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Abstract: Several studies have analyzed modulation of gene expression during physiological aging with interesting, but often contradictory results, depending on the model used. In the present report we compare age-related metabolic and synthetic parameters in human dermal fibroblasts (HDF) isolated from young and old subjects (ex-vivo ageing model) and cultured from early up to late cumulative population doublings (CPD) (in-vitro ageing model) in order to distinguish changes induced in vivo by the aged environment and maintained in vitro, from those associated with cell senescence and progressive CPD. Results demonstrate that fibroblasts from aged donors, already at early CPD, exhibit an impaired redox balance, highlighting the importance of this parameter during ageing, even in the presence of standard environmental conditions, which are considered optimal for cell growth. By contrast, several proteins, as those related to heat shock response, or involved in endoplasmic reticulum and membrane trafficking, appeared differentially expressed only during in-vitro ageing, suggesting that, at high CPD, the whole cell machinery becomes permanently altered. Finally, given the importance of the elastic component for a long-lasting connective tissue structural and functional compliance, this study focuses also on elastin and fibulin5 synthesis and deposition, demonstrating a close relationship between fibulin5 and ageing.

RESEARCH HIGHLIGHTS

a) the phenotype of human dermal fibroblasts is differently modulated in the ex-vivo and in the in-vitro ageing models
b) differences in fibroblast phenotype are mainly observed during in-vitro ageing, independently from donor's age
c) parameters of redox balance are markedly modified with in-vitro ageing and changes depend also on donor's age
d) extracellular elastin deposition is dramatically reduced with in vitro ageing and is associated to intracellular accumulation
e) fibulin-5 represents a very sensitive ageing marker and may be also involved in the redox balance.

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Comparison of ex-vivo and in-vitro human fibroblast ageing models

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Key words : aging, fibroblast, connective tissue, oxidative stress, protein expression, elastin

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Abstract

Several studies have analyzed modulation of gene expression during physiological aging with interesting, but often contradictory results, depending on the model used. In the present report we compare age-related metabolic and synthetic parameters in human dermal fibroblasts (HDF) isolated from young and old subjects (ex-vivo ageing model) and cultured from early up to late cumulative population doublings (CPD) (in-vitro ageing model) in order to distinguish changes induced in vivo by the aged environment and maintained in vitro, from those associated with cell senescence and progressive CPD. Results demonstrate that fibroblasts from aged donors, already at early CPD, exhibit an impaired redox balance, highlighting the importance of this parameter during ageing, even in the presence of standard environmental conditions, which are considered optimal for cell growth. By contrast, several proteins, as those related to heat shock response, or involved in endoplasmic reticulum and membrane trafficking, appeared differentially expressed only during in-vitro ageing, suggesting that, at high CPD, the whole cell machinery becomes permanently altered. Finally, given the importance of the elastic component for a long-lasting connective tissue structural and functional compliance, this study focuses also on elastin and fibulin5 synthesis and deposition, demonstrating a close relationship between fibulin5 and ageing.

INTRODUCTION

Ageing can be regarded as the accumulation, on a predetermined genetic background, of sequential changes leading to irreversible alterations of both cells and extracellular matrix. Moreover, advancing age has been considered to increase the incidence of cancer, neurodegenerative, and cardiovascular syndromes, possibly representing a risk factor for the occurrence of these disorders (Walker, 2002). Therefore, it is mandatory to improve the comprehension on the effects of ageing on cell behavior for a better knowledge also of the mechanisms and the molecular pathways in diseases often associated with ageing.

Many theories have been proposed to gain insights into causes and consequences of ageing, but no single theory is generally acceptable to satisfactorily explain the possible complex mechanisms, and despite the countless literature and the increased knowledge on ageing, never the less, it is still elusive what is the contribution of genetic and of environmental factors to the ageing phenotype (Kirkwood, 2002). Moreover, it is still a question of debate if cells from ageing donors or if in-vitro ageing cells represent a more suitable model for investigating this process (Schneider and Mitsui, 1976; Rubin, 1997 and 2002).

