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Hypoxia influences the cellular cross-talk of human dermal fibroblasts. A proteomic approach.

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Abstract

The ability of cells to respond to changes in oxygen availability is critical for many physiological and pathological processes (i.e. development, aging, wound healing, hypertension, cancer). Changes in the protein profile of normal human dermal fibroblasts were investigated in vitro after 96h in 5% CO_2 and 21% O_2 (pO_2=140mmHg) or 2% O_2 (pO_2=14mmHg), these parameters representing a mild chronic hypoxic exposure which fibroblasts may undergo in vivo. The proliferation rate and the protein content were not significantly modified by hypoxia, whereas proteome analysis demonstrated changes in the expression of 56 proteins. Protein identification was performed by mass spectrometry. Data demonstrate that human fibroblasts respond to mild hypoxia increasing the expression of hypoxia inducible factor (HIF1a) and of the 150 kDa oxygen regulated protein. Other differentially expressed proteins appeared to be related to stress response, transcriptional control, metabolism, cytoskeleton, matrix remodelling and angiogenesis. Furthermore, some of them, like galectin 1, 40S ribosomal protein SA, N-myc-downstream regulated gene-1 protein, that have been described in the literature as possible cancer markers, significantly changed their expression also in normal hypoxic fibroblasts. Interestingly, a bovine fetuin was also identified that appeared significantly less internalised by hypoxic fibroblasts. In conclusion, results indicate that human dermal fibroblasts respond to an in vitro mild chronic hypoxic exposure by modifying a number of multifunctional proteins. Furthermore, data highlight the importance of stromal cells in modulating the intercellular cross-talk occurring in physiological and in pathologic conditions.

1. Introduction

Fibroblasts are important stromal cells that synthesize the structural components of the extracellular matrix, but they also migrate within the stroma in order to interact with other cells and with the extracellular milieu according to different stimuli [1-3]. However, in physiological as well as in pathologic conditions, fibroblasts may be frequently found distant from blood vessels, where they are forced to adapt to mild hypoxia.

Hypoxia in fibroblasts is known to induce an upregulation of growth factors such as transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), insulin growth factor (IGF-1) and to favour extracellular matrix remodelling mainly through modulation of metalloproteases, activation of lysyl oxidase [4], stimulation of collagen type I synthesis [5,6] and reduced expression of elastin [7,8].

In addition, there are several other circumstances, as during wound healing, where capillary injury generates a hypoxic environment in which fibroblasts, and subsequently myofibroblasts, are attracted in order to activate the repair processes [9]. Experimental findings support the theory that fibroblasts play a significant role in the vascular response to injury, being capable to proliferate, transdifferentiate and migrate under hypoxic conditions through hypoxia inducible factor (HIF) activation [10,11].

Interestingly, there are evidence suggesting that HIF is functionally connected to senescence [12], and that aging is associated to high incidence of ischemic diseases where connective tissue homeostasis is severely compromised.

Moreover, during cancer progression, many tumors develop a hypoxic microenvironment in which oxygen delivery to neoplastic as well as to stromal cells is frequently reduced or even abolished. Tumor cells can survive and even grow in such a deteriorated microenvironment and their aggressiveness as well as their capacity to invade surrounding tissues are dependent

on the stroma produced by fibroblasts, suggesting that these cells may tightly interact and/or influence cancer cell behaviour [2,13,14].

Although a large number of studies have focused on the influence of hypoxia on the expression and the posttranslational modifications of a single protein or of a subset of functionally related proteins, only some of them have examined proteome-wide alterations during hypoxia [15-20] and few data are available for human fibroblasts [21,22].

Given the importance of fibroblasts in connective tissue homeostasis and their interactions with other cells in normal and pathological conditions [23-24], aim of the present study was to investigate changes in the protein profile of in vitro normal human dermal fibroblasts in primary cell culture exposed to mild chronic hypoxia [25].

2. Materials and Methods

2.1 Cells and treatments

Human dermal fibroblasts were taken from the upper thigh 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. Fibroblasts were used between 5^{th} and 7^{th} passages and routinely cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin, and 100 ug/ml streptomycin. Cells from each subject were kept separate during all experiments.

Hypoxic conditions were established by culturing fibroblasts in two different incubators as previously described [26]. In control experiments an incubator (KW Apparecchi Scientifici, Siena, Italy) set at 5% CO_2, 20% O_2 (atmospheric oxygen ≈ 140 mmHg) in a humidified environment at 37°C was used. Experiments under hypoxic conditions were performed using

a water-jacketed incubator (Forma Scientific, Marietta, OH, USA) providing a customized and stable humidified environment through electronic control of CO_2 (5%), O_2 and temperature (37°C). The O_2 tension was set and constantly maintained at 2% (≈ 14 mmHg) by automatically injecting N_2 in the chamber.

All experiments under hypoxia exposure were performed after 24 hours from seeding on synchronized cells (overnight in DMEM + 0.2% FBS). During experiments fibroblasts were cultured in DMEM + 10% FBS.

2.2 Cell proliferation

Proliferation was assessed using the CyQuant cell proliferation assay (Molecular Probes, OR, USA). In this assay, the CyQuant dye binds to DNA, and the emitted fluorescence is linearly proportional to the number of cells in the well. Fibroblasts were seeded at a density of 4000 cells/well in a 96-well black fluorescence micro-titre plates and allowed to attach for 4-8 h in DMEM + 10% FBS. After overnight starving (0,2% FBS), medium was replaced with 10% FBS and plates were simultaneously incubated for 48, 72 and 96 hours at 37°C in normoxic or hypoxic condition. At appropriate times, the medium was discarded, plates were washed with phosphate buffered saline (PBS) and frozen at -80°C until use. On the day of the analysis, plates with adherent cells were thawed and incubated with a buffer containing the CyQuant dye. Fluorescence was measured using a Fluostar optima (BMG LABTEACH Offenburg-Germany) multi-well plate reader with excitation 485 nm and emission 520 nm [27]. Three separate experiments were performed independently.

2.3 HIF quantitation

Equal amounts of proteins, determined using a kit from Pierce (Rockford, IL), were resolved on 10% SDS/polyacrylamide gels, and transferred to a nitrocellulose membrane

(Schleicher&Schuell, Keene, NH). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 hour and then incubated overnight at 4°C with a primary antibody for HIF-1α (BD Biosciences, San Jose, CA) diluted 1:250 and for β-actin (housekeeping gene, Cell Signaling Technology, Beverly, MA) diluted 1:2000, in 5% non-fat dry milk in TBST. Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (diluted 1:5000 in 5% non-fat dry milk in TBST) for 1 hour and the antigen-antibody complexes were visualized using an Immuno-star HRP kit (Bio-Rad Laboratories, Hercules, CA). Immunoreactive bands were digitalized with a charge-coupled device camera gel documentation system (ChemiDocXRS, Bio-Rad), and quantified with the Quantity One software (Bio-Rad). β-actin was used in the same gel to normalize the amounts of total protein present in the samples.

2.4 Proteome analysis

2.4.1. Sample preparation

Synchronized fibroblasts were grown for 96h in DMEM plus 10% FBS in normoxia and hypoxia. Afterwards, cells were detached from flasks by incubation in 0.25% Trypsin in PBS for 10 min at 37°C. After washes in DMEM plus FBS and proteinase inhibitors (1mM ethylenediaminetetraacetic acid (EDTA), 10μM ε-aminocaproic acid, 50mM benzamidine) cells were centrifuged at 1000g for 10 min. After supernatant removal, pellets were resuspended in PBS plus proteinase inhibitors, centrifuged at 1000g for 10min, and immediately resuspended in lysis buffer (8M urea, 2% 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate (CHAPS), 65mM dithioerythritol, 2% pharmalyte pH 3–10 and trace amount of bromophenol blue). Protein concentration was determined according to Bradford [28]. Cells from each subject were kept separate during all experiments.

2.4.2. Two-dimensional gel electrophoresis (2D-GE)

2D-GE was performed, essentially as described by Bjellqvist et al. [29], in two independent assays where the three different cell lines exposed to normoxia and hypoxia were run in duplicate.

Samples containing 60 µg (analytical gels) or 1 mg (preparative gels) of protein underwent 2D-GE using the Immobiline/polyacrylamide system [29]. Isoelectric focusing was performed on IPGphor system (GE- Healthcare, Uppsala, Sweden) at 16°C using two different protocols. For analytical gels: passive rehydratation 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). When the equilibration phase was finished, strips were loaded onto 12% acrylamide vertical gels using an Ettan DALTsix electrophoresis unit (GE- Healthcare, Uppsala, Sweden).

Analytical gels were stained with ammoniacal silver nitrate [30]; preparative gels for mass spectrometric analysis were silver-stained as described by Shevchenko et al. [31].

2.4.3. Data acquisition and analysis

To detect significant differences in protein abundance between the two experimental conditions, all silver-stained gel images were digitalized at 400 dpi resolution using ImageScanner (GE- Healthcare) and analyzed using Melanie 3.0 software (GE- Healthcare, Uppsala, Sweden). After background subtraction, protein spots were automatically defined and quantified with the feature detection algorithm [30]. Spot intensities were expressed as percentages (% vol) of relative volumes by integrating the optical density (OD) of each pixel in the spot area (vol) and dividing with the sum of volumes of all spots detected in the gel.

Only those spots that, within the same experimental condition, exhibited the same trend of expression in all gels underwent further quantitative analysis.

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

Statistical data were obtained using GraphPad software (San Diego, CA, USA) and data were compared by the unpaired *t*-test; differences between treatments were considered significant at $p < 0.05$.

Only those spots whose expression appeared significantly changed by hypoxia were selected for MS analysis.

