, pH 6.8, 30% glycerol, 2% SDS). After the equilibration phase, strips were loaded onto vertical SDS PAGE (12.5%) using an Ettan DALTsix electrophoresis unit (GE-Healthcare).

Analytical gels were stained with ammoniacal silver nitrate [15]. Preparative gels for mass spectrometric analysis were silver-stained as described by Shevchenko et al. [16].

Data acquisition and analysis

To detect significant differences in protein abundance between the two culture conditions, silver-stained gel images were digitalized at 400 dpi resolution using ImageScanner (GE- Healthcare) and analyzed using Melanie 3.0, software (GE- Healthcare).

After background subtraction, protein spots were automatically defined and quantified with the feature detection algorithm [17]. Spot intensities were expressed as percentages (%vol) of relative volumes by integrating the OD of each pixel in the spot area (vol) and dividing with the sum of volumes of all spots detected in the gel.

For 2-DE pattern analysis, each experimental condition (with or without serum) was represented by two independent assays done in triplicate for each cell lines.

Quantitative data were exported as a text file for further elaboration using Microsoft Excel program. Mean values, standard deviations and coefficients of variation were calculated using the Excel-provided formulas.

Statistical analyses were performed using the non-parametric Mann Whitney test (GraphPad software, San Diego, CA). Differences were considered statistically significant when $p<0.05$ and only those spots, whose expression levels appeared significantly changed by the hypoxic exposure, were selected for MS analysis.

In-gel Destaining and Digestion of Protein Samples

Spots of interest were manually excised from preparative silver- stained 2-DE gels, depending on their intensity and relative volume. Silver-stained gel pieces were destained as described by Gharahdaghi et al. [18]. Briefly, gel spots were incubated in 100 mM sodium thiosulfate and 30 mM potassium ferricyanide, rinsed twice in 25 mM ammonium bicarbonate (AmBic) and, once in water, shrunk with 100% acetonitrile (ACN) for 15 min, and dried in a Savant SpeedVac for 20–30 min. All excised spots were incubated with 12.5 ng/µl sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mM AmBic overnight at 37 °C. After digestion, the supernatants (crude extracts) were separated. Peptides were extracted from the gel pieces first into 50% ACN, 1% trifluoroacetic acid and then into 100% ACN. All extracts were pooled, and the volume was reduced by SpeedVac.

Mass Spectrometry

MALDI-TOF MS

The tryptic peptide extracts were dried in a vacuum centrifuge and redissolved in 12 µl of 0.1%TFA. The matrix (α-cyano-4-hydroxycinnamic acid, HCCA) was purchased from Laser BioLabs (Sophia-Antipolis, France). A saturated solution of HCCA (1µl) at 2mg/200µl in CH3CN/H2O (50/50 v/v) containing 0.1% TFA was mixed with 1µl of peptide solution on the MALDI target and left to dry.

MALDI-TOF mass spectra were recorded on a Voyager DE-PRO (Applied-Biosystems, Courtaboeuf, France) mass spectometer, in the 700-5000 Da mass range using a minimum of 200 shots of laser per spectrum. Delayed extraction source and reflector equipment allowed sufficient resolution to consider MH+ of monoisotopic peptide masses. Internal calibration was done using trypsin autolysis fragments at m/z 842.5100, 1045.5642 and 2211.1046 Da. PMF was compared to the theoretical masses from the Swiss-Prot or NCBI sequence databases using MS-Fit from ProteinProspector (http://www.expasy.org/tools/). Typical search parameters were as follows: ± 30ppm of mass tolerance; carbamidomethylation of cysteine residues; one missed enzymatic cleavege for trypsin; a minimum of four peptide mass hits was required for a match; methionine residues could be considered in oxidized form; no restriction was placed on the pI and molecular weight of the protein.

HPLC/MS

Peptides were resuspended in aqueous 5% FA and subsequently eluted onto a 150 mm x 75 µm Atlantis C18 column analytical (Waters, Milford, USA) and separated with an increasing ACN gradient from 10% to 85% in 30 min. using a Waters CapLC system. The analytical column (extimated flow approx. 200nanoL/min) was directly coupled, through a nanoES ion source, to a Q-TOF Ultima Global mass spectrometer (Waters). Multicharged ions (charge states 2, 3 and 4) were selected for fragmentation and the acquired MS/MS spectra were searched against the SWISS-PROT/TrEMBL non-redundant protein and NCBI database using the Mascot (www.matrixscience.com) MS/MS search engine.

Initial search parameters were as follows: enzyme (trypsin), maximum number of missed cleavages (1), fixed modification (carbamidomethylation of cysteines), variable modification parameters (oxidation Met), peptide tolerance (0.5 Da), MS/MS tolerance (0.3 Da), and charge state (2, 3, or 4). We basically selected the candidate peptides with probability-based MOWSE scores (total score) that exceeded its threshold, indicating a significant (or extensive) homology ($p<0.05$), and referred to them as "hits". The criteria were based on the manufacturer's definitions (Matrix Science) [19]. Proteins, that were identified with at least two peptides both showing a score higher than 40, were validated without any further manual validation. Proteins with at least two peptides, having a score lower than 40 and higher than 20, were systematically checked and/or interpreted manually to confirm or cancel the MASCOT suggestions. For proteins identified by only one peptide, the score had to exceed the value of 30, and the peptide sequence was systematically checked manually.

RESULTS

Cell viability

Serum withdrawal did not significantly modify the number of dermal fibroblasts (figure 1c). At the morphological level, serum-starved fibroblasts (figure 1b) appeared moderately more elongated after 48h of treatment, compared to control cells (figure 1a). No signs of cell necrosis or cell apoptosis were observed at the inverted light microscope. Moreover, at the and of treatment, fibroblasts were stained with propidium iodide and the percentage of dead cell was similar in control (8%) and in serum starved (10%) fibroblasts (data not shown).

ROS production

The intracellular content of reactive oxygen species (ROS), evaluated by the binding of the dihydroethidium probe to the anion superoxide, was significantly increased by serum withdrawal

already after 3 hours of serum deprivation. Values remained similarly increased also at 6, 24 and 48 hours (Figure 2).

F-actin staining

Fibroblasts, after 48 hours of serum withdrawal, were labelled with FITC-conjugated phalloidin and analysed by flow cytometry. Serum starved cells exhibited a significant increase of F-actin, compared to fibroblasts grown in the presence of 10% FBS (Figure 3).

2D gel electrophoresis

The cell monolayer total protein content did not reveal any significant difference between the two experimental conditions as evaluated by the Bradford assay. Similarly, the number of proteins that were separated by 2D gel electrophoresis was approximately of 1800 proteins from each primary cell culture, independently from the experimental conditions.

In order to exclude the influence of possible high intra-sample variability, the coefficient of variation (CV) (standard deviation normalized spot volume divided by mean) was evaluated for each cell line from triplicate parallel preparations.

As reported in the literature [20], we considered for further analyses spots with CV values of the normalized volumes lower than 0,3. Consistently with the observation that the great majority of proteins gave reproducible results in terms of sample preparation, extraction procedures and 2DE, CV values of the normalized volumes higher than 0,3 were only obtained for vary faint spots and for spots located close to the gel edges.