Fibroblasts play a key role in maintaining connective tissue homeostasis (Robert et al., 1992) and several humoral and cellular factors may activate or inhibit fibroblast activity or, after being released from fibroblasts, they may reciprocally act on other mesenchymal and epithelial cells (Abraham et al, 1989; Zalatnai, 2006) contributing to the complex network that modulates connective tissue homeostasis in physiological as well as in pathological conditions, such as during wound healing, inflammation or cancer (Robert and Labat-Robert, 2000).

Cultured fibroblasts represent a widespread in-vitro model for investigating genetic and acquired disorders and for exploring the importance of specific molecular pathways leading to differentiation, ageing and death (Holbrook and Byers, 1989; Van Gansen and Van Lerberghe, 1987).

It has been reported that fibroblasts, in vitro, start to develop progressive morphological changes shortly after cultures are established, regardless of the donor's age (Robbins et al., 1970), but also that cell cultures from old and young donors exhibit structural and metabolic differences quantitatively and qualitatively distinct from those observed at early and late passages. It has been therefore suggested that in-vitro ageing may represent a suitable system for examining the loss of replicative potential, whereas fibroblasts derived from old and young donors may be considered an appropriate cellular ageing model (Schneider and Mitsui, 1976).

Although it cannot be excluded that, by establishing a cell culture, a forced selection might be induced on the population of fibroblasts capable of extruding from the explant, never the less, there are some parameters that exhibit and maintain, in vitro, a peculiar behavior, according to donor's age (Boraldi et al., 2003).

Moreover, it has been suggested that fibroblast replicative lifespan in culture is inversely proportional to the age of donors (Martin et al., 1970), even though more recent, controlled and widespread investigations failed to validate this correlation (Cristofalo et al., 1998).

To our knowledge, a direct comparison of human fibroblasts from young and old donors at different cell population doublings (CPD) has been never performed.

The present study has been undertaken in order to compare the phenotype of human dermal fibroblasts (HDF) isolated and cultured from skin biopsies of young and old subjects (ex-vivo ageing model) with that of the same cells at early and late CPD (in-vitro ageing model) with special reference to proliferative capabilities, redox balance, stress response and expression of various ageing markers. Furthermore, since elastic fibers, due to their negligible turnover and their susceptibility to endogenous and exogenous noxae, represent the connective tissue component most severely affected by ageing (Robert et al., 2008), the expression of two major elastic fiber components (i.e. elastin and fibulin 5) has been evaluated.

MATERIALS AND METHODS

Skin biopsies and cell culture

According to the ethical guidelines approved by the Ethical Committee of the Modena University Faculty of Medicine, biopsies of skin minimally or not exposed to UV, and in the absence of diseases affecting connective tissues, were taken under local anesthesia from three young females (15.3±2.5 years) and three old females (83.3±1.5 years) who gave informed consent before undergoing surgery. Fibroblasts were allowed to extrude from biopsies during a period of 3-4 weeks. Thereafter all cells from each biopsy were trypsinized, cultured until confluence and stored in liquid nitrogen until use. Cells were grown in 25 and 75 cm^2 flasks (Falcon) (Quaglino et al. 2000) and serially passaged from 5 up to 32 CPDs (Cumulative Population Doublings). The number of population doublings (PD) was calculated using the formula:

PD = (ln[number of cells harvested] – ln[number of cells seeded]) /ln2 (van der Loo et al., 1998).

Cells from each individual were kept separate during all experimental procedures, always grown in parallel up to confluence and regularly observed under the inverted light microscope.

Flow cytometry

For each measurement, labelled fibroblasts were resuspended in PBS, transferred to polystyrene tubes and analyzed on an EPICS XL flow cytometer (Coulter, USA) with the excitation wavelength set at 488nm. Debris and dead cells were excluded by forward and side scatter gating. Ten thousand events were collected, compared with appropriate negative controls and evaluated for each cell line using WINMDI 2.8 program. Triplicate experiments were performed using all cell lines. Preparation of fibroblasts was performed according to the parameter to be investigated.

