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Short communication

# Identification of differentially expressed proteins in senescent human embryonic fibroblasts

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**Abstract**

Normal human fibroblasts undergo a limited number of divisions in culture, a process known as replicative senescence (RS). Although several senescence-specific genes have been identified, analysis at the level of protein expression can provide additional insights into the mechanisms that regulate RS. We have performed a proteomic comparison between young and replicative senescent human embryonic WI-38 fibroblasts and we have identified 13 proteins, which are differentially expressed in senescent cells. Some of the identified proteins are components of the cellular cytoskeleton, while others are implicated in key cellular functions including metabolism and energy production, $Ca^{2+}$ signalling, nucleo-cytoplasmic trafficking and telomerase activity regulation. In summary, our analysis contributes to the list of senescence-associated proteins by identifying new biomarkers and provides novel information on functional protein networks that are perturbed during replicative senescence of human fibroblast cultures.


*Keywords:* 2D PAGE; Ageing; Human fibroblasts; Mass spectrometry; Proteomics; Replicative senescence

## 1. Introduction

Ageing is the outcome of complicated interactions between genetic factors and the accumulation of a variety of deleterious stochastic changes overtime (Kirkwood, 2002). Human ageing can be studied in vitro. Specifically, normal human fibroblasts undergo a limited number of divisions in culture and progressively reach a state of irreversible growth arrest, a process termed as replicative senescence (RS). Replicative senescence occurs because, owing to the biochemistry of DNA replication, cells acquire one or more critically short telomere (Holliday, 1996). Senescent cells have recently been shown to accumulate with age in human tissues and it, thus, has been proposed that they contribute to organismal ageing (Campisi, 2000). Moreover, senescent cells acquire phenotypic changes that may contribute to certain age-related diseases, including late-life cancer (Campisi, 2005).

Several genes that are linked to senescence have been successfully identified and cloned on the basis of mRNA expression changes between young and senescent cells (Gonos et al., 1998; Lee et al., 1999; Ly et al., 2000). However, these studies do not consider the weakness of the correlation between a given level of transcripts and the abundance of the corresponding proteins. It is well established that analysis at the level of protein expression can provide additional and complementary information. The development of proteomic analysis methods using high-resolution two-dimensional gel electrophoresis (2DGE) and mass-spectrometry (MS) offers the advantage of identifying directly changes at the protein level. Regarding senescence, this methodology has been successfully applied in identifying new biomarkers during stress-induced premature senescence (SIPS) in human diploid fibroblasts (HDFs) (Dierick et al., 2002), as well as in conditionally immortalized rat embryo fibroblasts (Benvenuti et al., 2002). In this study, we have performed a proteomic comparison between

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young and senescent human embryonic WI-38 fibroblasts and we have identified 13 differentially expressed proteins.

## 2. Materials and methods

### 2.1. Cell culture

Human diploid WI-38 fibroblasts were obtained from the European Collection of Cell Cultures and were maintained in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Inc.), supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% non-essential amino acids. Proliferating WI-38 fibroblasts were sub-cultured at a split ratio 1:2 when they were confluent until they reached senescence at about 45 cell population doublings (CPD) (Petropoulou et al., 2001). In all experimental procedures, cells were fed approximately 16 h prior to the assay.

### 2.2. Sample preparation, 2DGE

Triplicates of WI-38 cell cultures at CPD21 or CPD45 were rinsed with ice-cold PBS and then lysed in 2% CHAPS, 15 mg/ml DTT and 50 mM Tris pH 7.4. Treatment with benzonase at 37 °C for 1 h removed interfering nucleic acids from cell extracts and proteins were further purified by acetone precipitation at −20 °C for 3 h. The protein pellet was finally re-suspended in 350 μl rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 10 mg/ml DTT, 2% IPG buffer, 0.01% bromophenol blue) and after the removal of any insoluble material, samples were loaded on immobilised pH gradient strips (pH 3–10 non-linear, Pharmacia Biotech). Isoelectric focusing on a MultiPhor II apparatus (Pharmacia Biotech) and preparation of the IPG strips for the second dimension were carried as per manufacturer's instructions. After the isoelectric focusing, the IPG strips were embedded on 12% polyacrylamide gel slabs for the second dimension run according to the discontinuous buffer system of Laemmli.