2.4.4. In-gel destaining and digestion of protein samples

Spots of interest were manually excised from preparative silver- stained 2-DE gels. Silver-stained gel pieces were destained as described by Gharahdaghi et al. [33]. 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, Basel, CH) in 25 mM AmBic overnight at 37°C. Peptide extraction was carried out twice using first 50% ACN, 1% trifluoroacetic acid (TFA) and then 100% ACN. All extracts were pooled, and the volume was reduced by SpeedVac.

2.5 Mass Spectrometry

2.5.1. MALDI-TOF MS

The tryptic peptide extracts were redissolved in 12µl 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 CH_3CN/H_2O (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 49.1 or the NCBI (march 2006 database release) databases using MS-Fit 3.1.1 from ProteinProspector 3.2.1 (http://www.expasy.org/tools/). Typical search parameters were as follows: ± 30ppm of mass tolerance; carbamidomethylation of cysteine residues; one missed enzymatic cleavage for trypsin; a minimum of four peptide mass hits was required for a match (when the matched peptide number was low, the delta mass difference between experimental measured masses and exact masses from the data base was evaluated and it was checked if masses of peptides were in the same range); methionine residues could be considered in oxidized form; no restriction was placed on the pI and molecular weight of the protein. The minimum signal/noise ratio was generally between 5/1 to 10/1, depending on the spectrum quality. Finally, triptic digests that did not produced unambiguous protein identification were successively subjected to HPLC/MS.

2.5.2. HPLC/MS

Peptides were resuspended in aqueous 5% formic acid and subsequently eluted onto a 150mm x 75µm Atlantis C18 column analytical (Waters, Milford, MA, 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, Milford, MA,

USA). 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 the 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; charge state, 2, 3, or 4.

We basically selected the candidate peptides with probability-based MOWSE scores 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, Boston, MA, USA) [32]. Proteins that were identified with at least two peptides both showing a score higher than 40, were validated without any manual processing. Those with at least two peptides whose score was lower than 40 and higher than 20 were systematically checked and/or interpreted manually to confirm or cancel the MASCOT suggestions. For protein identified by only one peptide, its score has to exceed 30, and its peptide sequence was systematically checked manually.

2.6 Immunoblot

Protein extracts were processed, electrophoresed (30µg proteins/lane) on 10-lane 1-DE 10% polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane. The membrane was blocked in TBST + 5% non fat dry milk for 1 h at room temperature. The primary antibodies, all purchased from Abcam (Cambridge, UK), were diluted in TBST + 2,5% non fat dry milk as follows: a) filamin 1:1000 (goat polyclonal, ab11074); b) protein disulfide isomerase 1:1000 (mouse monoclonal, ab2792); c) heat shock

protein60 1:10000 (Mouse monoclonal, ab13532); d) enolase-1 1:30000 (rabbit polyclonal, ab49343); e) heat shock protein-27 1:1000 (mouse monoclonal, ab2790); f) calmodulin 1:500 (mouse monoclonal, ab2860); g) galectin 1 1:5000 (rabbit polyclonal, ab25138); h) thioredoxin 1:2000 (rabbit polyclonal, ab16835). The membrane were incubated with primary antibodies at room temperature for 60 min. The following secondary antibodies were used after washing three times in TBST: horseradish peroxidase (HRP)-conjugated sheep anti-mouse immunoglobulin antibody 1:5000 (GE Healthcare) or donkey anti-goat IgG 1:15000 (ab6885, Abcam, Cambridge, UK) or donkey anti-rabbit IgG 1:20000 (ab6802, Abcam, Cambridge, UK). Subsequently, membranes were washed three times in TBST and Western blots were visualized using the ECL plus detection system (GE Healthcare) as described in the technical manual provided by the company. Images were analysed using ImageQuant TL v2005 software in order to automatically determine the band volumes.

3. Results

3.1 Cell proliferation

In our experimental conditions, hypoxia did not significantly modified the proliferation of dermal fibroblasts, as evaluated by the CyQuant cell proliferation assay after 48, 72 and 96h of treatment (data not shown).

Moreover, cells in the two experimental conditions were similar as far as their morphology.

3.2 HIF determination

HIF accumulation was evaluated by Western blot, using antibodies recognizing HIF1α and HIF2α (figure 1a). Densitometric evaluation of HIF bands normalised to β-actin was performed in all cell lines and confirmed a significant ($p<0.05$) increase of HIF-1α after 96

hours of hypoxia (figure 1b), whereas HIF2α did not changed in the two experimental conditions (data not shown).

3.3 Protein profile evaluation

By 2D-GE approximately 2500 proteins were separated from each cell line, independently from the experimental conditions.

The experimental intra-samples variability was measured by analysing changes in the volume of 600 protein spots on duplicate 2D gels from the same sample. Spot location and intensities were very similar between gels from the same sample. The coefficient of variation (CV% calculated by the standard deviation of the normalized spot volumes divided by the mean of values, expressed as a percent) was also analysed in order to quantify intra-sample (20%) and inter-samples (32%) variability. Values were similar to those already described in the literature [35].

The hypoxic condition determined statistically significant changes ($p<0.05$) in the expression of 56 proteins, indicated by the arrows on two representative gels obtained from normoxic and hypoxic fibroblasts (figure 2). In particular, 32 proteins appeared significantly upregulated under hypoxia, whereas 24 proteins were significantly downregulated (figure 3).

By mass spectrometry we have identified about 63% 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 the case of spot 16, MS revealed the presence of two different proteins in the same spot: namely actin 1/2 and protein disulfide-isomerase.

It has to be mentioned that, for some proteins, we identified spots that did not match with the predicted Mr or pI, indicating once more the presence of different isoforms and/or fragments as already shown by other Authors [36].

Surprisingly, all identified proteins were produced by human fibroblasts, with the exception of fetuin, that was of bovine origin. Even though cells were placed in the same culture medium, hypoxic fibroblasts showed, i.e. retained, a significantly reduced amount of bovine fetuin within the cell monolayer.

Each identified protein was assigned to a functional classification based on the Gene Ontology annotation system using the DAVID database bioinformatic resources (http://david.abcc.ncifcrf.gov) (table 2). GO is a structured, controlled vocabulary that describes gene products in terms of their associated biological processes, cellular components and molecular functions. The protein distribution into functional categories is reported in figure 4. It can be noted that proteins were grouped into 15 different categories, some proteins belonging to more than one category due to their multifunctional properties.

3.4 Immunoblot verification of protein changes

To validate results of proteome analysis, immunoblot experiments were performed on 1D SDS-PAGE using specific antibodies for filamin, protein disulfide isomerase, heat shock protein-27, heat shock protein-60, enolase-1, calmodulin, galectin 1 and thioredoxin (figure 5). Data were generally in agreement with those observed at proteome level; however in the case of filamin C, where two isoforms, by proteome analysis (spot 1 and 2), exhibited different protein changes, one being upregulated, wheres the other was downregulated, immunoblot revealed that filamin C was globally downregulated upon hypoxia.

Furthermore, in the case of spot 16, that MS demonstrated to be actually formed by two proteins (i.e. actin 1/2 and protein disulfide isomerase), immunoblot data revealed a marked upregulation of protein disulfide isomerase in hypoxic conditions. Therefore, the global downregulation of spot 16 could be attributable to a downregulation of the actin isoform,

consistently with the reduced expression also of the other actin isoform corresponding to spot 11.

4. Discussion

Albeit hypoxia is a stress condition that in some circumstances can compromise cell viability leading to growth arrest and apoptosis [37], human fibroblasts, similarly to other cell lines [18] are highly resistant to the hypoxic condition, being even capable to heighten proliferation capabilities [38,39]. In the experimental conditions described in this study, differences between normoxic and hypoxic fibroblasts were negligible as far as their growth capabilities and cell morphology, indicating that fibroblasts in the presence of chronic mild hypoxia exposure can well adapt themselves.

In the present investigation, cells were grown for 96 hour at 140 (normoxia) or 14 mmHg (hypoxia) pO_2. These parameters have been selected because oxygen pressure in tissues decreases with increasing distance from blood vessels reaching values of pO_2 as low as 0.5 – 2.5 kPa (4–20 mm Hg) [25]**,** and therefore a mild (2%) chronic (up to four days) hypoxia can be considered a condition that stromal cells may frequently experience, depending on local physiological and pathological stimuli.

Furthermore, a four day exposure allows cell to stabilize their phenotype in the new environment, and changes should not be regarded as a transient response, but the result of a real adaptation to hypoxia.

4.1. Oxygen responsive proteins

Human dermal fibroblasts responded to mild chronic hypoxia exposure by increasing the expression of HIF1α, whereas HIF 2α, as expected [40,41], did not exhibited any significant

change. Therefore, changes in fibroblast protein profile are the consequence of low oxygen availability.

In addition to HIF1α accumulation, hypoxia determined a significant up-regulation of the 150 kDa oxygen regulated protein (ORP150), an inducible chaperone that facilitates the protein transport/processing in the endoplasmic reticulum under low oxygen tension [42]. It is expressed in a range of pathologic situations such as ischemic brain, atherosclerotic plaques and malignant tumours, suggesting that it may contribute to cellular response to environmental stress. Moreover, in low ambient oxygen concentrations, ORP150, is likely to subserve a cytoprotective role at the level of the endoplasmic reticulum, enhancing cellular ability to sustain oxygen deprivation [43], as indicated by the ability of fibroblasts to adapt to hypoxia. Consistently with its up-regulation upon hypoxia, it has been demonstrated that OPR150 promotes angiogenesis favouring the transport and secretion of VEGF [42], a cytokine which plays a fundamental role in wound healing and in cancer progression. This finding further supports the role of hypoxia in modulating fibroblast behaviour and tissue remodelling.