Serum withdrawal determined significant changes in the expression of 41 proteins or protein fragments, as indicated by the markers on two representative gels obtained from confluent fibroblasts grown in the presence or in the absence of serum (figure 4).

In particular, 11 proteins appeared significantly more expressed upon serum deprivation, whereas 30 proteins were significantly decreased (Table 1).

Mass spectrometry

By mass spectrometry we have identified 31 of the differentially expressed proteins in the whole cell lysate (Table 1). The remaining proteins were either in insufficient amount to be analyzed by MS or MS/MS or the MS-compatible staining procedure failed to reveal them.

Only in one case MS revealed the presence of two different proteins in the same spot: namely phosphoglycerate mutase 1 and peptidyl-prolys cis-trans isomerase B (Table 1).

It has to be mentioned that, for some proteins, we identified spots that did not match with the predicted Mr or p*I*, indicating once more the presence of different isoforms and/or fragments as already shown by other Authors [21].

Due to the very low molecular weight and to the position of identified peptides within the whole protein length (see also supplementary material), we may suggest that the following spots can be protein fragments: heat shock protein 71kDa, mannose 6 phosphate receptor binding protein, glyceraldehyde 3 phosphate, T-complex protein 1, beta subunit, fructose-bisphosphate aldolase A, elongation factor 1 - alpha1. It could be suggested that these protein fragments may be caused either experimentally or in vivo. Interestingly, these fragments appeared to be reproducibly and significantly differentially expressed and, although they might not be functionally proteins, it cannot be excluded that these fragments might have a biological role and/or that they are the result of proteolytic activities after serum starvation [22].

In the case of two actin isoforms, the identified sequences that were spread through the whole molecule and the experimental molecular weight, lower than expected, could indicate the presence of two short forms of the protein [23].

Functional classification of identified proteins:

Protein distribution into functional categories and the cellular localization are reported in figure 5. The majority of differentially expressed proteins belongs to cell metabolism, cellular cytoskeleton and protein synthesis and folding.

In particular, we have demonstrated that serum deprivation caused a significant reduction of several proteins involved in the glycolytic pathway (figure 6).

Furthermore, among the cytoskeletal proteins, both tubulin alpha and beta were significantly increased in serum starved fibroblasts, whereas two actin isoforms appeared to be differently influenced by the absence of serum (Table 1).

The total protein content, as measured by the Bradford assay, was unaffected by serum deprivation (data not shown). However, by proteome analysis we demonstrated a down-regulation in the expression of proteins regulating protein assembly and folding, i.e. elongation factor 1 alpha, triosephosphate isomerase, peptidyl-prolyl-cis-trans isomerase (Table 1).

Finally, serum withdrawal causes a significant increase of calreticulin and a downregulation of the far upstream element-binding protein 1, and of annexin 2.

Discussion

The importance of serum factors for cell growth and cell maintenance in vitro is well known [24] as well as the influence of serum withdrawal on cell cycle [1, 2]. By contrast, few data are available on the effect of serum deprivation on quiescent cells in primary cell culture. Since fibroblasts may frequently experience, beside reduce oxygen tension, also changes in growth factors availability, as during wounding, aging or as a result of fibrotic proceses, aim of the present study was to investigate the response of human dermal fibroblasts in primary cell culture to serum withdrawal applied for 48 hours after cell confluence. We have selected quiescent fibroblasts, in order to avoid the effect of serum deprivation on the cell cycle and because quiescent cells are in a condition more similar to the in vivo situation.

Firstly, we have demonstrated that, in our experimental model, serum deprivation does not increase the number of dead cells. Interestingly, there are data in the literature indicating that few hours of serum withdrawal, in different cell lines, causes cell death either by necrosis or apoptosis [5, 7, 25]. In the present experimental model, 48 hours of serum deprivation does not seem to affect cell viability, as shown by cell counting, cell morphology, trypan blue and propidium iodide staining, thus demonstrating that dermal fibroblasts, in primary cell culture, can quite well adapt themselves to changes of the environmental conditions, at least for the time-length of these experiments.

One of the most significant changes occurring upon serum withdrawal is the reduced expression, at the protein level, of numerous enzymes involved in the glycolytic pathway, allowing to hypothesize that glycolysis is reduced in serum starved fibroblasts. A similar reduction of glycolysis has been demonstrated after dietary restriction [26], this decrement contributing to control and to favourably modulate senescence [27]. Glycolysis, in fact, may represent a source of endogenous molecular toxicity since most glycolytic intermediates are potentially toxic and capable to modify (i.e.glycate) proteins and other macromolecules non-enzymatically, producing advanced glycosylation end-products (AGEs) implicated in age-related pathologies [28] and in increased reactive oxygen species (ROS) [29]. Therefore, one of the beneficial effect of dietary restriction on the aging process is the reduced production of ROS [30]. By contrast, serum starvation, although exhibiting a down-regulation of glycolysis, is associated to increased production of ROS, indicating that insufficient availability of serum factors causes oxidative stress through ROS production and/or accumulation [31]. It is conceivable to hypothesize that a prolonged serum deprivation may interfere with cell viability. Since, reduced and/or inadequate nutrients supply may frequently occur during aging or in fibrotic processes, insufficient growth factors availability may further increase ROS accumulation, synergistically contributing to oxidative stress-induced cell damages [32]. Furthermore, it has been demonstrated that, under oxidative stress, glycolysis is down regulated [33], therefore serum withdrawal may lower glycolysis through oxidative stress occurrence.

Interestingly, reactive oxygen species, like superoxide, may represent important signalling molecules, altering for instance DNA binding and the transactivation properties of transcription factors [34]. Remodelling and regulation of the acting cytoskeleton has been recently linked to the effects of ROS, as indicated by the observation that superoxide could be transported through actin microfilaments from the plasma membrane, or from the mitochondria, towards the nucleus [35], specifically influencing the transport into the nucleus of transcription-related proteins [36]. It could be the case of the far upstream element binding protein 1 [37], that was significantly reduced in fibroblasts after serum withdrawal.

Aging, as well as serum deprivation, have been described to induce a decrease in the rate of DNA synthesis and an extension of the cytoplasmic microtubular complex [38] accompanied by loss of F-actin fibres [9]. Consistently with these data, we have demonstrated that serum starved dermal fibroblasts showed a significant upregulation of alpha and beta tubulins; by contrast, in our experimental model we observed a significant increase of falloidin-stained actin molecules. These apparently contradictory results could be due to the different response of human dermal fibroblasts compared to Balb/c 3T3 cells, as already revealed in the case of cell death [25]. Interestingly, by proteome analysis we demonstrated that two actin isoforms were differently affected by serum deprivation, one being up-regulated and the other down-nregulated. It is not yet fully established if differential expression of actin isoforms is functionally relevant or if they play a role in human diseases [39]. We may suggest that changes in the expression of actin isoforms could be also sustained by reduced expression of cofilin, thus contributing to unbalanced cytoskeletal dynamics [40]. Furthermore, cytoskeletal changes may influence interactions with superoxide as well as cell shape and integrin cell adhesion receptors for proteins of the extracellular matrix that are known to be physically linked to the actin cytoskeleton at the focal adhesion plaques influencing communication with growth factors pathways [41].