### 2.3. In-gel tryptic digestion

Protein spots were visualised after staining of the gels with silver nitrate. Protein patterns in distinct gels were manually compared. We deliberately avoided the use of automated, software-driven procedures (e.g. the Melanie software), because in our experience they increase the number of artefacts and are useful only in high-throughput studies where manual inspection of a large number of gels is very difficult. We excluded features showing subtle changes in protein expression or those not repeated in all three independent experiments and, thus we selected only for those with a high level of confidence. An example is given in Fig. 1A. Spots which were senescence-specific, were detected and their expression levels (individual spot intensities) were compared from scanned gel images by using the software Image Quant (Molecular Dynamics).

Spots were then excised from gels and cut into small pieces. The gel particles were destained with acetonitrile and proteins were reduced with 10 mM DTT at 56 °C for 1 h and alkylated with 10 mg/ml iodoacetamide for 45 min in the dark before being subjected to digestion with 5 ng/μl trypsin overnight. The resulting peptides were eluted from gel particles following successive washes with 50% acetonitrile/5% formic acid and then concentrated by centrifugal lyophilisation that removed the organic solvent. The volume of the sample was adjusted to 10 μl with 0.1% formic acid and injected in the reversed-phase nano-high performance liquid chromatography (nano-HPLC) system.

### 2.4. Nano-HPLC separation, nano-spray ion-trap mass spectrometer (NSI-MS) analysis

Nano-HPLC was performed for the separation of the tryptic peptides using the ultimate HPLC system (LC Packings) and a PepMap reversed phase C18 column (75 μm × 15 cm, LC Packings). The injected samples were eluted at 150 nl/min with a 5–80% (v/v) acetonitrile/water gradient containing 0.1% formic acid over 35 min. The separated tryptic fragments were visualised by detection of the absorbance at 214 nm and were introduced online into a LCQ Deca NSI-MS equipped with a nano-electrospray source (ThermoFinnigan). For the analysis of peptides, three scanning events were performed successively. During the first scanning event, the spectrum representing the mass to charge ratio ($m/z$) of all the ions detected was collected on a real-time basis. The mass of the most abundant ion was then calculated based on the data obtained by the second zoom scan and once the parent ion was identified, the mass spectrometer was set up to obtain the collision-induced dissociation MS–MS spectrum.

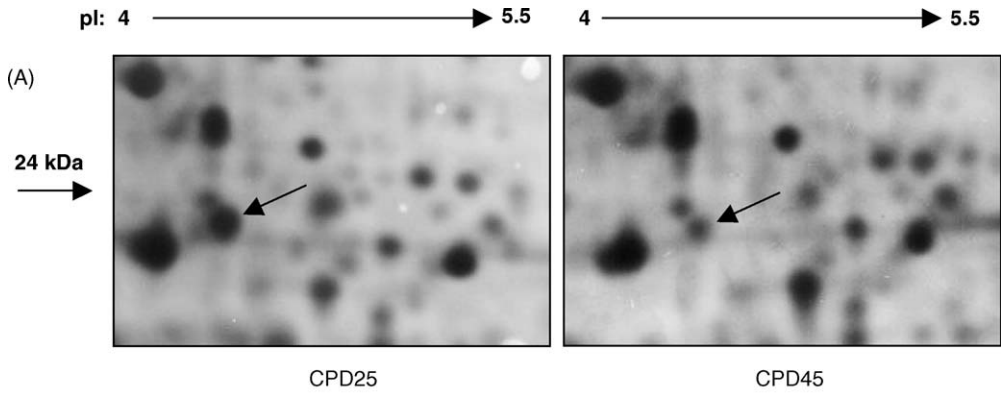
The data were collected using the Xcalibur software (Finnigan) and were subsequently used to search protein databases with the TurboSequest software. The following default score values were used as cut-off parameters during the search: $X_{corr} > 1.0, \Delta C_n > 0.1, S_p > 500, R_{sp} < 10$ and a peptide mass tolerance of 1.0. Following this initial filtering, all candidate peptides were further evaluated by manual inspection of the spectra and the Sequest parameters. The ions produced were checked for each peptide, with an acceptance limit of >60% coverage. The Xcorr values were also evaluated depending on the length of the peptide, since longer peptides produce greater numbers. Only peptides ending with a lysine or arginine residue were included. Finally, for each identified protein the predicted or published molecular weight was checked with that observed on the gels. An example of such analysis is given in Fig. 1B.