Determination of β-galactosidase (β-gal) activity

The fluorogenic substrate C_{12}FDG (imaGene Green) was used in the presence or absence of chloroquine, an inhibitor of endogenous β-gal. C_{12}FDG is a membrane permeable, nonfluorescent substrate of β-gal, which after hydrolysis of the galactosyl residues emits a green fluorescence that remains confined within the cell. Fibroblasts were plated in 35 mm dishes at the density of $1.2x10^5$ cells. After two days from seeding, the majority of cells were still in a proliferative state. Therefore, some dishes were pre-treated with 300μM chloroquine for 90 minutes at 37°C. All dishes were then incubated with 300μM of C_{12}FDG for 1 h. Cells were washed in ice-cold PBS, detached with trypsin and centrifuged. The obtained pellets were resuspended in 300μl of PBS. Fluorescence was measured at the emission wavelength of 520 nm.

F-actin staining

Trypsinized cells were centrifuged for 10 min at 1000g, washed in PBS, 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 0.05% Triton X-100 in PBS for 10min at 4°C. After a rapid centrifugation, cells were incubated with phalloidin-FITC labelled for 30 min at 4°C. After washing with PBS, pellets were resuspended in 500 μl of PBS. Fluorescence was measured at the emission wavelength of 520 nm.

Elastin determination

Cells were trypsinized, centrifuged and fixed in cold methanol for 10 min at 4°C. After washes in PBS, cells were incubated for 30 min in blocking solution (1% BSA in PBS), centrifuged and incubated for 1 h at room temperature with primary rabbit anti-elastin (Abcam) antibodies diluted 1:80 in PBS plus 1% BSA. After washes with PBS, incubation with a secondary TRITC conjugate goat anti-rabbit IgG antibody (Sigma) was performed for 1 h at room temperature. Cells were finally centrifuged for 5 min at 4°C and resuspended in 300μl of PBS. Fluorescence was measured at the emission wavelength of 575 nm.

Reactive oxygen species (ROS) measurements

Intracellular levels of ROS as superoxide anion (O_2•-) and hydrogen peroxide (H_2O_2) were estimated using the dihydroethidium (DH_2, 1 µM) and the H_2DCF-DA (2µM) probes (Molecular Probes, Eugene, OR) (Luo et al., 2002). Fibroblasts treated with DH_2 for 60 minutes at 37 °C were then trypsinized and collected in 500 µl of PBS, whereas cells to be stained with H_2DCF-DA were trypsinized, centrifuged for 10 min at 1000g, washed in PBS, suspended in 500µl of PBS, incubated with the probe for 30 min at 37°C, centrifuged for 5 min at 4°C and finally resuspended in 250µl of PBS. DH_2 and H_2DCF-DA fluorescence was measured at the emission wavelength of 575 and 520 nm, respectively.

Total antioxidant status (TAS)

The total antioxidant activity was measured using the Antioxidant Assay kit (Cayman, Ann Arbor, MI) according to the manufacturer's instructions. Briefly, fibroblasts were cultured at confluence, detached with a cell scraper and centrifuged at 1000 x g at 4°C. Pellets were homogenized on ice in 5mM potassium phosphate buffer, pH 7.4, centrifuged at 10.000 x g at 4°C for 15 minutes and supernatants were collected for analyses. The assay relies on the ability of the antioxidants present in the sample to inhibit the oxidation of ABTS® to ABTS® + metmyoglobin. The ability of the antioxidants in the sample to prevent ABTS oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and quantified as molar Trolox equivalents by reading the assorbance at 405 nm.