Changes in signalling pathways can be further influenced by changes in membrane stability. As mentioned before, dermal fibroblasts after 48 hours of serum withdrawal did not show signs of cell death, neither membrane damages as demonstrated by trypan blu and by propidium iodide exclusion. However, increased ROS production may cause lipid peroxidation [42] in addition to the dramatic reduction of annexin 2. As other annexins, annexin 2 binds to negatively charged phospholipids and when is relocated to the plasma membrane contributes to its mechanical stability. Annexin 2 acts as a calcium-dependent promoter of lipid microdomains required for structural and spatial organization of the exocytic machinery [43]. Reduced expression of annexin 2 may cause altered distribution of lipid microdomains on plasma membrane, thus causing a weakness of

membrane resistance and/or altered secretion of molecules from fibroblasts into the extracellular space.

It has been already reported that serum deprivation affects the turnover of nascent or newly synthesized proteins [44]. In the present study, we revealed a significant decrease of elongation factor 1α. Interestingly, loss of expression of EF1α is accompanied by the onset of senescence in human fibroblasts [45] confirming, once more, that altered growth factors availability may contribute to the aging phenotype. Talapatra and coworkers have demonstrated that elongation factor 1-alpha is a selective regulator of ER stress and of growth factor withdrawal-induced apoptosis [46]. A reduced expression of elongation factor 1, as well as of other proteins involved in protein folding, such as peptidyl prolyl cis-trans isomerase, may increase the frequency of proteins that are no longer able to adopt their native conformation. Accumulation of unfolded proteins can initiate an ER specific unfolded protein response and ultimately apoptosis [47] or, before reaching an irreversible point of no return, can be responsible for changes in proteolytic activities of proteasomes and lisosomes [22]. At present, it is not clear if the relatively high number of protein fragments that were down-regulated in serum-starved fibroblasts is the consequence of changes in cellular protein degradations or is due to an altered susceptibility to proteolysis in vitro as a result of conformational damages.

Moreover, it has to be mentioned that serum starvation caused an upregulation of calreticulin, a molecular chaperone being part of the primary quality control of protein folding within the ER [48]. However, beside the role as chaperone, calreticulin may be also considered a stress response protein [49]. In the present study we demonstrated that serum withdrawal represents a stress condition, as shown by the increased production of ROS, however, classical stress-related proteins (i.e. HSP 27, 70, 90) were not upregulated in serum-deprived fibroblasts. Interestingly, we demonstrate significant changes in the expression of calreticulin, suggesting that calreticulin may be a specific target of serum withdrawal. Moreover, it has been recently hypothesized that calreticulin can modulate cell apoptosis [50]. Therefore, increased expression of calreticulin may represent a first sign of the apoptotic pathway activation that may occur for longer serum starvation.

In conclusion, we have presented evidence that human dermal fibroblasts in primary cell culture, upon serum starvation, exhibited a significant increase of ROS production, and changes in the protein profile, mainly affecting glycolysis, membrane stability, protein folding and the cytoskeleton, as summarized in figure 7. It could be suggested that dermal fibroblasts can adapt themselves to environmental changes and that these cells represent a good experimental model allowing to investigate the response of quiescent cells to serum deprivation, highlighting the

pathways that may prelude to irreversible changes and to cell death after a prolonged serum withdrawal.

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Legend to figures

Figure 1. Light microscopy of human dermal fibroblasts cultured in the presence (a) and in the absence (b) of 10% FBS show that serum starved cells appeared more elongated compared to controls. Serum withdrawal for 48 hours does not significantly changed the number of cells evaluated by cell counting (c). Original magnification: 40x (a,b)

Figure 2. Flow cytometry of ROS accumulation in human dermal fibroblasts as revealed by the DH2 probe. Serum deprivation increases the amount of ROS already after 48h from serum starvation. A representative experiment based on the analysis of 10.000 cells is reported in panel a. Histograms represent the mean values of three experiments done in duplicate with all cell lines (b). Data are expressed as fluorescence percentage variation ± SD of serum deprived fibroblasts compared to cells grown in the presence of serum (*$p<0.05$).

Figure 3. Flow cytometry of dermal fibroblasts labelled with FITC- labelled falloidin. Serum withdrawal is associated to a significant increase of F-actin. A representative experiment based on the analysis of 10.000 cells is reported in panel a. Histograms represent the mean values of three experiments done in duplicate with all cell lines (b). Data are expressed as fluorescence percentage variation ± SD of serum deprived fibroblasts compared to cells grown in the presence of serum (*$p<0.05$).

Figure 4. Representative silver-stained 2-D electropherograms of the cell layer of human dermal fibroblasts cultured in the presence of 10% FBS (left) or in the absence of serum for 48h (right). Identified differentially expressed proteins are indicated by the symbol name. Unidentified proteins are marked with open circles or with arrows when up-regulated an down-regulated after serum withdrawal, respectively.

Figure 5. Differentially expressed proteins, identified by MS, are grouped according to their cellular localization (a) and their biological function (b).

Figure 6. Proteome analysis reveal that eight proteins are down-regulated in serum deprived cells: their name and role within the glycolytic pathway are reported. Changes in protein expression are visualized by bar charts close to the enzyme name: Triosephosphate isomerase 1 (TPIS1), Fructose

bisphosphate aldolase A (ALDOA), Glyceraldehyde 3 phosphate (G3P), Phosphoglycerate Kinase 1 (PGK1), Phosphoglycerate Mutase 1 (PGAM1), Alpha enolase (ENOA).

Figure 7. Scheme drawing that summarizes the effect of serum withdrawal on human dermal fibroblasts in primary cell culture. Outlined by the oval shape are changes supported by data from the present investigation.

Table I

N°	Protein name N° Acces	Symbol	Theor Mass (kDa)/pI	Δ#	Identification method For MS/MS sequencing,% coverage, peptide sequence, ion score, charge state For PMF, % coverage and peptide sequence matched are given M* - Methionine oxidation; *C# - Cysteine carboxyamidomethylation*
1	Unknown			**3X**	unidentified
2	Unknown			**4X**	unidentified
3	Unknown			**4X**	unidentified
4	Far upstream element-binding protein1 Q96AE4	FUBP1	67.4/7.1	4X	MS/MS – 8% K.IQIAPDSGGLPER.S (Ions score 38) (2+) R.IGGNEGIDVPIPR. (Ions score 24) (2+) R.FAVGIVIGR. (Ions score 24) (2+) IAQITGPPDR (Ions score 25) (2+) R.GTPQQIDYAR.Q (Ions score 24) (2+)
5	Unknown			3X	unidentified
6	Unknown			**2X**	unidentified
7	Calreticulin P27797	CALR	48.1/4.2	**4X**	MS/MS – 19% R.FYALSASFEPFSNK.G (Ions score 63) (2+) K.HEQNIDCGGGYVK.L (Ions score 66) (2+) K.IDNSQVESGSLEDDWDFLPPKK.I (Ions score 36) (3+) K.IKDPDASKPEDWDER.A (Ions score 34) 3+ R.QIDNPDYK.G (Ions score 23) (2+)
8	Alpha-enolase P06733	ENOA	47.0/6.9	2X	MS/MS – 56% R.GNPTVEVDLFTSK.G (Ions score 27) (2+) R.AAVPSGASTGIYEALELR.D (Ions score 43) (2+) K.LNVTEQEKIDK.L (Ions score 33) (2+) K.DATNVGDEGGFAPNILENK.E (Ions score 33) (2+) K.AGYTDKVVIGMDVAASEFFR.S (Ions score 20) (3+) K.YDLDFKSPDDPSR.Y (Ions score 20) (3+) R.YISPDQLADLYK.S (Ions score 36) (2+) K.SFIKDYPVVSIEDPFDQDDWGAWQK.F (Ions score 26) (3+) K.FTASAGIQVVGDDLTVTNPKR.I (Ions score 41) (3+) K.VNQIGSVTESLQAC#K.L (Ions score 28) (2+) K.LAQANGWGVMVSHR.S (Ions score 55) (3+) K.IGAEVYHNLK.N (Ions score 35) (2+) R.LMIEMDGTENK.S (Ions score 28) (2+)