4.2. Stress responsive proteins

Hypoxia can be considered a stress condition capable to modulate cell phenotype through activation of several stress-response pathways [44]. In our experimental conditions, we have shown an up-regulation of Hsp27, which is probably mediated through HIF-1 activation [45]. Hsp27 has been demonstrated to act as an anti-apototic protein with a cytoprotective effect [46], consistently with the high resistance of fibroblasts to various stress conditions (personal observation).

By contrast, upon mild chronic hypoxia exposure, Hsp60 appeared significantly reduced both by proteome analysis and by immunoblot. Hsp60 is a mitochondrial protein that is important

for folding key proteins after import into the mitochondria. It has been demonstrated that, during hypoxia, Hsp60 cellular distribution changes, with Hsp60 leaving the cytosol and translocating to the plasma membrane [47]. Since it is well known that analysis of membrane proteins may offer some difficulties when two-dimensional gel electrophoresis conventional protocols are used, it cannot be excluded that reduced expression of Hsp60 is simply the consequence of cellular redistribution of the protein to the plasma membrane, or, possibly, the consequence of functional differences between various stress proteins [44].

In addition to Hsp(s), also some oxidative stress-related proteins, namely peroxiredoxin and thioredoxin, appeared to be significantly less expressed by hypoxic fibroblasts. In particular, peroxiredoxin belongs to a family of multifunctional antioxidant thioredoxin-dependent peroxidases, thus exerting a cellular protection against oxidative stress, modulating intracellular signalling cascades and regulating cell proliferation [48]. The observation that peroxiredoxin is homologous to the natural killer enhancing factor, allowed to hypothesize that hypoxic fibroblasts have a lower capability to protect cells from oxidative damage, or to selectively promote NK citotoxicity against certain tumor cells [49]. Furthermore, recent evidence highlighted the role of peroxiredoxin as important tumor suppressors [48], playing a role in preventing the oxidative damage which may activate pathways leading to aggressive tumors [50].

Similarly to peroxiredoxin, also thioredoxin was significantly less expressed in human fibroblasts upon hypoxia. Thioredoxin is an ubiquitous oxidoreductase with strong cytokine, chemoattractant and anti-apoptotic activities [51]. These data seem to support the hypothesis that hypoxia may modulate the cellular cross-talk within stromal connective tissue, and that, regarding oxidative stress, hypoxia may prevent the production of free radicals, thus down-regulating the expression of anti-oxidant molecules.

4.3. Proteins related to cell metabolism

There are several papers indicating that one of the major metabolic consequences of the hypoxic condition is the activation of glycolitic enzymes such as aldolase A and C, enolase, lactate dehydrogenase, phosphofructokinase L, phoshoglycerate kinase, pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase, possibly through HIF-mediated transcription induction [52]. In our experimental conditions, some of these enzymes were only moderately increased under mild hypoxia, without reaching a statistical significance. In particular, four glyceraldehyde-3-phosphate dehydrogenase isoforms were identified increasing their expression from 10% to 15% in hypoxic condition, the phosphoglycerate kinase raises of 20% and fructose aldolase increases 2,5 fold. These findings indicate that in hypoxic fibroblasts several glycolytic enzymes are only moderately affected.

In the case of triosephosphate isomerase (TPI), this protein was significantly increased upon hypoxia, as already demonstrated by other Authors at both mRNA and protein levels [53], suggesting that this upregulation, by increasing the flow of triosephosphate through the glucolytic cascade, may lead to anaerobic energy generation. Unlike the previously described enzymes, it could be suggested that TPI is a glycolytic enzyme particularly sensitive to hypoxia.

As far as enolase, a statistically significant up-regulation of two isoforms has been shown ($p<0.05$). Enolase catalyzes the formation of phosphoenolpyruvate from 2-phosphoglycerate, but evidence indicates that it may function, other than a glycolytic enzyme, as a modulator of growth control as well as of thermal and hypoxia tolerance. Moreover, enolase may act as a cell surface receptor for plasminogen, suggesting that fibroblasts in a hypoxic environment favour proteolytic activities on the cell surface [54], thus contributing to matrix remodelling.

Interestingly, we have shown that hypoxic fibroblasts upregulate the expression of parathymosin, a zinc-binding protein, which is known to interacts with several enzymes

involved in carbohydrate metabolism and to inhibit the binding of the activated glucocorticoid receptor to nuclei. More recently, parathymosin has been demonstrated to act modulating H1 interactions with chromatin and affecting the condensation state of chromatin fibers allowing to hypothesize that parathymosin may participate in global chromatin remodeling during gene activation and perhaps during the transcription initiation process itself [55-57]. Therefore, it is conceivable to suggest that increased expression of parathymosin may influence the protein profile, as a consequence of chromatin structure remodelling.

4.4. Cytoskeletal related proteins

A large network of physically interconnected cellular components, starting from the structural components of the cell nucleus, via cytoskeleton filaments to adhesion molecules and the extracellular matrix, constitutes an integrated matrix that functions as a scaffold allowing the cell to cope with changes of the microenvironment. Several cytoskeletal and cytoskeletal-related molecules are significantly affected by hypoxia, most of these proteins appeared to be upregulated, i.e. tubulins, transgelin and lamins. Lamins, for instance, are intranuclear class of intermediate filament proteins being part of the nuclear envelope and playing a role in nuclear integrity maintenance, in chromatin organization and in transcriptional control modulation [58]. The upregulation of different lamin isoforms may contribute to hypoxia-related changes in the protein profile through increased nuclear strength and activation of transcription factors [59].

More intriguing is the different response of two filamin C isoforms, as revealed by proteome analysis. Interestingly, validation of protein changes by immunoblot, demonstrated that filamin C was globally down-regulated upon hypoxia. Filamin-C belongs to the filamin family of actin binding proteins. Beside its role in crosslinking actin filaments into a 3D structure, filamin has been reported to directly interact with more than 30 cellular proteins,

among which membrane receptors for cell signalling molecules [60]; nevertheless, the physiological significance of most of these interactions is still unknown. Given the multifunctional role of filamins [61], the occurrence of isoform switching as well as of alternative mRNA splicing and of different post-translational modifications, namely phosphorylation [62], it could be assumed that filamin C isoforms: i) may exert various biological role by interacting with various molecules, ii) may have a different susceptibility to hypoxia, iii) may interact with some, but not all actin filaments, indicating that hypoxia can be responsible for filamin redistribution and actin rearrangement, as observed in other stress conditions [63].

4.5. Tumour-related proteins

The microenvironment of solid tumours present hypoxic regions and several hypoxia-regulated genes may contribute to tumour progression and treatment resistance [64]. Within this context, stromal cells, being regulated by reduced oxygen tension, may actively contribute to modulate the transformed phenotype [24].

Mild chronic hypoxia, for instance, caused a down-regulation of the N-myc downstream regulated gene1 protein (NDRG1), that shuttles between cytoplasm and nucleus upon several insults [65]. This protein has been shown to be markedly up-regulated in several tumour cell lines. Surprisingly, our data indicate that NDRG1 is expressed also by normal fibroblasts, although its expression, differently from tumour cells, is decreased upon hypoxia. These data may indicate that oxygen-responsive proteins can be differentially regulated depending on cell type and that stromal cells may interfere with the overall evaluation of this protein as a potential cancer marker.

Galectin 1 is involved in numerous biological functions, i.e. cell-cell and cell-substrate interactions and induction of apoptosis of activated T-lymphocytes, and is over-expressed in

tumours and/or in the tissue surrounding neoplastic proliferation [66]. Previous data already demonstrated that galectin 1 is expressed by endothelial cells from capillaries infiltrating tumours such as prostate carcinoma [67] and it has been hypothesized that galectin 1 expression in the endothelium close to tumours could provide cancer cells with increased abilities to interact with endothelial cells as well as a defence against the host immune system. Data from the present study, by proteome analysis as well as by immunoblot, indicate that galectin 1 is over-expressed in hypoxic fibroblasts, and therefore it could be suggested that in hypoxic conditions, as in cancer progression, stromal cells may contribute, as endothelial cells [67] and cancer cells [68], to the aggressiveness of tumours.

Several factors are known to contribute to severity and aggressiveness of tumour cells and hypoxia has been shown to reduce the efficacy of conventional radiotherapy and to diminish survival prognosis [69].

In our experimental model, hypoxia up-regulated the expression of the 40S ribosomal protein SA also known as a multidrug resistance associated protein MGr1-Ag or as a human 34-67 kDa laminin receptor, thus highlighting the involvement of mesenchymal cells, such as fibroblasts, in the stromal tissue response to hypoxia.

The strong relationship between hypoxia, stroma and cancer development is further supported by the up-regulation of elongation factor 1α (EF1α), an ubiquitous cellular protein, responsible for the GTP-dependent recruitment of aminoacyil-tRNAs to the ribosome during the elongation cycle of protein translation [70], even though, several other non-canonical functions have been ascribed to EF-1α, including microtubule severing, actin filament bounding, oncogenic transformation and ubiquitin-dependent proteolysis of N-terminus proteins [71-73].

Furthermore, it has to be mentioned that hypoxia-induced matrix remodelling may be further supported by the increased expression of annexin I and II. Annexin I inhibits the expression

and/or the activity of inflammatory enzymes such as the inducible nitric oxide synthase and cyclooxigenase and contribute to the anti-inflammatory signalling allowing safe-post-apoptotic clearance of dead cells [74]. Moreover, it has been demonstrated that annexin II serves as a profibrinolytic coreceptor for both plasminogen and tissue plasminogen activator, and it has been shown that the abundant presence of annexin II on the cell surface [75] may contribute to the invasive potential through the extracellular matrix [76], to the activation of other metalloproteases and/or to the release of matrix-bound angiogenic growth factors. There is in fact evidence that some proteases and proteases receptor expression are under the control of tumour hypoxia, which is the result of an imbalance in oxygen supply and demand [77]. Present data underline, once more, that hypoxia modulates fibroblast protein profile and that stromal cells are active and crucial players in several pathologic processes [78].