Superoxide dismutase (SOD) activity

SOD activity was measured on trypsinized cells using the Superoxide Dismutase Assay (Cell Technology, Inc, USA) according to manufacturer's instructions. Briefly, the enzyme activity was determined using the highly water soluble tetrazolium salt WST-1 (= 2-(4-Iodophenyl) -3- (4-nitrophenyl)-5-(2,4-disulfophenyl) -2H-tetrazolium, monosodium salt) assay. This method is based on the production of a water-soluble formazan dye upon reduction by superoxide anions (Ukeda et al., 1999) produced by xanthine-xanthine oxidase added in order to generate a reproducible flux of $O_2^{\cdot-}$. SOD competes with WST-1 for $O_2^{\cdot-}$, resulting in the inhibition of WST-1 reduction. The percentage of inhibition of WST-1 reduction is therefore a measure of SOD activity. The amount of reduced WST-1 (corresponding to formazan dye formation) was followed spectrophotometrically at 450 nm and values were obtained using the following equation:

1/ {[(A control 1 - A control 3) -(A sample)]/ (A control 1 - A control 3)} x 100

Western blot

Cells were washed several times with phosphate-buffered saline and homogenized in RIPA buffer (50 mM Tris, pH 7.5, 0.1% Nonidet P-40, 0.1% deoxycholate, 150 mM NaCl, and 4 mM EDTA) in the presence of protease inhibitors (Sigma). Cellular lysates were centrifuged at 15000 rpm for 20 minutes to clear cell debris, and supernatants were colleted and stored at −80 °C until analysis. Protein concentration in the cellular extracts was determined using the Bradford method (Bradford, 1976). Proteins (30µg proteins/lane) were separated on 10-lane 1-DE 10% or 12% polyacrylamide gel (see Table 1), under reducing conditions and transferred onto nitrocellulose membranes. Membranes were blocked in TBS+ 0.1% Tween 20 (TBST) + 5% non fat dry milk for 1 h at room temperature (RT) and incubated with primary antibodies diluted in TBST + 2,5% non fat dry milk (see Table 1). Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cambridge, UK, diluted 1:5000) were used after 3 washes of membranes in TBST. Western blots were visualized using Super Signal West Pico (Pierce) according to manufacturer's protocols.
Densitometric analysis of protein bands was performed using the ImageQuant TL v2005 software (GE Healthcare).

RNA preparation and quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated using the RNeasy Protect cells Mini kit (Qiagen, Valencia, CA). Quality and quantity of RNA were checked by spectrophotometry and agarose gel electrophoresis. According to manufacturer's instructions, 3µg of total RNA were reverse transcribed using Superscript III (Invitrogen) and Oligo dT primers (Invitrogen) and 2µl of cDNA were amplified on a iCycler (BioRad) using SYBR® GreenER™qPCR SuperMix (Invitrogen). Characteristics of primers are shown in Table 2. Thermal cycling parameters were set to 50°C for 2 min, 95°C for 3min, 45 cycles of 95°C for 30s, an annealing temperature of 60°C for 30 s and 72°C for 30 s, followed by melting curve analysis with a temperature ranging from 95 to 65°C.
Experiments were carried out in triplicate and gene expression in each sample was normalized against the housekeeping gene (CLK2) and quantified with the Pfaffl method (Pfaffl, 2001).

Confocal microscopy

Fibroblasts, plated in 2 well- chamber slides at a density of 3 X 10^4 cells, were grown for 5 days in 2 ml of DMEM with 10% FBS. Confluent cells were grown for further 48h in the presence or absence of 100µM β-aminopropionitrile. Cells were fixed in cold methanol for 10 min at 4°C. After washes with PBS, the cell monolayer was treated with a blocking solution (1% BSA in PBS) for 30 min and incubated for 2 h at room temperature with rabbit polyclonal anti-elastin (Abcam) and goat

polyclonal anti-fibulin-5 antibodies (Santa Cruz Biotechnology), diluted 1:100 in PBS. After extensive washes with PBS, fibroblasts were incubated, for 1h at room temperature, with secondary antibodies conjugated with either goat anti-rabbit IgG TRITC (Sigma) diluted 1:200 or rabbit anti-goat IgG FITC (Sigma) diluted 1:100. After a final wash, cells were visualized with a Leica TCS SP2 confocal microscope. Negative controls were routinely performed by omitting the primary antibody incubation step.