					R.SGETEDTFIADLVVGLC#TGQIK.L (Ions score 32) (2+)
9	Phosphoglycerate kinase 1 P00558	PGK1	44.4/8.3	2X	MS/MS – 7% K.ACANPAAGSVILLENLR.F (Ions score 20) (2+) K.LGDVYVNDAFGTAHR.A (Ions score 37) (2+)
10	Fructose-bisphosphate aldolase A P04075	ALDOA	39.2/8.3	4X	MS/MS – 9% K.ELSDIAHR.I (Ions score 40) (2+) R.LQSIGTENTEENRR.F (Ions score 26) (3+) R.ALANSLACQGK.Y (Ions score 30) (2+)
11	Tubulin α 6 chain/ Tubulin α 1 chain Q9BQE3/ P05209	TBA6/ TBA1	49.8/4.9	**4X**	MS/MS – 19% R.AVFVDLEPTVIDEVR.T (Ions score 84) (2+) R.GHYTIGKEIIDLVLDR.I (Ions score 38) (3+) K.EIIDLVLDR.I (Ions score 48) (2+) R.LISQIVSSITASLR.F (Ions score 67) (2+) R.FDGALNVDLTEFQTNLVPYPR.I (Ions score 24) (2+) R.IHFPLATYAPVISAEK.A (Ions score 45) (3+)
12	Unknown			3X	unidentified
13	Annexin 2 P07355	ANXA2	38.4/7.5	10X	MS/MS – 13% K.GVDEVTIVNILTNR.S (Ions score 29) (2+) K.TPAQYDASELK.A (Ions score 31) (2+) R.TNQELQEINR.V (Ions score 32) (2+) R.DLYDAGVKR.K (Ions score 21) (2+)
14	Actin beta P60709	ACTB	41.7/5.2	2X	MS/MS – 45% K.DSYVGDEAQSK.R (Ions score 41) (3+) R.VAPEEHPVLLTEAPLNPK.A (Ions score 31) (2+) R.GYSFTTTAER.E (Ions score 22) (2+) K.LC#YVALDFEQEMATAASSSSLEK.S (Ions score 23) (3+) K.SYELPDGQVITIGNER.F (Ions score 38) (2+) K.DLYANTVLSGGTTMYPGIADR.M (Ions score 41) (2+) K.EITALAPSTMK.I (Ions score 31) (2+) K.IKIIAPPER.K (Ions score 22) (2+) K.QEYDESGPSIVHR.K (Ions score 35) (3+)
15	Unknown			3X	unidentified
16	Elongation factor 1 alpha 1 P68104	EF1A1	50.1/9.1	3X	MS/MS 22% K.YYVTIIDAPGHR.D (Ions score 65) (2+) R.LPLQDVYK.I (Ions score 29) (2+) K.IGGIGTVPVGR.V (Ions score 44) (2+) K.SGDAAIVDMVPGKPMCVESFSDYPPLGR.F (Ions score 28) (2+)

					R.QTVAVGVIK.A (Ions score 26) (2+)
17	Unknown			3X	unidentified
18	Glyceraldehyde-3-phosphate dehydrogenase P04406	G3P	35.9/8.5	4X	MS/MS – 24% K.LVINGNPITIFQER.D (Ions score 42) (2+) K.IISNASCTTNCLAPLAK.V (Ions score 105) (2+) K.VIPELNGK.L (Ions score 37) (2+) K.LTGMAFRVPTANVSVVDLTCR.L (Ions score 48) (2+) R.VPTANVSVVDLTCR.L (Ions score 52) (2+) R.LEKPAKYDDIK.K (Ions score 20) (2+) R.VVDLMAHMASKE.- (Ions score 18) (2+)
19	Heat shock protein 71kDa * P11142	HSP7C	70.8/5.3	3X	MS/MS – 25% K.VEIIANDQGNR.T (Ions score 40) (2+) R.TTPSYVAFTDTER.L (Ions score 87) (2+) K.NQVAMNPTNTVFDAK.R (Ions score 79) (2+) R.RFDDAVVQSDMK.H (Ions score 18) (2+) K.VQVEYKGETK.S (Ions score 42) (2+) K.SFYPEEVSSMVLTK.M (Ions score 65) (2+) K.EIAEAYLGK.T (Ions score 50) (2+) K.TVTNAVVTVPAYFNDSQR.Q (Ions score 53) (2+) K.DAGTIAGLNVLR.I (Ions score 55) (2+) R.IINEPTAAAIAYGLDKK.V (Ions score 57) (2+) K.STAGDTHLGGEDFDNR.M (Ions score 48) (2+) R.MVNHFIAEFK.R (Ions score 18) (2+)
20	Actin beta P60709	ACTB	41.7/5.2	**2X**	MS/MS – 36% R.TTGIVMDSGDGVTHTVPIYEGYALPHAILR.L (Ions score 36) (2+) R.GYSFTTTAER.E (Ions score 34) (2+) K.SYELPDGQVITIGNER.F (Ions score 43) (2+) K.DLYANTVLSGGTTMYPGIADR.M (Ions score 46) (2+) K.EITALAPSTMK.K (Ions score 33) (2+) K.IKIIAPPER.K (Ions score 26) (2+) K.QEYDESGPSIVHR.K (Ions score 39) (2+)
21	Tubulin beta-2 chain P07437	TBB2	49.6/4.7	**3X**	MS/MS – 26% -MREIVHIQAGQCGNQIGAK.F (Ions score 53) (3+) R.EIVHIQAGQCGNQIGAK.F (Ions score 39) (3+) R.ISVYYNEATGGK.Y (Ions score 70) (2+) R.AILVDLEPGTMDSVR.S (Ions score 74) (2+) K.GHYTEGAELVDSVLDVVRK.E (Ions score 79) (2+)