4.6. Serum-derived proteins

All identified proteins were produced by human fibroblasts, with the exception of fetuin, that was of bovine origin. Even though cells were placed in the same culture medium, hypoxic fibroblasts seemed to retain a significant reduced amount of bovine fetuin within the cell monolayer, suggesting that fibroblasts, in vitro, can incorporate the fetuin present in the culture media as already observed in human vascular smooth muscle cells [79].

Fetuin is a protein synthesized by the liver and abundantly present in serum, and in several tissues and organs where acts as an important inhibitor of ectopic calcification [80].

Present data indicate that hypoxic fibroblasts have a reduced serum fetuin uptake. It could be speculated that, in vivo, hypoxic cells could internalise a significantly lower amount of fetuin from the extracellular compartment, thus perturbing calcium homeostasis, as observed during atherosclerosis, chronic renal failure and cancer, leading to ectopic calcifications.

4.7. Concluding remarks

The maintenance of oxygen homeostasis is crucial during embryonic development and in postnatal life [81] and a better comprehension of the pathways involved in the response to changes in oxygen availability might have important biological and therapeutic implications [82]. Cells can respond to changes in oxygen availability with a rapid feedback mediated through post-translational modifications or membrane depolarisation [83] and with a "late hypoxic response pathway" affecting gene and protein expression over several hours. These last changes are mediated, at least in part, through the induction of hypoxia-inducible transcription factors as HIF [84], which is considered a marker of the ability of the cells to respond to the hypoxic condition. Even though several genes, that are modulated by HIF, have been described in numerous cell lines undergoing hypoxia [85], a large-scale analysis of changes occurring in the protein profile of hypoxic normal human fibroblasts is still absent.

Fibroblasts, one of the most abundant cells of connective tissues, are responsible for protein synthesis and turnover and produce factors possibly modulating other cell types [86-89].

A better comprehension of the pathways affected in human fibroblasts upon mild chronic hypoxia exposure may get further light on the intercellular cross-talk and on tissue remodelling occurring in physiological and in pathological conditions [2,3].

Data indicate that mild chronic hypoxia modulates fibroblast protein profile by inducing significant changes in the expression of 56 proteins. The 35 identified proteins fell into 15 different functional categories, according to the GO annotation system, indicating that hypoxia might interfere with a broad range of functional activities such as transcriptional control, angiogenesis, matrix remodelling, stress response and energy metabolism.

In conclusion, although further studies are necessary in order to deeper examine each potentially activated pathway, this study clearly indicates that human dermal fibroblasts respond to a mild chronic hypoxic condition by modifying several proteins that might

influence the intercellular cross-talk occurring in several physiologic and pathologic conditions, as during embryogenesis, aging, wound healing and tumor progression.

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LEGEND TO FIGURES

Figure 1. HIF quantitation. Representative Western blot (a) showing the expression of HIF-1α, HIF-2α and β-actin in human dermal fibroblasts grown in normoxia (N) and hypoxia (H). Hypoxia significantly increased the protein accumulation of HIF-1α, but not HIF-2α. Data in panel b are mean values ± SD of densitometric evaluations of HIF-1α normalized to β-actin in Western blots made with three different cell lines. Three different experiments were performed. * p<0,05 Hypoxia vs Normoxia

Figure 2. 2DGE. Representative silver-stained 2-D electropherograms of the cell layer of human dermal fibroblasts cultured in normoxia (left) and hypoxia (right). Arrows and numbers denote the position of differentially expressed proteins.

Figure 3. Differentially expressed proteins. Changes in protein expression are represented in the chart according to the value of the expression factor (R) according to the equation $R=\log_2 (H/N)$. H is the % mean volume of the spot in hypoxia and N is the % mean volume of the spot in normoxia. Proteins that did not changed their expression have R=0. Proteins are identified by numbers, as in figure 2.

Figure 4. Functional classification. Distribution of all human identified protein isoforms, according to the Gene Ontology (GO) annotation system, was performed using the DAVID database bioinformatic resources (http://david.abcc.ncifcrf.gov). The percentage of upregulated/downregulated proteins in hypoxia is reported for each function category.

Figure 5. Western blots. Immunoblots were performed in order to validate changes in the expression of filamin (FLN), protein disulfide isomerase (PDI), heat shock protein 60

(HSP60), heat shock protein 27 (HSP27), enolase 1 (ENO1), calmodulin (CALM), galectin 1 (LEG1), thioredoxin (TRX) in normoxia (N) and hypoxia (H). A quantitative representation of changes are visualized by histograms adjacent to immunoblots.

Figure 1

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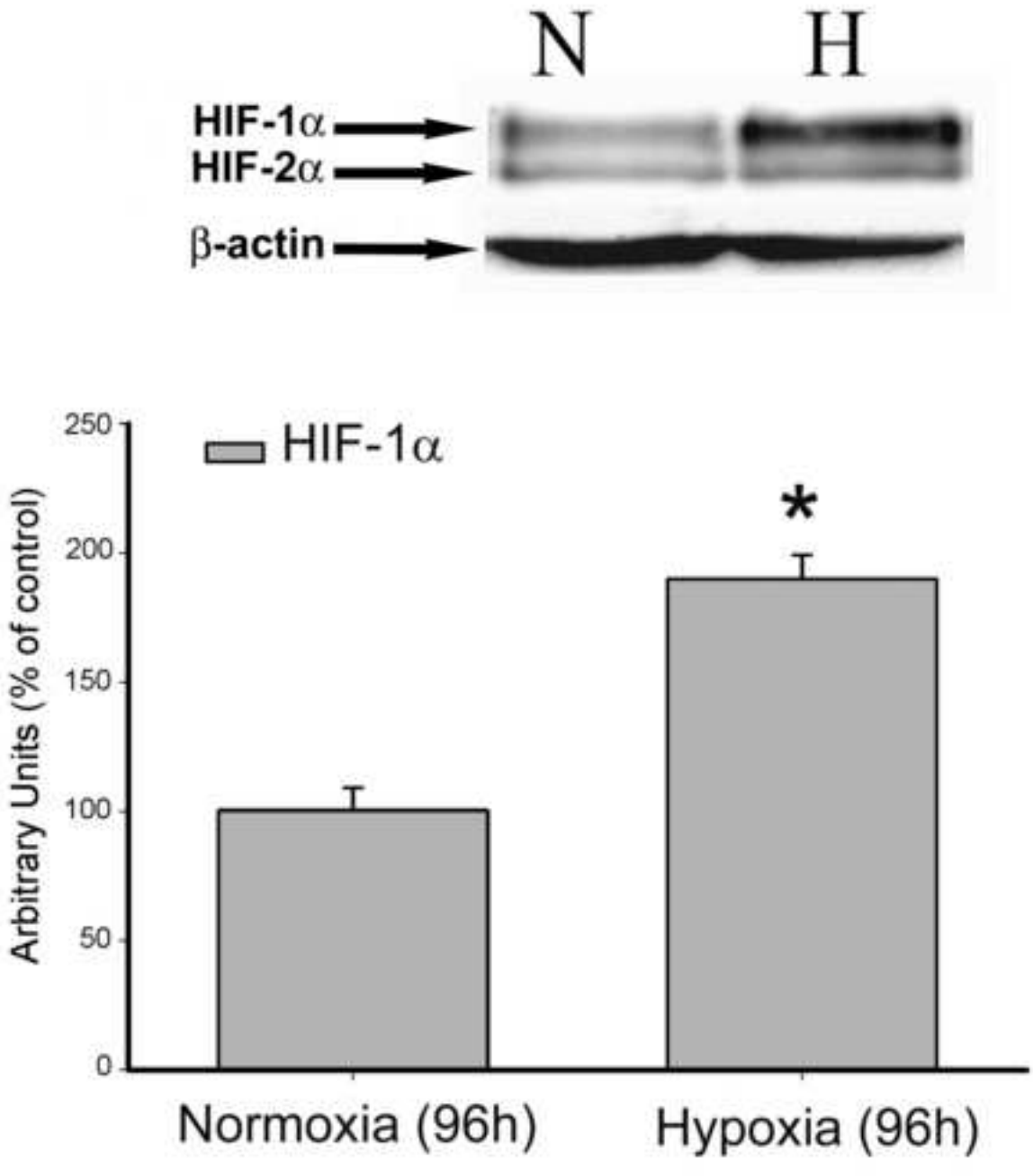