Data analysis

Experiments were performed independently at least three times using all cell lines (i.e. dermal fibroblasts obtained from biopsies of 3 young and 3 old donors) kept separated during all experiments. Data were expressed as mean values ± SEM of all measurements and compared by Anova test with significance at $p<0.05$.

Statistical data were obtained using GraphPad software (San Diego, CA, USA).

RESULTS

Morphology, cell growth and determination of β-galactosidase activity

Activity of β-galactosidase, a widespread used marker of replicative senescence, was detectable in fibroblasts already at early CPD, and values progressively increased with passages, although independently from donor's age (Figure 1a, right panel). At higher CPD, a greater heterogeneity between cell lines was observed. Cells pre-treated with chloroquine were completely negative (Figure 1a, left panel), indicating that beta-galactosidase activity was only of intracellular origin.

Consistently, fibroblasts isolated from young (yHDF) and old donors (oHDF), independently from donor's age, exhibited good growth capabilities at early passages and, by light microscopy, all cells strains were characterized by a typical elongated shape and were oriented in a parallel array (Figure 1b, upper panels). Around CPD 30 cell growth started to decline, although with a certain variability between cells derived from different individuals. Morphologically, fibroblasts at high passages appeared more polymorphous and progressively larger with numerous intracellular vacuoles, suggesting an accumulation of proteins and/or metabolites (Figure 1b, lower panels).

Phenotypic changes were also confirmed by FACS analysis, since forward and side scatter values progressively increased with in-vitro ageing (data not shown).

Changes in cell shape appeared, at least in part, associated with cytoskeletal modifications. Staining for F-actin revealed only a moderate increase of fluorescence intensity when yHDF and oHDF were

compared at early passages; by contrast, at high CPD, the F-actin content significantly increased in all cell lines, independently from donor's age (Figure 1c).

Parameters of redox balance

TAS measurement, taken as an indicator of the total antioxidant potential of cell cultures, revealed a progressive decline in the in-vitro aged cells as well as in oHDF at low CPD, compared with fibroblasts from young donors (Figure 2a).

Similarly, superoxide dismutase (SOD) activity was lower in in-vitro aged cells and, at low CPD, also in oHDF (Figure 2b) compared to yHDF.

The intracellular content of reactive oxygen species (ROS) was evaluated by measuring the fluorescence intensity of the H_2-DCFDA (Figure 2c) and the DH_2 (Figure 2d) probes sensitive to the presence of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$), respectively. Values appeared significantly higher in cells from old donors, and the DH_2-related signal was further increased during in-vitro ageing (Figure 2d). By contrast, staining with H_2-DCFDA was not modified by in-vitro ageing since values observed at early CPD were similar to those at late CPD (Figure 2c).

Taken together, these data indicate that reduced antioxidant properties (TAS, SOD) and consequently higher ROS levels were induced by in-vitro ageing and were present in oHDF already at low CPDs.

Protein expression

As revealed by the Bradford assay, the total amount of proteins produced by cells, in both experimental models, was not significantly different, suggesting that ageing, at least in the in-vitro cell culture model, is more associated to a different expression of specific proteins than to a significant reduction of the whole protein synthesis.

<u>Proteins related to redox balance and cell stress</u>

Looking at molecules related to changes in the redox status, we have firstly investigated thioredoxin-1 (Trx), an antioxidant enzyme that, being localized in the cytosol and in the nucleus, controls many transcription factors (Holmgren, 2010) either alone or in combination with peroxiredoxin (Prdx), protects against environmental stress and promotes longevity (Olahova et al., 2008). At early CDP cells from oHDF expressed significantly reduced levels of TRX (Figure 3a) and only slightly less PRDX2 compared to yHDF (Figure 3b). During in-vitro ageing, yHDF exhibited significantly reduced PRDX2 expression, whereas the low levels in oHDF were very

similar at early and late CPD (Figure 3b). By contrast, TRX expression was no further reduced at high CPD compared to values at low CPD (Figure 3a). These findings sustain once more that altered redox balance is a key point not only in in-vitro ageing, but also in oHDF at low CPD.