					R.KEAESCDCLQGFQLTHSLGGGTGSGMGTLLISK.I (Ions score 34) (4+) K.IREEYPDR.I (Ions score 31) (2+) R.IM*NTFSVVPSPK.V (Ions score 69) (2+)
22	Triosephosphate isomerase P60174	TPIS	26.5/6.5	3X	MS/MS – 77% R.KQSLGELIGTLNAAK.V (Ions score 35) (3+) K.QSLGELIGTLNAAK.V (Ions score 69) (2+) K.VPADTEVVCAPPTAYIDFAR.Q (Ions score 23) (2+) K.IAVAAQNCYK.V (Ions score 59) (2+) K.VTNGAFTGEISPGMIK.D (Ions score 47) (2+) R.HVFGESDELIGQK.V (Ions score 37) (2+) K.VAHALAEGLGVIACIGEK.L (Ions score 51) (3+) K.VVLAYEPVWAIGTGK.T (Ions score 66) (2+) K.TATPQQAQEVHEKLR.G (Ions score 33) (3+) K.SNVSDAVAQSTR.I (Ions score 74) (2+) R.IIYGGSVTGATCK.E (Ions score 86) (2+) K.ELASQPDVDGFLVGGASLKPEFVDIINAK.Q (Ions score 24) (3+)
23	Unknown			2X	unidentified
24	Transgelin Q01995	TAGL	22.4/8.8	3X	MS/MS – 77% K.YDEELEER.L (Ions score 38) (2+) R.LVEWIIVQCGPDVGRPDR.G (Ions score 15) (2+) R.LGFQVWLK.N (Ions score 11) (2+) K.NGVILSK.L (Ions score 20) (2+) K.LVNSLYPDGSKPVK.V (Ions score 39) (2+) K.VPENPPSMVFK.Q (Ions score 32) (2+) K.QMEQVAQFLK.A (Ions score 34) (2+) K.AAEDYGVIK.T (Ions score 26) (2+) K.TDMFQTVDLFEGK.D (Ions score 64) (2+) R.TLMALGSLAVTK.N (Ions score 67) (2+) R.EFTESQLQEGK.H (Ions score 44) (2+) K.HVIGLQMGSNR.G (Ions score 28) (2+) R.GASQAGM*TGYGRPR.Q (Ions score 21) (3+)
25	Mannose 6 phosphate receptor binding protein* O60664	M6PBP	47.0/5.3	2X	MS/MS – 7% K.LHQMWLSWNQK.Q (Ions score 31) (2+) K.QLQGPEKEPPKPEQVESR.A (Ions score 37) (3+)

26	P4HB protein 15680282	P4HB	21.2/4.4	**3X**	PMF – 23% (K) NFEDVAFDEK(K) (K) VHSFPTLK(F) (K) FFPASADR(T) (R) TVIDYNGER(T) (R) TLDGFKK(F)
27	Glyceraldehyde 3 phoshate* P04406	G3P	35.9/8.5	2X	MS/MS – 29% K.LVINGNPITIFQER.D (Ions score 33) (2+) K.IKWGDAGAEYVVESTGVFTTMEK.A (Ions score 39) (2+) K.AGAHLQGGAK.R (Ions score 32) (2+) R.VIISAPSADAPMFVMGVNHEK.Y (Ions score 37) (3+) K.VIHDNFGIVEGLM*TTVHAITATQK.T (Ions score 26) (3+)
28	Cofilin P23528	COF1	18.3/8.2	4X	MS/MS – 53% -.ASGVAVSDGVIK.V (Ions score 68) (2+) K.AVLFCLSEDKK.N (Ions score 20) (2+) K.NIILEEGK.E (Ions score 36) (2+) K.EILVGDVGQTVDDPYATFVK.M (Ions score 31) (2+) R.YALYDATYETK.E (Ions score 44) (2+) K.LGGSAVISLEGKPL.- (Ions score 57) (2+)
29	TUBB protein 38511503	TUBB	14.4/4.2	**3X**	MS/MS – 56% K.EVDEQMLNVQNK.N (ions score 20) (+2) K.NSSYFVEWIPNNVK.T (ions score 31) (+2) K.TAVCDIPPR.G (ions score 44) (+2) K.MAVTFIGNSTAIQELFKR.I (ions score 58) (+3) R.ISEQFTAMFR.R (ions score 57) (+2)
30	Nascent polypeptide-associated complex subunit alpha Q13765	NACA	23.3/4.5	**4X**	MS/MS – 19% K.SPASDTYIVFGEAK.I (Ions score 37) (2+) K.IEDLSQQAQLAAAEK.F (Ions score 39) (2+) K.DIELVMSQANVSR.A (Ions score 41) (2+)
31	Peptidyl-prolyl-cis-trans isomerase A P62937	PPIA	17.8/7.8	3X	MS/MS – 32% -VNPTVFFDIAVDGEPLGR.V (Ions score 45) (2+) R.IIPGFMCQGGDFTR.H (Ions score 71) (2+) K.TEWLDGKHVVFGK.V (Ions score 21) (3+)
32	Glyceraldehyde 3 phoshate* P04406	G3P	35.9/8.5	2X	MS/MS – 21% K.LVINGNPITIFQER.D (Ions score 38) (2+) K.WGDAGAEYVVESTGVFTTMEK.A (Ions score 30) (2+) R.VIISAPSADAPMFVMGVNHEK.Y (Ions score 26) (3+)

33	Triosephosfate isomerase P60174	TPIS	26.5/6.5	3X	MS/MS – 62% R.KQSLGELIGTLNAAK.V (Ions score 40) (3+) K.VPADTEVVCAPPTAYIDFAR.Q (Ions score 42) (3+) K.IAVAAQNCYK.V (Ions score 54) (2+) K.VTNGAFTGEISPGMIK.D (Ions score 62) (2+) K.VAHALAEGLGVIACIGEK.L (Ions score 56) (3+) K.TATPQQAQEVHEKLR.G (Ions score 52) (2+) K.SNVSDAVAQSTR.I (Ions score 83) (3+) R.IIYGGSVTGATCK.E (Ions score 87) (2+) K.ELASQPDVDGFLVGGASLKPEFVDIINAKQ.- (Ions score 22) (3+)
34	T-complex protein 1, eta subunit* Q99832	TCPH	59.3/7.5	4X	MS/MS – 11% R.GMDKLIVDGR.G (Ions score 45) (2+) K.ATISNDGATILK.L (Ions score 17) (2+) K.LLDVVHPAAK.T (Ions score 59) (2+) K.TLVDIAK.S (Ions score 36) (2+) K.SQDAEVGDGTTSVTLLAAEFLK.Q (Ions score 30) (2+)
35	Phosphatidylethanolamine-binding protein P30086	PEBP1	20.9/7.4	3X	MS/MS – 60% K.WSGPLSLQEVDEQPQHPLHVTYAGAAVDELGK.V (Ions score 31) (3+) K. VLTPTQVK.N (Ions score 26) (2+) K.NRPTSISWDGLDSGK.L (Ions score 46) (2+) K.LYTLVLTDPDAPSR.K (Ions score 35) (2+) K.GNDISSGTVLSDYVGSGPPK.G (Ions score 54) (2+) R.YVWLVYEQDRPLK.C (Ions score 39) (2+)
36	Fructose-bisphosphate aldolase A* P04075	ALDOA	39.2/8.3	3X	MS/MS – 6% K.GILAADESTGSIAK.R (Ions score 41) (2+) R.QLLLTADDR.V (Ions score 31) (2+)
37	Transgelin Q01995	TAGL	22.5/8.8	4X	MS/MS – 38% K.TDMFQTVDLFEGK.D (Ions score 22) (2+) R.TLM*ALGSLAVTK.N (Ions score 43) (2+) R.EFTESQLQEGK.H (Ions score 38) (2+) K.HVIGLQMGSNR.G (Ions score 27) (2+) R.GASQAGMTGYGRPR.Q (Ions score 31) (3+)
38	Peptidyl-prolyl cis-trans isomerase B P23284	PPIB	22.7/9.3	3X	MS/MS – 19% K.DFMIQGGDFTR.G (Ions score 28) (2+) K.VLEGMEVVR.K (Ions score 55) (2+) R.DKPLKDVIIADCGK.I (Ions score 45) (3+)
39	Phosphoglycerate mutase 1	PGAM1	28.6/6.7	2X	MS/MS – 15%