Figure 2

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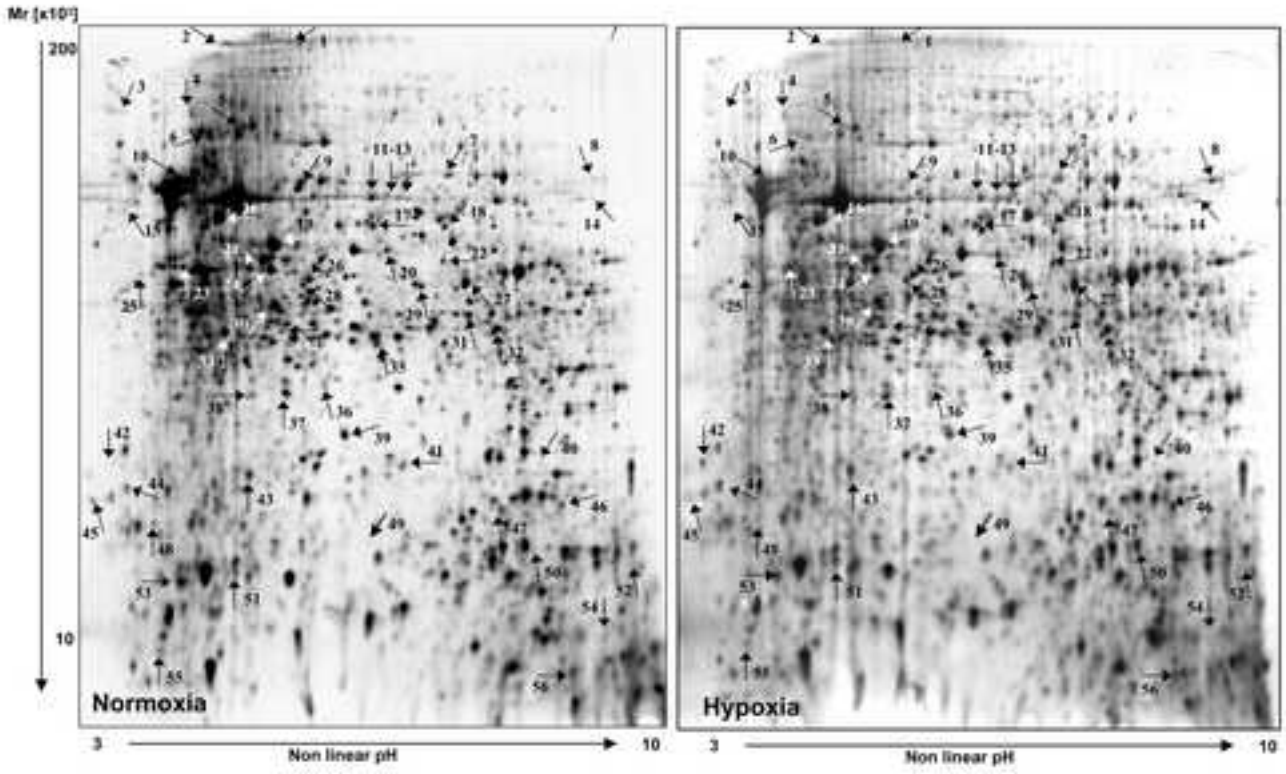


Figure 3 final revision

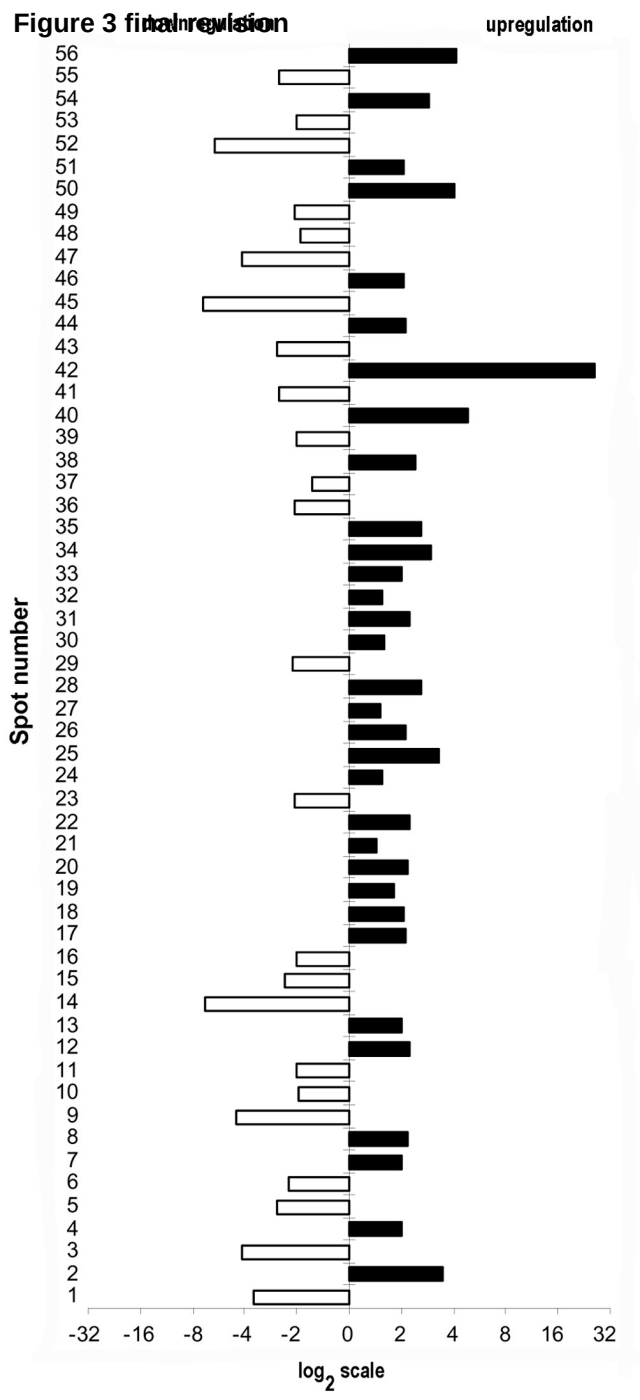


Figure 4

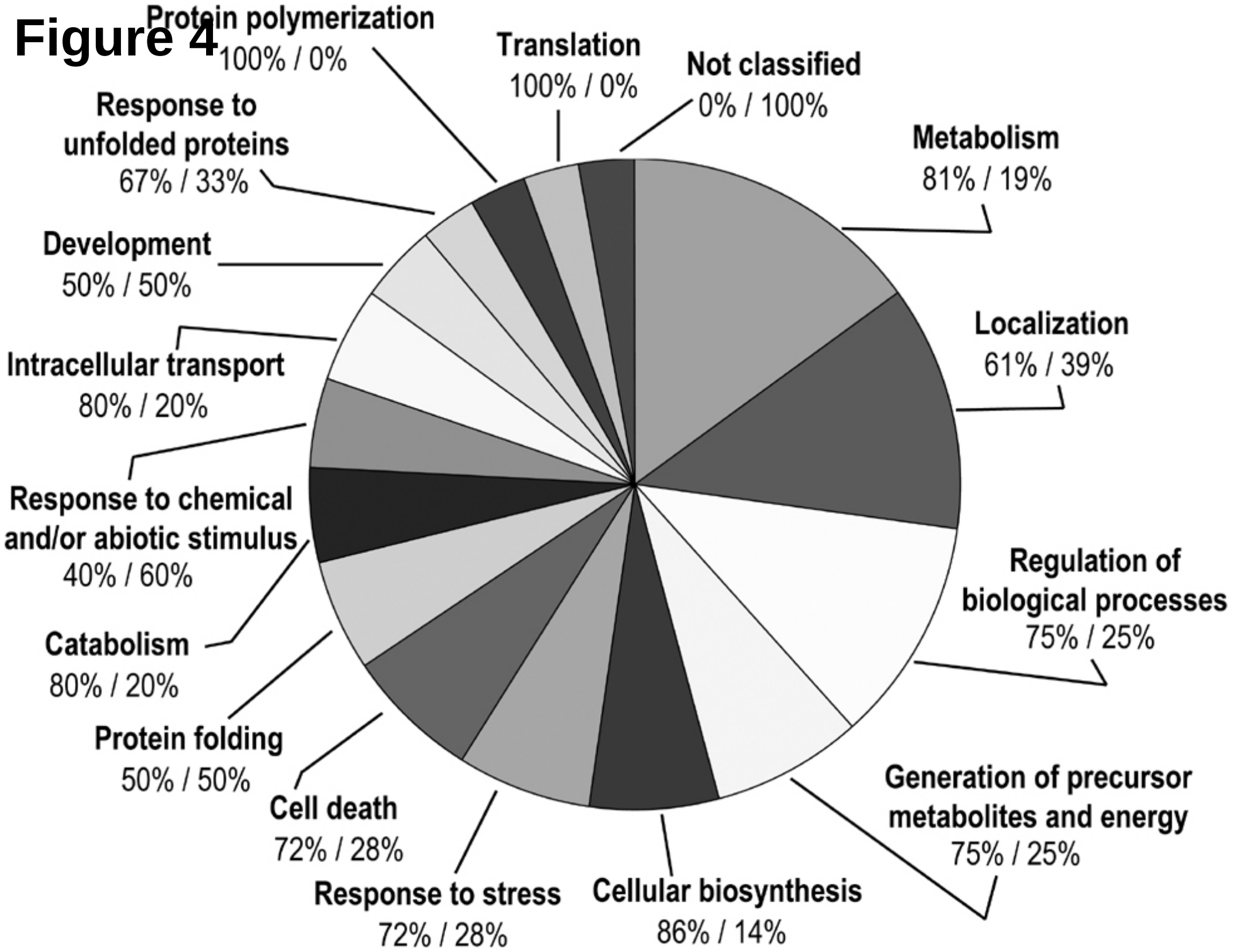

Protein polymerization
100% / 0%
Translation
100% / 0%
Not classified
0% / 100%
Response to unfolded proteins
67% / 33%
Metabolism
81% / 19%
Development
50% / 50%
Localization
61% / 39%
Intracellular transport
80% / 20%
Response to chemical and/or abiotic stimulus
40% / 60%
Regulation of biological processes
75% / 25%
Catabolism
80% / 20%
Protein folding
50% / 50%
Generation of precursor metabolites and energy
75% / 25%
Cell death
72% / 28%
Response to stress
72% / 28%
Cellular biosynthesis
86% / 14%