Cells try to protect themselves by activating a heat shock response (Feder and Hofmann, 1999) in order to repair moderate protein misfolding and to prevent inter- or intra-molecular aggregation of damaged proteins. Hsp27 and Hsp60 appeared significantly decreased during the in-vitro ageing, independently from donor's age (Figure 3d,e). By contrast, Hsp90 was reduced not only with in-vitro ageing, but already in oHDF at early CPD. At high CPD, expression of Hsp90 was markedly reduced in yHDF, whereas levels in oHDF were not further diminished (Figure 3f). Surprisingly, expression of FKBP52, a cochaperone of Hsp90, was significantly up-regulated during in-vitro ageing in all cell lines. Differences according to donor's age were always negligible (Figure 3c).

Endoplasmic reticulum and plasma membrane proteins

Oxidative damage is known to affect not only plasma membrane constituents, but also molecules located in other organelles and/or cellular compartments such as those in endoplasmic reticulum (ER). The ER contains a number of molecular chaperones including GRP78, calreticulin (CALR) and protein disulfide isomerase (PDI) that, upon decrease in their expression or activity, could directly contribute to the age-dependent accumulation of misfolded proteins. CALR, GRP78 and PDI were always less expressed in in-vitro aged fibroblasts, being the influence of donor's age generally not significant (Figure 3g-i).

Furthermore, since oxidative stress may have consequences on cell membrane structure and function, we have also investigated the expression of two important constituents of cell membranes, as caveolin 1 (CAV 1) and annexin II (ANXII). Both proteins were up-regulated in the ex-vivo and in the in-vitro ageing models, although changes appeared more dependent on CPDs (Figure 3j,k).

By contrast, the expression of galectin 1 (LEG1), a carbohydrate binding protein present on the cell surface and capable of activating several intracellular signaling pathways, did not change in fibroblasts from donors of different ages as well as at early or late CPDs (Figure 4l).

Proteins related to synthetic and metabolic pathways

As far as cellular metabolic pathways, α-enolase (ENO1A1), a highly conserved cytoplasmic glycolytic enzyme, has been linked in-vivo and in-vitro to the ageing process (Kanski et al., 2005; Dierick et al., 2002) and is upregulated by oxidative stress (Trougakos et al., 2006; Baty et al., 2005). In the present study, ENO1A1 was similarly expressed in yHDF and oHDF at early CPD,

whereas there was a marked up-regulation with in-vitro ageing and changes were more evident in oHDF than in yHDF (Figure 3m).

Consistently with the occurrence of modified energy production and/or altered metabolism with in-vitro ageing (Corstjens et al., 2007), we have observed that the expression of receptors for advanced glycation end products (RAGE) exhibited a significant in-vitro age-dependent increase. Differences between cells from young and old donors were negligible at all CPDs (Figure 3o).

By contrast, expression of elongation factor 1A1 (eEF1A1), that is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome, but has been also related to cell senescence (Byun, 2009), was significantly down-regulated during in-vitro ageing, being differences due to donor's age irrelevant at all CPDs (Figure 3n).

Elastic fiber-related gene and protein expression

In vivo elastin is physiologically expressed and synthesized especially in the perinatal period (Quaglino et al., 1996). In this in vitro study, elastin mRNA expression was not significantly modified in fibroblasts from donors of different age and during the in-vitro ageing (Figure 4a). By contrast, elastin protein production, evaluated by Western blot and flow cytometry with antibodies recognizing both soluble and insoluble forms of tropoelastin (Figure 4b and 4c), independently from donor's age, was significantly up-regulated at high CPD. However, immunolabelling of in-vitro aged-cells with the same antibodies failed to reveal increased amounts of elastic fibers, whereas there was a more intense intracellular fluorescence. The occurrence of an unspecific staining was excluded by appropriate negative controls (data not shown). The intracellular staining started to be evident in oHDF already at low passages (Figure 4f-g), when the elastin filamentous network was always less evident and less organized (Figure 4e) compared to yHDF (Figure 4d). In in-vitro aged fibroblasts, immunofluorescence was mainly intracellular, especially in the nuclear/perinuclear region and in small dots spread in cytoplasm or at the close periphery of the cells.