## 3. Results and discussion

We visualised approximately 1500 protein spots after gel staining with silver nitrate (not shown), of which 13 were senescence-specific. As shown in Table 1, eight proteins (α-enolase, β-actin, annexins I and VI, creatine kinase B chain, glutathione transferase omega-1, tubulin β-1 chain and vimentin) were consistently found up-regulated in senescent fibroblasts, whereas five proteins (RAN specific GTPase-activating protein, type II keratin subunit, telomerase binding protein p23, L-lactase dehydrogenase A chain and the ATP-dependent RNA helicase p47) were found to be down-regulated in all samples preparations. Few of these proteins have been linked previously to ageing and/or cellular senescence in various tissues and animal species. For instance, α-enolase, β-actin, annexin I and creatine kinase B chain were also isolated by Dierick et al. (2002) after a proteomic analysis of HDFs undergoing RS.

The observed differential up-regulation of the cytoskeletal proteins during RS, is in accordance with the changes in the cytoskeleton structure and cell morphology which accompany the senescence phenotype. More specifically, β-actin is known to play a role in the reorganization of the cytoskeleton in conditions generating stress fibres such as SIPS (Chen et al., 2000). Tubulins contribute to the formation of microtubules, which are known to be modified during cellular ageing (Raes, 1991). Vimentin has been directly linked to the senescence phenotype since its over-expression has been shown to induce a senescence-like morphology in human fibroblasts (Nishio et al., 2001). Finally, considering that the primary function of type I and type II keratins is to impact mechanical strength to cells (Kirfel et al., 2003), the observed down-regulation of the type II keratin subunit may be related to the fragility of the senescent cells.

Three proteins involved in energy production and/or metabolism were identified as senescence-specific. In particular, α-enolase, a highly conserved cytoplasmic glycolytic enzyme, has been linked to ageing of the heart of Fisher 344/Brown Norway F1 rats (Kanski et al., 2005). Interestingly, α-enolase is down-regulated during non-small cell lung cancer




(B)

| Seq | # | b′ | y′ | # |
|---|---|---|---|---|
| K | 1 | 129.2 | - | 10 |
| G | 2 | **186.2** | **1004.0** | 9 |
| E | 3 | 315.3 | **947.0** | 8 |
| S | 4 | 402.4 | **817.9** | 7 |
| G | 5 | **459.5** | **730.8** | 6 |
| Q | 6 | 587.6 | **673.7** | 5 |
| S | 7 | **674.7** | **545.6** | 4 |
| W | 8 | **860.9** | **458.5** | 3 |
| P | 9 | 958.0 | **272.3** | 2 |
| R | 10 | - | 175.2 | 1 |

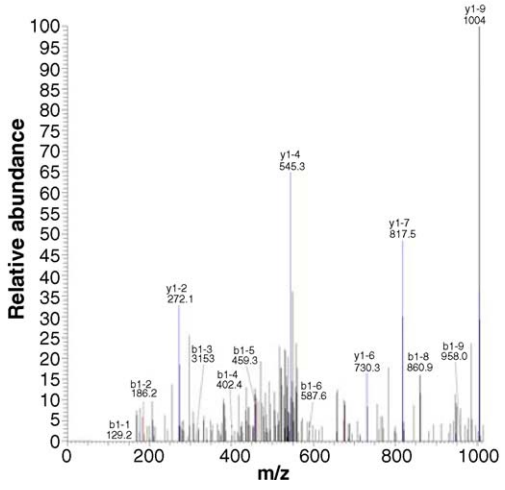



Fig. 1. (A) Details of representative 2D gels of CPD21 and CPD45 cells, showing a characteristic change in protein expression (arrowed). As is evident by comparing the intensity of the surrounding spots, the differences in the arrowed spots are very clear. Molecular weight is shown on the left, p*I* ranges are approximates since these are non-linear gels. (B) An example of b′ and y′ fragments identified by MS/MS analysis of a Telomerase Binding Protein peptide, the b′ and y′ fragments are peptide-bond fragments and are the most relevant in identifying the protein sequence. The table on the left panel depicts in bold type ions identified in the MS/MS scan, shown in the right panel.

Table 1
Proteins identified in the proteomic analysis of replicative senescent WI-38 human fibroblasts

| | Fold change[a] | ESI spot identification | Accession number |
|---|---|---|---|
| Up-regulated in senescence | | | |
| 1 | +++ | α-Enolase | P06733 |
| 2 | +++ | β-Actin | P02570 |
| 3 | ++ | Annexin I | P04083 |
| 4 | ++ | Creatine kinase B chain | P12277 |
| 5 | ++ | Glutathione transferase omega 1 | P78417 |
| 6 | ++ | Tubulin β-1 chain | P07437 |
| 7 | ++ | Vimentin | P08670 |
| 8 | + | Annexin VI | P08133 |
| Down-regulated in senescence | | | |
| 9 | +++ | RAN-specific-GTPase-activating protein | P43487 |
| 10 | +++ | Type II keratin subunit | AAA36153 |
| 11 | ++ | Telomerase-binding protein p23 | Q15185 |
| 12 | + | L-Lactate dehydrogenase A chain | P00338 |
| 13 | + | Probable ATP-dependent RNA helicase p47 | Q13838 |