Figure 5

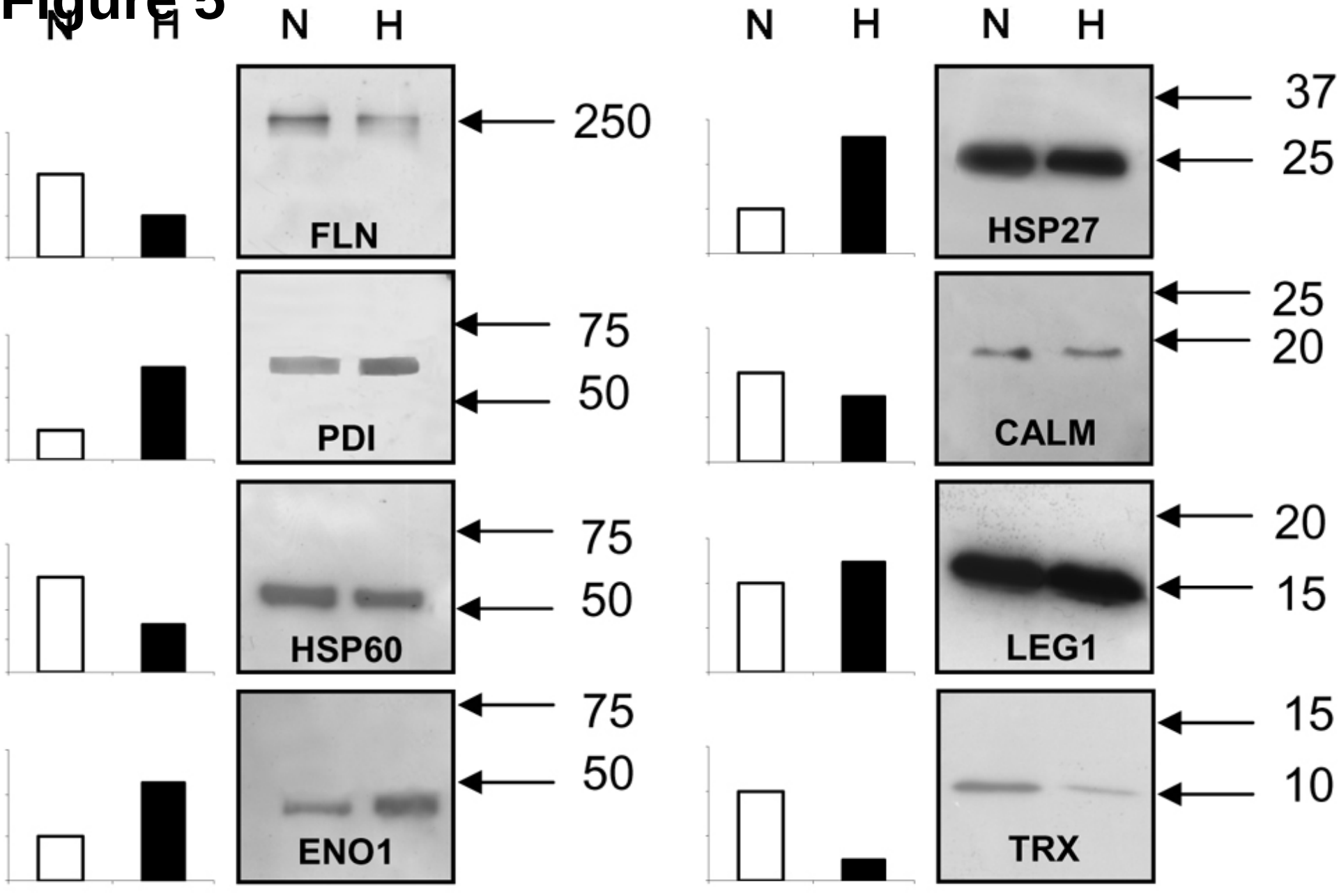

N
H
N
H
250
FLN
75
50
PDI
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HSP60
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ENO1
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N
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37
25
HSP27
25
20
CALM
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LEG1
15
10
TRX

TABLE 1

List of identified proteins whose expression significantly (p<0.05 Normoxia vs Hypoxia) changed upon hypoxia

# [a)]	Name and acc. no. [b)]	pI/ MW [c)]	Identification method [d)]
1	**Filamin C** Q14315	5.4/ 200	MS/MS – 161; 6; 3%
2	**Filamin C** Q14315	5.2/ 200	PMF - 7.44e+011; 26/32; 11%
3	**Fetuin-A** P12763	3.8/ 90	MS/MS – 225; 4; 19%
4	**150 kDa oxygen-regulated protein** Q9Y4L1	4.8/ 105	MS/MS –68; 3; 5%
5	**HSP 60** P10809	5.4/ 65	PMF - 6.58e+004; 10/18; 24%
7	**Lamin A/C** P02545	6.7/ 56	PMF - 5.07e+004; 12/14; 20%
8	**Elongation factor 1-alpha 1** P68104	9.0/ 50	MS/MS – 192; 5; 22%
9	**NDRG1 protein** Q92597	5.4/ 45	MS/MS – 38; 1, 3%
11	**Actin 1** P60709/ **Actin 2** P63261	6.4/ 42	PMF - 1.91e+004; 7/8; 22%
16a + 16b	**Actin1/2** P60709/P63261 + **Protein disulfide-isomerase** P07237	4.8/ 41	PMF - 1.59e+08; 14/ 36; 53% + PMF - 3.37e+003; 9/36; 19%
17	**Alpha enolase** P06733	6.4/ 40	PMF - 1.13e+007; 9/11; 28%
19	**Tubulin alpha-3 chain** Q71U36	5.0/ 40	MS/MS –505; 8; 27%
21	**Tubulin beta-2 chain** P07437	4.7/ 38	MS/MS – 610; 10; 35%
23	**Tropomyosin 1 alpha chain** P09493	4.7/ 37	PMF - 3.95e+006; 11/25; 40%
25	**40S ribosomal protein SA** P08865	4.0/ 36	MS/MS – 113; 2; 12%
26	**Alpha enolase** P06733	5.6/ 36	MS/MS – 651; 14; 48%
27	**Annexin A2** P07355	7.0/ 35	PMF - 1.51e+010; 17/24; 58%
28	**Protein disulfide isomerase A3** P30101	5.4/ 30	MS/MS – 189; 4; 11%
30	**Actin 1** P60709/ **actin 2** P63261	5.0/ 28	PMF - 7.2e+004; 6/20; 20%
31	**Heat shock cognate 71kDa protein** P11142	6.8/ 28	PMF - 2.93e+006; 13/18; 26%
32	**Triosephosphate isomerase** P60174	7.2/ 26	PMF - 4.13e+007; 13/18;65%
33	**Annexin A1** P04083	4.8/ 26	PMF - 7.63e+009; 15/22; 57%
35	**HSP 27** P04792	6.4/ 24	PMF - 5.25e+004; 8/10; 43%
36	**Pyruvate kinase M1/M2 isozyme** 66910342	5.9/ 22	MS/MS – 171; 4; 24%

37	**Peroxiredoxin 2** P32119	5.3/ 22	PMF - 1.07e+005; 9/18; 44%
39	**HSP 90-beta** P08238	6.0/ 20	MS/MS – 130; 4; 7%
41	**Lamin A protein** 386856	5.3/ 19	MS/MS – 84; 2; 6%
42	**Parathymosin** P20962	3.7/ 18	MS/MS – 71; 1; 15%
43	**T-complex protein 1 subunit beta** P78371	5.1/ 17	MS/MS – 120; 4; 12%
44	**Tubulin beta 2 chain** 38511503	3.8/ 16	MS/MS – 210; 5; 56%
45	**Calmodulin 1** P62158	3.6/ 16	MS/MS – 115; 3; 43%
46	**Transgelin** Q01995	8.8/ 15	PMF - 3.96e+004; 7/13; 41%
49	**Prolyl 4-hydroxylase alpha-1 subunit precursor** 190786	6.3/ 16	MS/MS – 243; 5; 7%
50	**Pyruvate kinase M1/M2 isozyme** Q8WUW7	7.2/ 15	MS/MS – 204; 4; 14%
51	**Galectin 1** P09382	5.1/ 14	PMF - 6.15e+0.003; 5/11; 43%
53	**Thioredoxin** P10599	4.7/ 11	MS/MS – 97; 3; 32%

Detailed protein identification data are available from Table 1 supplementary material

a) Protein number as reported on gels in figure 2
b) Protein name and accession number according to Swiss_Prot/TrEMBL and NCBI databases
c) Experimental p*I* and M*r* (kDa)
d) For **MS/MS** sequencing the following parameters are given: total score (i.e. the sum of scores of all peptides), number of matched peptides; % coverage
For **PMF** the following parameters are given: Mowse score; number of matched peptides/ total peptides; % coverage

TABLE 2
Distribution of identified human proteins into functional categories according to the GO annotation system (http://david.abcc.ncifcrf.gov).

Category	Function	Proteins and protein isoforms [a)]
Macromolecule metabolism	The chemical reactions and pathways involving macromolecules, large molecules including proteins, nucleic acids and carbohydrates.	4, 5, 8, 16b, 17, 19, 21, 25, 26, 31, 32, 35, 36, 42, 44, 49, 50
Regulation of biological process	Any process that modulates the frequency, rate or extent of a biological process. Biological processes are regulated by many means; examples include the control of gene expression, protein modification or interaction with a protein or substrate molecule.	5, 8, 17, 21, 23, 25, 26, 33, 35, 37, 42, 44, 51
Localization	The processes by which a cell, a substance or a cellule entity, such as a protein complex or organelle, is transported to, and/or maintained in a specific location.	5, 11, 16a, 16b, 19, 21, 23, 28, 30, 33, 35, 44, 53
Generation of precursor metabolites and energy	The chemical reactions and pathways resulting in the formation of precursor metabolites, substances from which energy is derived and the processes involved in the liberation of energy from these substances.	4, 16b, 17, 26, 28, 32, 36, 50, 53
Cellular biosynthesis	The chemical reactions and pathways resulting in the formation of substances carried out by individual cells.	8, 17, 25, 26, 32, 35, 36, 50
Response to stress	A change in the state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a stimulus indicating the organism is under stress. The stress is usually, but not necessarily, exogenous (e.g. temperature, humidity, ionizing radiation).	4, 5, 31, 33, 35, 37, 42
Cell death	The specific activation or halting of processes within a cell so that its vital functions markedly ceases, rather than simply deteriorating gradually over time, which culminates in cell death.	5, 21, 33, 35, 37, 44, 51
Catabolism	The chemical reactions and pathways resulting in the breakdown of substances, including the breakdown of carbon compounds with the liberation of energy for use by the cell or organism.	4, 17, 26, 32, 36, 50
Protein folding	The process of assisting in the covalent and noncovalent assembly of single chain polypeptides or multisubunit complexes into the correct tertiary structure.	4, 5, 31, 35, 39, 43
Response to chemical and/or abiotic stimulus	A change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc) as a result of a chemical and/or abiotic stimulus.	5, 9, 31, 35, 37
Intracellular transport	The directed movement of substances within a cell.	5, 19, 21, 28, 44
Development	A biological process whose specific outcome is the progression of an integrated living unit: a cell, tissue, organ, or organism over time from an initial condition to a later condition.	7, 27, 41, 46
Response to unfolded proteins	A change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of an unfolded protein stimulus.	5, 31, 35
Protein polymerization	The process creating protein polymers, compounds composed of a large number of component monomers; polymeric proteins may be made up of different or identical monomers. Polymerization occurs by the addition of extra monomers to an existing poly-or oligomeric protein.	19, 21, 44
Translation	A ribosome-mediated process in which the information in messenger RNA (mRNA) is used to specify the sequence of amino acids in a polypeptide chain.	8, 25, 35
Not classified		1, 2, 45

a) proteins present in each functional category are indicated by the corresponding number on gels.