Incubation of the cells with beta-aminopropionitrile (100μM), an inhibitor of the crosslinking enzyme lysyl oxidase (Keeley, 1976), did not change the intensity nor the pattern of the immunostained elastic network (data not shown), indicating that fibers were not crosslinked, consistently with previous observation that, at physiological temperature, due to its coacervation properties (Bressan et al., 1986), elastin forms amorphous extracellular aggregates also in the absence of crosslinks.

By contrast, both at mRNA and protein levels (Figure 5a and 5b), fibulin 5 appeared significantly reduced during the in-vitro ageing and in oHDF already at early CPDs. At low CPD, fibroblasts from young donors appeared all positively labelled by anti-fibulin 5 antibodies (Figure 5c).

Negative controls allowed to assess the specificity of the reactions (data not shown). Immunolabelling of oHDF at early CPD revealed a reduced staining with a marked heterogeneity between cells (Figure 5d), since few fibroblasts were still positive, but the great majority of cells exhibited only a weak fluorescence (Figure 5 d). At high CPD, independently from donor's age, fluorescence was barely detectable in all cells (Figure 5 e and f).

DISCUSSION

The present study was performed in order to explore if fibroblast phenotype is differently modulated depending on the experimental ageing model. Therefore, on the basis of findings from experimental models, as well as from in vivo data (Boraldi et al., 2003; Weinert and Timiras, 2003; Ljubuncic and Reznick, 2009), we have compared ex-vivo and in-vitro aged human fibroblasts by investigating a selection of parameters that represent and reflect the behaviour of different cell responses and cellular compartments.

In agreement with the observations of Cristofalo et al. (1998), we could not discriminate, in vitro, fibroblasts from young or old donors, since no significant differences were observed as far as morphological features, F-actin expression and proliferative lifespan in culture. By contrast, all these parameters appeared significantly modified during in-vitro ageing.

Since, β-Gal has been considered by several Authors a marker of replicative senescence and possibly of ageing (Dimri et al., 1995; Campisi, 2005), we have investigated its expression in our experimental models. The observed CPD-dependent increase of β-Gal, the presence of positive cells already at low passages and the absence of differences related to donor's age are in agreement with previously reported data (Ravelojaona et al., 2000) and further support the hypothesis that senescence cannot be regarded as an anti-oncogene mediated process enabling fibroblasts to escape malignancy (Campisi 2005). Consistently with the observation that β-Gal is expressed by several cancer cell lines (Krishna et al., 1999) and in a variety of conditions (Severino, 2000), present findings suggest that, even in a newly established cell culture system, there are cells positive to β-Gal, suggesting that senescent or quiescent cells may coexist with cell clones still capable to proliferate and to respond to growth factors (Kurz et al., 2000; Maier et al., 2007). Therefore, β-Gal is probably not a suitable indicator for making a distinction between a long quiescence and a terminal post-mitotic state (Macieira-Coelho, 2010).

It is generally accepted that ageing is associated with a progressive loss of functions and that changes in the regulation of protein synthesis, post-translational modifications and turnover may

represent key factors in the age-related decline of maintenance, repair and survival of cells, tissues and organisms (Rattan, 1996).

Within this context, the free radical theory of aging (Harman, 1956) has received, for more than four decades, great support and popularity, although, more recently, an expanded criticism has arisen (Bokov et al., 2004; Muller et al., 2007) leading to controversial hypotheses (Barouki, 2006). In the present study, the redox balance, as the ratio between antioxidant and oxidant species, decreased during in-vitro ageing and was lower in fibroblasts from old compared to young donors. This finding indicates that, despite the presence of optimal environmental conditions and/or the possible selection of more active cell clones, oHDF and in-vitro aged fibroblasts exhibit and maintain in vitro parameters of oxidative stress, a condition that is known to favour protein, lipid and DNA damages (Jung et al., 2009).