	P18669/ Peptidyl-prolyl cis-trans isomerase B P23284	/ PPIB	/ 22.7/9.3		R.FSGWYDADLSPAGHEEAK.R (Ions score 25) (3+) R.HGESAWNLENR.F (Ions score 31) (2+) R.HYGGLTGLNK.A (Ions score 31) (2+) MS/MS – 11% K.DFMIQGGDFTR.G (Ions score 34) (2+) K.DTNGSQFFITTVK.T (Ions score 40) (2+)
40	Elongation factor 1alpha1* P68104	EF1A1	50.1/9.1	2X	MS/MS – 13% K.YYVTIIDAPGHR.D (Ions score 28) (2+) K.NMITGTSQADC#AVLIVAAGVGEFEAGISK.N (Ions score 21) (3+)
41	Unknown			10X	unidentified

* Protein fragment
# in bold upregulated proteins after serum withdrawal

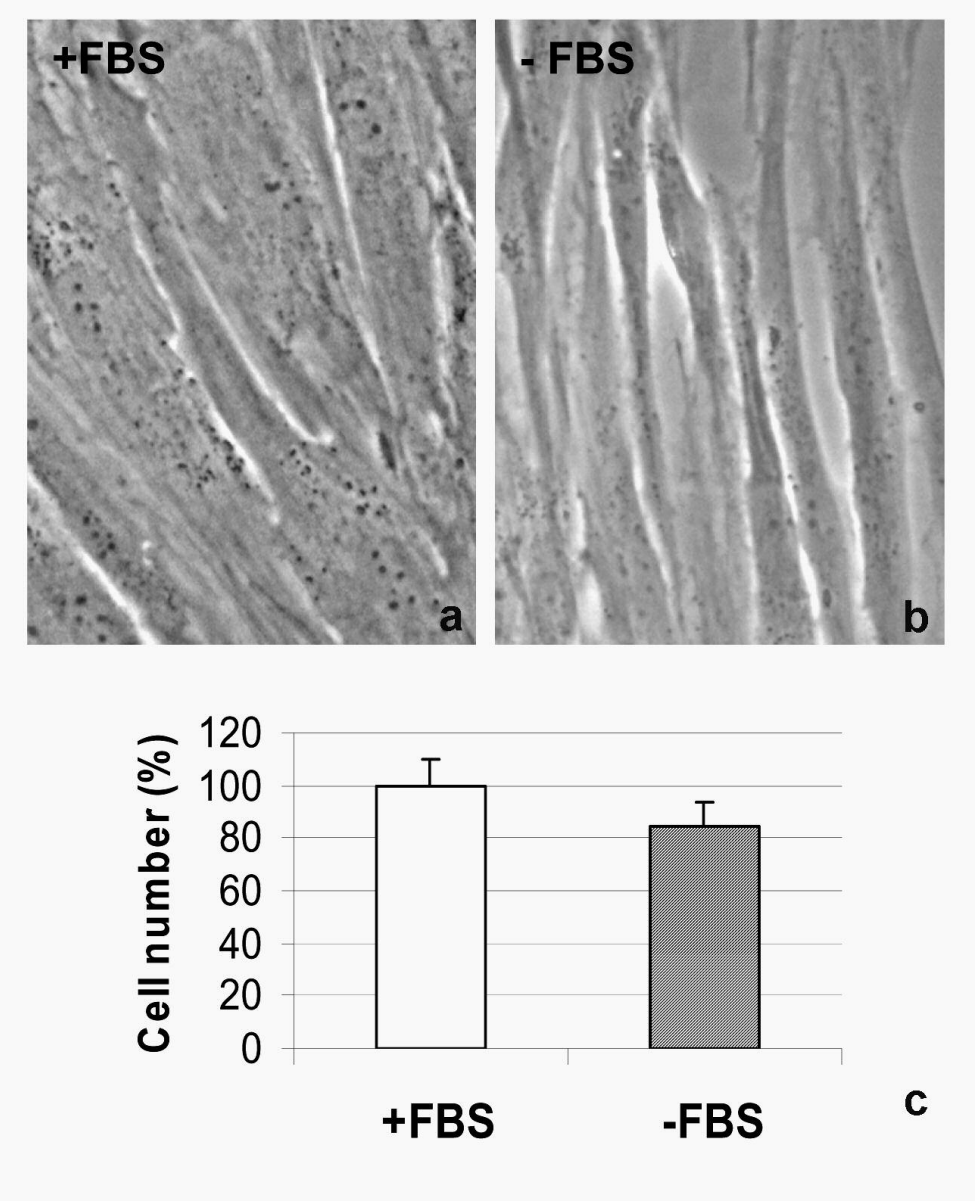
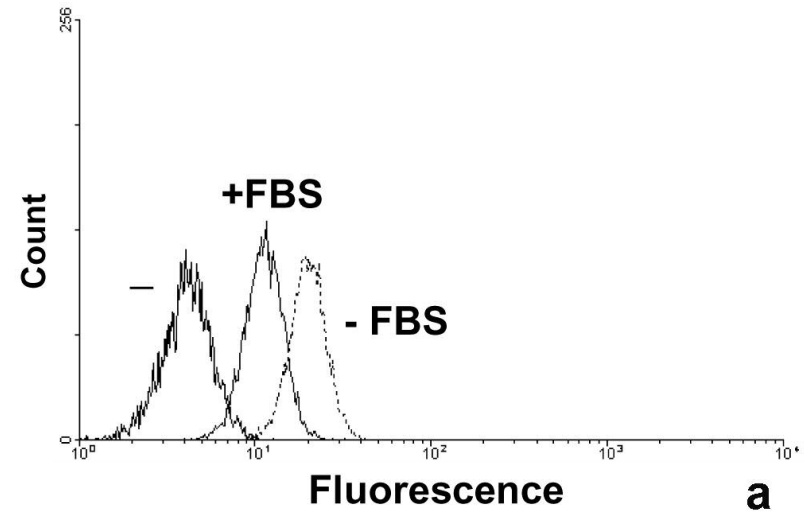


Figure 1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+FBS
- FBS
0
10^0
10^1
10^2
10^3
10^4
Fluorescence
a