Supplementary Material
Click here to download Supplementary Material (for online publication): Table 1 Supplementary material revised.doc

TABLE 1S

List of identified proteins whose expression significantly changed (p<0.05) upon hypoxia

# [a)]	**Name and acc. no.** [b)]	**pI/ MW** [c)]	**Identification method** For **MS/MS** sequencing: % coverage, peptide sequence, ion score, charge state For **PMF**: Mowse score; % coverage and peptide sequence matched are given M* - Methionine oxidation
1	**Filamin C** Q14315	5.4/ 200	MS/MS – 3% R.AFGPGLEGGLVNK (ion score 33) (+2) R.APLQVAVLGPTGVAEPVEVR (ion score 22) (+2) R.EVTTEFTVDAR (ion score 22) (+2) R.GAGGQGQLDVR (ion score 34) (+2) R.SLTATGGNHVTAR (ion score 20) (+2) R.SPFEVQVSPEAGVQK (ion score 30) (+2)
2	**Filamin C** Q14315	5.2/ 200	PMF - 7.44e+011; 11% (K) LKPGAPVR(S) (K) VVPNNDKDR(T) (K) VTVLFAGQNIER(S) (R) DTVEVALEDKGDSTFR(C) (K) GTEEPVKVR(E) (R) SPFEVQVSPEAGVQK(V) (R) VNVGEGSHPER(V) (K) YTPPGAGR(Y) (K) VAVGQEQAFSVNTR(G) (R) GAGGQGQLDVR(M) (R) MTSPSRRPIPCK(L) (K) ATIRPVFDPSK(V) (R) ASGPGLER(G) (K) VSGPGVEPHGVLR(E) (R) EVTTEFTVDAR(S) (R) SLTATGGNHVTAR(V) (K) TDTYVTDNGDGTYR(V) (R) FTVETR(G) (R) APLQVAVLGPTGVAEPVEVR(D) (K) YADQEVPR(S) (R) ASGPGLNASGIPASLPVEFTIDAR(D) (K) YGGDEIPYSPFR(I) (R) IQIGQETVITVDAK(A) (K) YVITIR(F) (R) FDDKHIPGSPFTAK(I) (K) ITESDLSQLTASIR(A)
3	**Fetuin-A** P12763	3.8/ 90	MS/MS -19% K.HTLNQIDSVK.V (ions score 58) (+2) K.CDSSPDSAEDVR.K (ions score 82) (+2) R.HTFSGVASVESSSGEAFHVGK.T (ions score 21) (+3) K.TPIVGQPSIPGGPVR.L (ions score 64) (+2)
4	**150 kDa oxygen-regulated protein** Q9Y4L1	4.8/ 105	MS/MS – 5% K.LGNTISSLFGGGTTPDAK.E (ions score 29) (+2) K.AEAGPEGVAPAPEGEKK.Q (ions score 20) (+3) K.AANSLEAFIFETQDK.L (ions score 19) (+2)
5	**HSP 60** P10809	5.4/ 65	PMF - 6.58e+004; 24% (K) LVQDVANNTNEEAGDGTTTATVLAR(S) (K) GANPVEIRR(G) (K) ISSIQSIVPALEIANAHR(K) (R) KPLVIIAEDVDGEALSTLVLNR(L) (K) APGFGDNRK(N) (K) AQIEKR(I) (R) IQEIIEQLDVTTSEYEKEK(L) (R) VTDALNATR(A)

			(R) AAVEEGIVLGGGCALLR(C) (K) IGIEIIKR(T)
7	**Lamin A/C** P02545	6.7/ 56	PMF - 5.07e+004; 20% (R) SGAQASSTPLSPTR(I) (R) LQEKEDLQELNDR(L) (R) LAVYIDR(V) (R) SLETENAGLR(L) (K) EAALSTALSEKR(T) (R) TLEGELHDLR(G) (K) NIYSEELRETK(R) (R) LADALQELR(A) (R) NSNLVGAAHEELQQSR(I) (R) IRIDSLSAQLSQLQK(Q) (K) LRDLEDSLAR(E) (K) LALDMEIHAYR(K)
8	**Elongation factor 1-alpha 1** P68104	9.0/ 50	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) (+3) R.QTVAVGVIK.A (ions score 26) (+2)
9	**NDRG1 protein** Q92597	5.4/ 45	MS/MS – 3% R.TASGSSVTSLDGTR.S (ions score 38) (+2)
11	**Actin 1/ Actin 2** P60709/ P63261	6.4/ 42	PMF - 1.91e+004; 22% (K) AGFAGDDAPR(A) (R) AVFPSIVGRPR(H) (R) VAPEEHPVLLTEAPLNPK(A) (R) GYSFTTTAER(E) (K) SYELPDGQVITIGNER(F) (K) IIAPPER(K) (K) QEYDESGPSIVHR(K)
16a + 16b	**Actin1/2 + Protein disulfide-isomerase** P60709/ P63261 + P07237	4.8/ 41	PMF - 1.59e+08; 53% (-) DDDIAALVVDNGSGMCK(A) (K) AGFAGDDAPR(A) (R) AVFPSIVGRPR(H) (K) RGILTLK(Y) (R) VAPEEHPVLLTEAPLNPK(A) (R) TTGIVM*DSGDGVTHTVPIYEGYALPHAILR(L) (R) LDLAGRDLTDYLMK(I) (R) GYSFTTTAEREIVR(D) (R) DLTDYLMK(I) (K) LCYVALDFEQEMATAASSSSLEK(S) (K) SYELPDGQVITIGNER(F) (K) CDVDIRK(D) (K) DLYANTVLSGGTTMYPGIADR(M) (K) IIAPPERK(Y) + PMF - 3.37e+003; 19% (K) LKAEGSEIR(L) (K) VDATEESDLAQQYGVR(G) (K) YQLDKDGVVLFK(K) (K) KFDEGR(N) (K) HNQLPLVIEFTEQTAPK(I) (K) IFGGEIK(T) (K) ILFIFIDSDHTDNQR(I) (K) KEECPAVR(L) (R) ITEFCHR(F)
17	**Alpha enolase** P06733	6.4/ 40	PMF - 1.13e+007; 28% (R) EIFDSR(G) (R) GNPTVEVDLFTSK(G) (R) AAVPSGASTGIYEALELR(D)

			(K) FGANAILGVSLAVCK(A) (K) DATNVGDEGGFAPNILENK(E) (K) VVIGMDVAASEFFR(S) (R) YISPDQLADLYK(S) (K) FTASAGIQVVGDDLTVTNPK(R) (R) IEEELGSK(A)
19	**Tubulin alpha-3 chain** Q71U36	5.0/ 40	MS/MS – 27% K.TIGGGDDSFNTFFSETGAGK.H (ions score 96) (+2) R.AVFVDLEPTVIDEVR.T (ions score 85) (+2) R.QLFHPEQLITGKEDAANNYAR.G (ions score 68) (+3) K.EIIDLVLDR.I (ions score 45) (+2) R.LSVDYGK.K (ions score 20) (+2) R.NLDIERPTYTNLNR.L (ions score 36) (+2) R.LIGQIVSSITASLR.F (ions score 64) (+2) R.IHFPLATYAPVISAEK.A (ions score 91) (+2)
21	**Tubulin beta-2 chain** P07437	4.7/ 38	MS/MS – 35% -.M*REIVHIQAGQCGNQIGAK.F (ions score 26) (+3) R.ISVYYNEATGGK.Y (ions score 76) (+2) R.AILVDLEPGTMDSVR.S (ions score 51) (+2) R.SGPFGQIFRPDNFVFGQSGAGNNWAK.G (ions score 62) (+3) K.GHYTEGAELVDSVLDVVR.K (ions score 77) (+3) R.IM*NTFSVVPSPK.V (ions score 86) (+2) R.FPGQLNADLR.K (ions score 66) (+2) K.LAVNM*VPFPR.L (ions score 43) (+2) R.LHFFMPGFAPLTSR.G (ions score 65) (+3) R.ALTVPELTQQVFDAK.N (ions score 58) (+2)
23	**Tropomyosin 1 alpha chain** P09493	4.7/ 37	PMF - 3.95e+006; 40% (K) QLEDELVSLQK(K) (K) ATDAEADVASLNR(R) (R) IQLVEEELDR(A) (R) IQLVEEELDRAQER(L) (R) LATALQKLEEAEK(A) (K) LEEAEKAADESER(G) (R) AQKDEEKMEIQEIQLK(E) (K) HIAEDADR(K) (K) YEEVARK(L) (K) LVIIESDLER(A) (R) AEFAER(S)
25	**40S ribosomal protein SA** P08865	4.0/ 36	MS/MS – 12% R.AIVAIENPADVSVISSR.N (ions score 56) (+2) K.FAAATGATPIAGR.F (ions score 57) (+2)
26	**Alpha enolase** P06733	5.6/ 36	MS/MS – 48% R.AAVPSGASTGIYEALELR.D (ions score 36) (+3) K.TIAPALVSKK.L (ions score 36) (+2) K.LMIEMDGTENK.S (ions score 58) (+2) K.FGANAILGVSLAVCK.A (ions score 41) (+2) K.GVPLYR.H (ions score 23) (+2) R.HIADLAGNSEVILPVPAFNVINGGSHAGNK.L (ions score 62) (+4) K.LAMQEFMILPVGAANFR.E (ions score 36) (+2) R.IGAEVYHNLK.N (ions score 49) (+2) K.DATNVGDEGGFAPNILENK.E (ions score 63) (+2) K.EGLELLK.T (ions score 40) (+2) K.VVIGMDVAASEFFR.S (ions score 61) (+2) R.SGKYDLDFK.S (ions score 39) (+2) R.YISPDQLADLYK.S (ions score 53) (+2) K.FTASAGIQVVGDDLTVTNPK.R (ions score 54) (+2)
27	**Annexin A2** P07355	7.0/ 35	PMF - 1.51e+010; 58% (-)STVHEILCK(L) (K) AYTNFDAER(D) (K) TKGVDEVTIVNILTNR(S) (R) QDIAFAYQR(R)