[a] Estimate of the fold-difference in protein spot intensity; +++, >5-fold; ++, >2–5-fold; +, 2-fold.

and it has been suggested that it may function as a tumour suppressor (Chang et al., 2003). Creatine kinase isoenzymes play a central role in energy transduction in tissues with large, fluctuating energy demands, such as skeletal muscle, heart, brain and spermatozoa. Creatine kinase was found to be up-regulated after a proteome analysis in the brains of the aged senescence-accelerated-prone-mouse-strain 8 (SAMP8) (Poon et al., 2004). In support to our data, both α-enolase and creatine kinase B chain enzymes were found by Dierick et al. (2002) to be up-regulated in HDFs undergoing RS. L-Lactate dehydrogenase A chain (LDHA) is involved in anaerobic glycolysis. In recent studies, it has been found to be deregulated in cancer (Ishikawa et al., 2004), while its expression in human fibroblasts is suppressed by the catalytic subunit of telomerase (hTERT) and is induced by the Ataxia telangiectasia mutated (ATM) gene (Baross et al., 2004).

The function of glutathione-transferase omega-1 (GSTO1) is not well understood but recent data have associated GSTO1 with the Alzheimer and Parkinson diseases (Li et al., 2003) which are both related to advance ageing. ATP-dependent RNA helicase p47 protein encodes for a nuclear member of the DEAD protein family of ATP-dependent RNA helicases with unknown function (Peelman et al., 1995). The DEAD protein family has more than 40 members, including the eukaryotic translation initiation factor-4A (eIF-4A), the human nuclear protein p68 and the Drosophila oocyte polar granule component vasa (Peelman et al., 1995).

Annexins are a family of calcium and membrane-binding proteins that have been involved in diverse cellular functions including vesicle trafficking, cell division, apoptosis, growth regulation and calcium signalling (Hayes and Moss, 2004). Annexin I, which was found to be up-regulated during ageing in a proteome analysis of in vitro cultured fibroblasts from healthy subjects of different ages (Boraldi et al., 2003), has an anti-inflammatory effect and exerts profound inhibitory effects on both neutrophil and monocyte migration. It was also recently identified as one of the ''eat-me'' signals on apoptotic cells to be recognised and ingested by the phagocytes (Parente and Solito, 2004). This latter function is of particular interest, if one considers the necessity for removal of the senescent cells that gradually accumulate in a living organism during ageing (Krtolica and Campisi, 2002). Notably, annexin I was previously found to be down-regulated in senescent HDFs (Dierick et al., 2002). This discrepancy can be attributed to the complexity of the systems being analysed (i.e. whole proteomes), differences between independent clones of the same cell line as well as methodological variations.

Two other RS biomarkers, which are both involved in key cellular functions, are the RAN-specific GTPase-activating protein (RanGAP) and the telomerase-binding protein p23. RanGAP binds to the GTPase Ran and, along with RCC1 and RanBP1, regulate the action of this protein, which is a well known regulator of protein transport across the nuclear envelope (Steggerda and Paschal, 2002). In agreement with our data which suggest a reduced nucleo-cytoplasmic trafficking in senescent cells, other factors of the nucleo-cytoplasmic traffic system such as RanBP1 were found to be down-regulated during ageing in human dermal fibroblasts obtained from young, mature and old donors, both at the mRNA and protein level (Ly et al., 2000; Pujol et al., 2002). Considering that this down-regulation was accompanied by a reduction in protein import in fibroblasts derived from old donors (Pujol et al., 2002), it seems that diminished nucleo-cytoplasmic trafficking is a major characteristic of the RS phenotype. p23 is a molecular chaperone that along with other five subunits compose the telomerase complex. Blockade of the interaction between p23 and hTERT inhibits the assembly of active telomerase both in vitro and in vivo (Holt et al., 1999), while antisense treatment of the p23 mRNA results in decreased or abolished telomerase activity (Chang et al., 2002). Since telomerase activation represents a key event towards cellular immortalisation and eventual transformation (Rangarajan et al., 2004), p23 down-regulation in senescent cells may represent an additional blockade to the pro-oncogenic event of telomerase activation.

In conclusion, our analysis has identified novel biomarkers of RS, providing information on functional protein networks that are perturbed during RS of human fibroblast cultures.

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