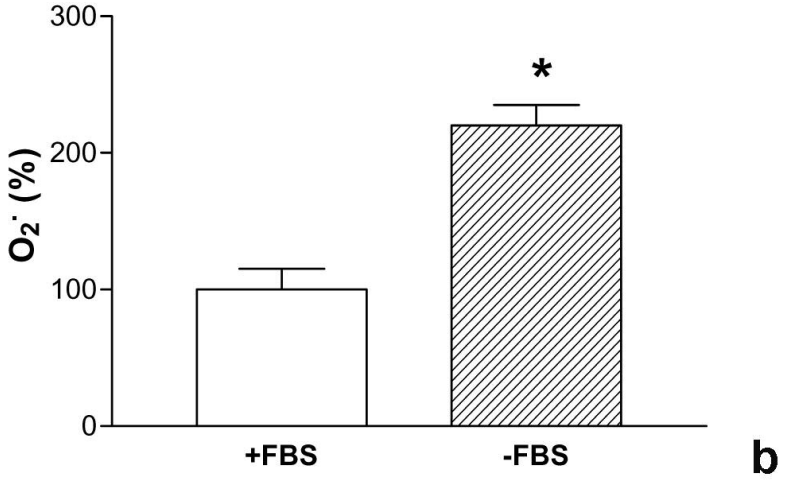
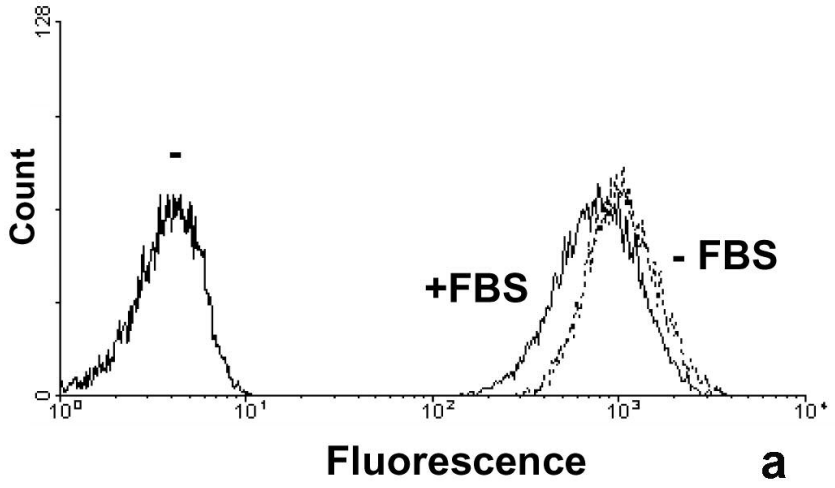

300
200
100
0
$O_2^{\cdot}$ (%)
*
+FBS
-FBS
b

Figure 2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Count
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-
+FBS
- FBS
10^0
10^1
10^2
10^3
10^4
Fluorescence
a