			(K) SALSGHLETVILGLLK(T) (K) TPAQYDASELK(A) (K) GLGTDEDSLIEIICSR(T) (R) TNQELQEINR(V) (K) TDLEKDIISDTSGDFR(K) (K) LMVALAK(G) (R) AEDGSVIDYELIDQDAR(D) (R) DLYDAGVK(R) (K)WISIMTER(S) (K) VFDRYK(S) (K) SYSPYDMLESIR(K) (R) LYDSMK(G) (R) DKVLIR(I)
28	**Protein disulfide isomerase A3** P30101	5.4/ 30	MS/MS – 11% R.LAPEYEAAATR.L (ions score 69) (+2) K.YGVSGYPTLK.I (ions score 45) (+2) R.DGEEAGAYDGPR.T (ions score 52) (+2) R.TADGIVSHLKK.Q (ions score 23) (+3)
30	**Actin 1/ actin 2** P60709/ P63261	5.0/ 28	PMF - 7.2e+004; 20% (R) GYSFTTTAER(E) (K) SYELPDGQVITIGNER(F) (K) CDVDIRK(D) (K) DLYANTVLSGGTTMYPGIADR(M) (K) IIAPPER(K) (K) QEYDESGPSIVHR(K)
31	**Heat shock cognate 71kDa protein** P11142	6.8/ 28	PMF - 2.93e+006; 26% (K) GPAVGIDLGTTYSCVGVFQHGK(V) (K) VEIIANDQGNR(T) (R) TTPSYVAFTDTER(L) (K) NQVAMNPTNTVFDAK(R) (K) VQVEYKGETK(S) (K) MKEIAEAYLGK(T) (K) TVTNAVVTVPAYFNDSQR(Q) (K) DAGTIAGLNVLR(I) (R) IINEPTAAAIAYGLDKK(V) (K) STAGDTHLGGEDFDNR(M) (R) MVNHFIAEFK(R) (K) DISENKR(A) (R) GTLDPVEKALR(D)
32	**Triosephosphate isomerase** P60174	7.2/ 26	PMF - 4.13e+007; 65% (K) VPADTEVVCAPPTAYIDFAR(Q) (R) QKLDPK(I) (K) IAVAAQNCYK(V) (K) DCGATWVVLGHSER(R) (R) RHVFGESDELIGQK(V) (K) VAHALAEGLGVIACIGEK(L) (K) LDEREAGITEK(V) (K) VVFEQTK(V) (K) VIADNVKDWSK(V) (K) VVLAYEPVWAIGTGK(T) (K) TATPQQAQEVHEK(L) (K) SNVSDAVAQSTR(I) (R) IIYGGSVTGATCK(E)
33	**Annexin A1** P04083	4.8/ 26	PMF - 7.63e+009; 57% (-)AMVSEFLK(Q) (K) QAWFIENEEQEYVQTVK(S) (K) GGPGSAVSPYPTFNPSSDVAALHK(A) (K) GVDEATIIDILTK(R) (K) RNNAQR(Q) (K) AAYLQETGKPLDETLKK(A) (K) ALTGHLEEVVLALLK(T)

			(K) TPAQFDADELR(A) (K) GLGTDEDTLIEILASR(T) (R) TNKEIR(D) (R) DLAKDITSDTSGDFR(N) (R) SEDFGVNEDLADSDAR(A) (R) ALYEAGER(R) (K) GTDVNVFNTILTTR(S) (R) SYPQLR(R)
35	**HSP 27** P04792	6.4/ 24	PMF - 5.25e+004; 43% (R) VPFSLLR(G) (R) LFDQAFGLPR(L) (R) QLSSGVSEIR(H) (R) HTADRWR(V) (R) VSLDVNHFAPDELTVK(T) (K) DGVVEITGKHEER(Q) (R) QDEHGYISR(C) (K) LATQSNEITIPVTFESR(A)
36	**Pyruvate kinase M1/M2 isozyme** 66910342	5.9/ 22	MS/MS – 24% R.TATESFASDPILYRPVAVALDTK.G (ions score 27) (+3) K.ITLDNAYMEK.C (ions score 20) (+2) K.IYVDDGLISLQVK.Q (ions score 82) (+2) K.GADFLVTEVENGGSLGSK.K (ions score 42) (+2)
37	**Peroxiredoxin 2** P32119	5.3/ 22	PMF - 1.07e+005; 44% (R) IGKPAPDFK(A) (K) ATAVVDGAFKEVK(L) (R) AEDFRK(L) (K) EGGLGPLNIPLLADVTR(R) (R) LSEDYGVLK(T) (K) TDEGIAYR(G) (R) GLFIIDGK(G) (R) QITVNDLPVGR(S) (R) SVDEALR(L)
39	**HSP 90-beta** P08238	6.0/ 20	MS/MS – 7% K SIYYITGESK (ions score 36) (+3) K EQVANSAFVER (ions score 30) (+2) K.LGIHEDSTNR (ions score 34) (+2) R YHTSQSGDEMTSLSEYVSR (ions score 30) (+3)
41	**Lamin A protein** 386856	5.3/ 19	MS/MS – 6% R.TALINSTGEEVAM*R.K (Ions score 26) (+2) R.SVGGSGGGSFGDNLVTR.S (Ions score 58) (+2)
42	**Parathymosin** P20962	3.7/ 18	MS/MS – 15% -.SEKSVEAAAELSAK.D (ions score 71) (+2)
43	**T-complex protein 1 subunit beta** P78371	5.1/ 17	MS/MS – 12% R DASLMVTNDGATILK (ions score 28) (+2) R EALLSSAVDHGSDEVK (ions score 41) (+2) R QDLMNIAGTTLSSK (ions score 21) (+2) R VQDDEVGDGTTSVTVLAAELLR (ions score 30) (+3)
44	**Tubulin beta 2 chain** 38511503	3.8/ 16	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)
45	**Calmodulin 1** P62158	3.6/ 16	MS/MS – 43% K.DGNGFISAAEL.R (score ione 35) (+2) K.EAFSLFDKDGDGTITT.K (score ione 40) (+2) K.DTDSEEEI.R (score ione 40) (+2)
46	**Transgelin** Q01995	8.8/ 15	PMF - 3.96e+004; 41% (K) YDEELEER(L) (R) LVEWIIVQCGPDVGRPDR(G) (K) QMEQVAQFLK(A)

			(K) TDMFQTVDLFEGKDMAAVQR(T) (K) NDGHYR(G) (K) AQEHKR(E) (R) GASQAGMTGYGRPR(Q)
49	**Prolyl 4-hydroxylase alpha-1 subunit precursor** 190786	6.3/ 16	MS/MS – 7% R.LQDTYNLDTDTISKGNLP.G (ions score 80) (+2) K.SFLTAEDCFELGK.V (ions score 31) (+2) K.LLELDPEHQR.A (ions score 49) (+3) K.GVAVDYLPER.Q (ions score 49) (+2) R.QKYEMLCR.G (ions score 34) (+2)
50	**Pyruvate kinase M1/M2 isozyme** Q8WUW7	7.2/ 15	MS/MS – 14% K.GDYPLEAVR.M (ions score 46) (+2) R.MQHLIAR.E (ions score 25) (+2) R.LAPITSDPTEATAVGAVEASFK.C (ions score 68) (+2) K.CCSGAIIVLTK.S (ions score 65) (+2)
51	**Galectin 1** P09382	5.1/ 14	PMF - 6.15e+0.003; 43% (-)ACGLVASNLNLKPGECLR(V) (R) VRGEVAPDAK(S) (K) DSNNLCLHFNPR(F) (K) DGGAWGTEQR(E) (K) LPDGYEFK(F)
53	**Thioredoxin** P10599	4.7/ 11	MS/MS – 32% TAFQEALDAAGDK (ions score 41) (+2) EKLEATINELV (ions score 30) (+2) VGEFSGANK (ions score 26) (+2)

a) Protein number as reported on gels in figure 2
b) Protein name and accession number according to Swiss_Prot/TrEMBL and NCBI databases
c) Experimental p*I* and M*r* (kDa)