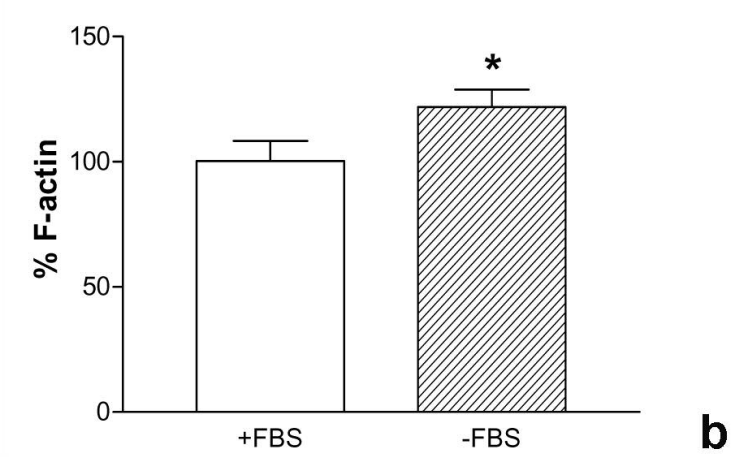

150
100
50
0
% F-actin
*
+FBS
-FBS
b

Figure 3

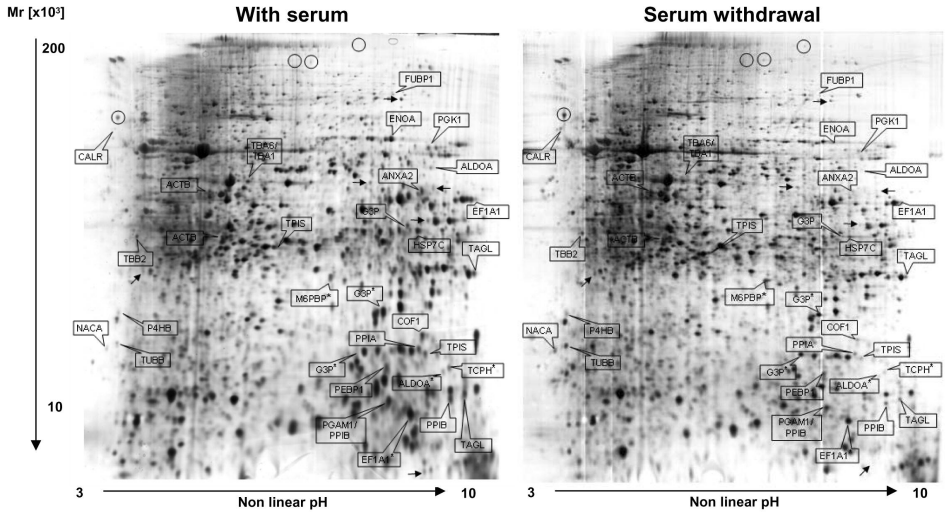


Figure 4

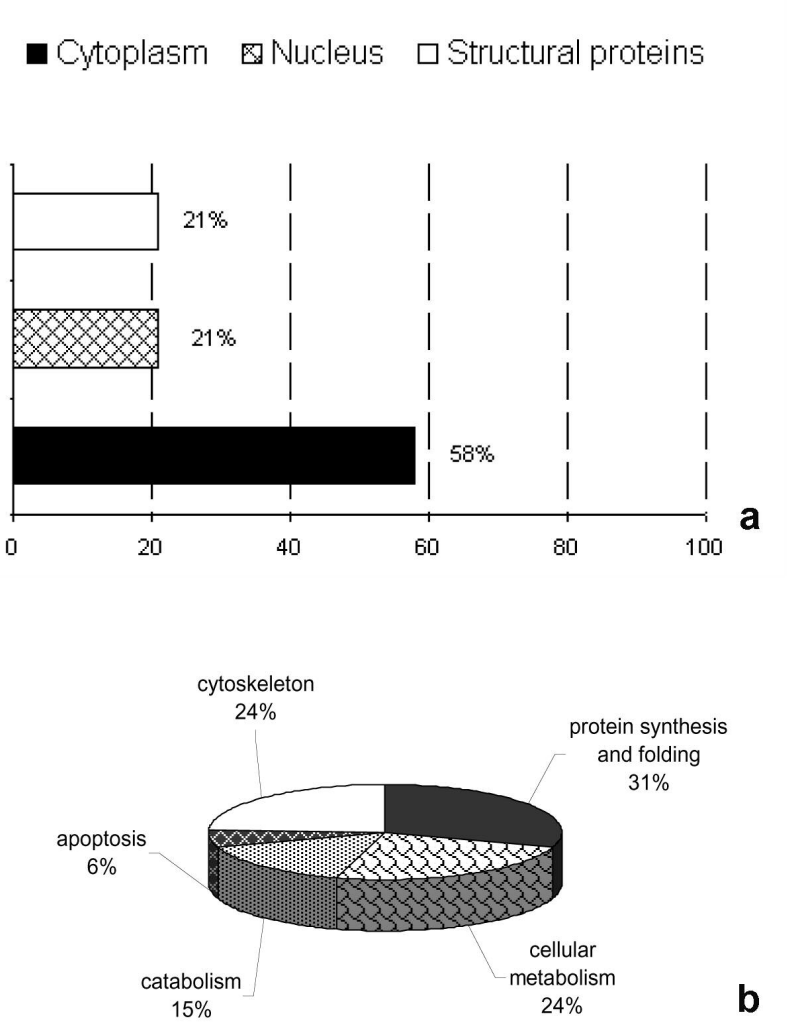
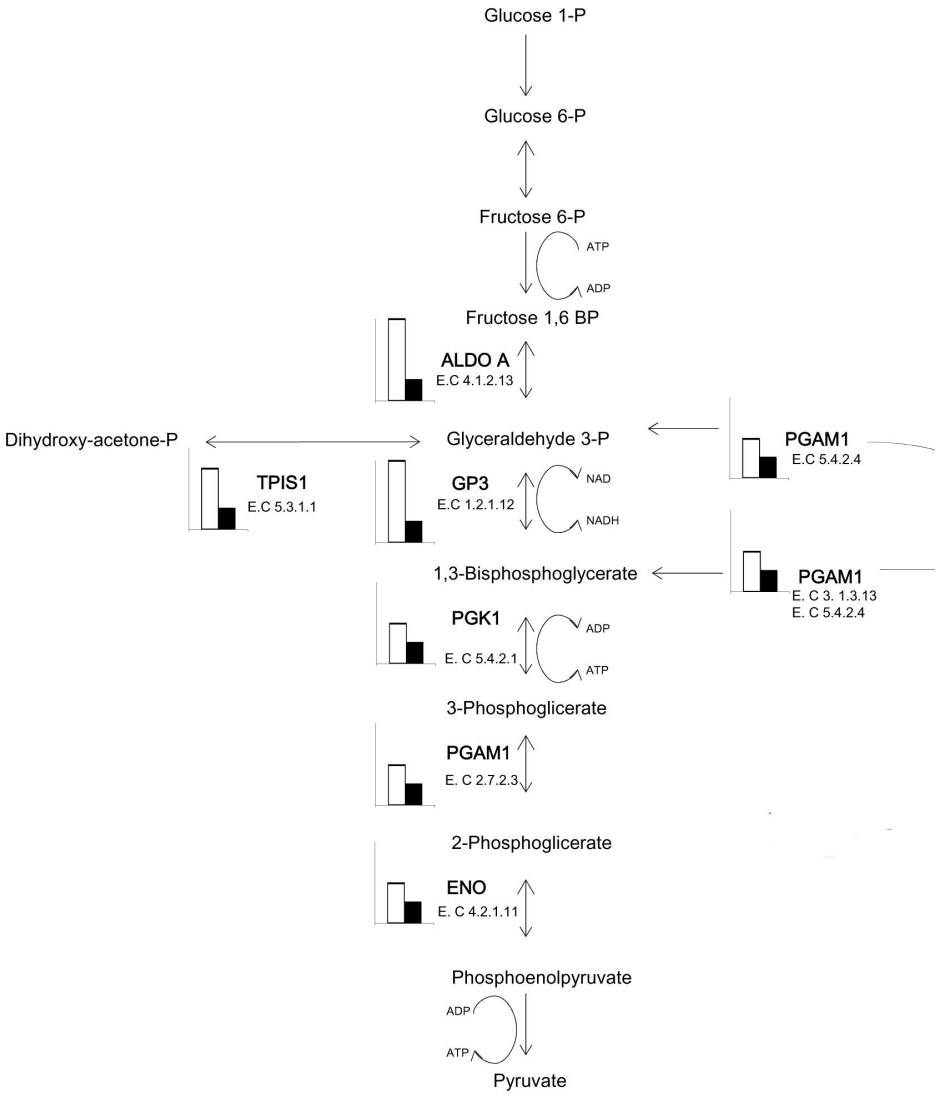

Cytoplasm
Nucleus
Structural proteins
21%
21%
58%
0
20
40
60
80
100
a
cytoskeleton
24%
protein synthesis
and folding
31%
apoptosis
6%
catabolism
15%
cellular
metabolism
24%
b

Figure 5


Glucose 1-P
Glucose 6-P
Fructose 6-P
ATP
ADP
Fructose 1,6 BP
ALDO A
E.C 4.1.2.13
Dihydroxy-acetone-P
Glyceraldehyde 3-P
PGAM1
E.C 5.4.2.4
TPIS1
E.C 5.3.1.1
GP3
E.C 1.2.1.12
NAD
NADH
1,3-Bisphosphoglycerate
PGAM1
E. C 3. 1.3.13
E. C 5.4.2.4
PGK1
E. C 5.4.2.1
ADP
ATP
3-Phosphoglicerate
PGAM1
E. C 2.7.2.3
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Phosphoenolpyruvate
ADP
ATP
Pyruvate

Figure 6

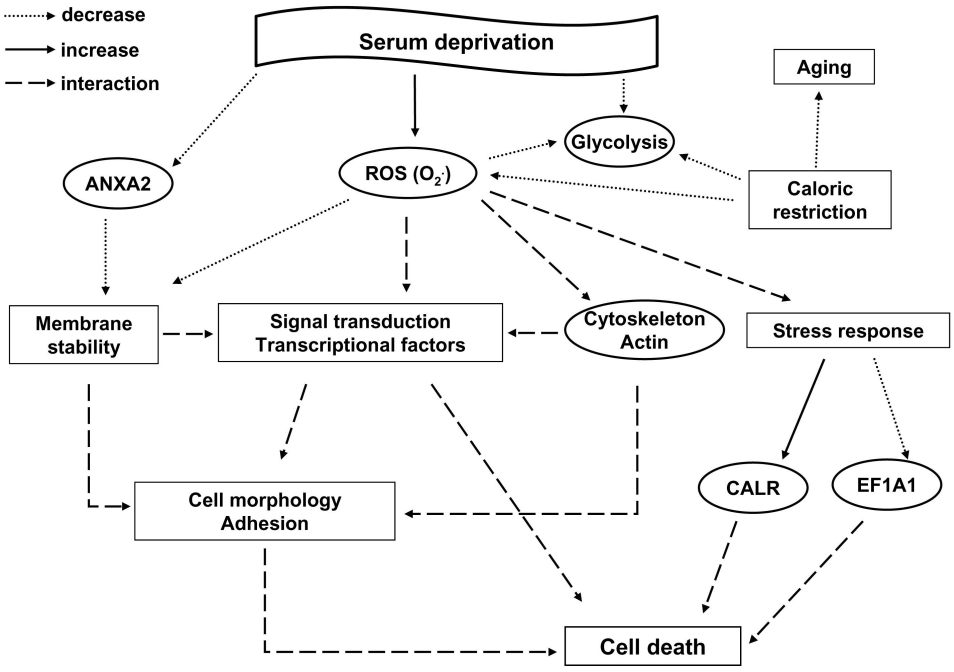

Figure 7