As suggested in a model of oxidative stress-induced premature ageing (Dasari et al., 2006; Volonte et al., 2002), changes in the expression of membrane molecules as annexin II and caveolin 1 might have a great influence on the ageing process, duet to their ability of modulating the plasticity of the membrane-associated actin cytoskeleton (Hayes et al., 2006) and of interacting with a variety of regulatory and structural molecules (Park, 2006). In accordance with the proposed role of caveolin 1 as a potential target of the aging process (Park, 2006) and with the observations that changes in annexin II expression may have consequences on caveolae formation and localization (Parkin et al., 1996; Sagot et al., 1997), we have provided evidence that both caveolin 1 and annexin II are significantly upregulated during in-vitro-ageing.

By contrast, expression of the polyvalent molecule galectin-1 remained quite constant in all experimental conditions. Although the biological activity of galectin-1 depends on the properties/characteristics of the binding partners (Camby et al., 2006), data from the present study indicate that in physiological conditions, such as aging, galectin 1 expression is a rather stable parameter, whereas significant changes are associated with pathologic conditions (Demydenko and Berest, 2009) or to modified environmental conditions, as during hypoxia (Boraldi et al., 2007).

A further marker of protein damage is the presence of advanced glycation end products (AGEs) that, either directly or by interacting with RAGE, may trigger intracellular signalling pathways, rapid generation of reactive oxygen species (ROS) and up-regulation of inflammatory pathways (Ramasamy et al, 2005), thus playing a pivotal role in the development and acceleration of age-related diseases (Bierhaus et al, 2005). The marked increase of RAGE expression, observed during in-vitro ageing, may contribute to worsening the redox balance and further supports the concept that, at high CPD, the cell machinery is permanently and irreversibly modified. In addition, during in-vitro ageing, we have demonstrated a significant decrease of GRP78, calreticulin and PDI, three

proteins of the endoplasmic reticulum that, if not adequately present or active, significantly contribute to the age-dependent accumulation of misfolded proteins.

Cells counteract protein misfolding and aggregation by activating a heat shock response (Feder and Hofmann, 1999). The observed reduced expression of Hsp 27, 60 and 90 at high CPD, suggests that in-vitro aged cells loose the ability of actively counteracting stress conditions. Interestingly, the expression of Hsp90 appeared to be particularly sensitive to the ageing process, being down-regulated in in-vitro aged cells as well as in oHDF at early CPD. Moreover, Hsp90 interacts with different proteins including FKBP52, an immunophilin also known as Hsp56, that functions in the glucocorticoid receptor system increasing the receptor avidity for hormones (Ratajczak et al., 2003). During in-vitro ageing FKBP52 was significantly upregulated and this finding could be further investigated in future studies in order to better understand the relationships between ageing, Hsp and metabolic pathways, through hormone regulation.

Changes in mesenchymal cell phenotype and behaviour significantly affect connective tissue homeostasis. Loss of elasticity is a well known paradigm of ageing connective tissue, and data are present in the literature demonstrating that tropoelastin production is dramatically decreased with in-vitro ageing or in cells from 70 to 90 year old donors (Sephel and Davidson, 1986) compared to cells from young subjects. In the present investigation, differences in elastin mRNA expression were negligible both in the ex-vivo and the in-vitro ageing models, whereas a significant up-regulation was detected at protein level in in-vitro aged fibroblasts. However, by confocal microscopy, the intricate elastin network present at early passages was less evident in cultures from old donors and completely disappeared during in vitro ageing, leaving a marked intracellular immunostaining. The presence of intracellular tropoelastin, especially in the perinuclear area, has been already demonstrated in vitro, suggesting that it may correlate to the elastogenic potential of the cells (Grosso et al., 1990). It may also represent the substrate for endogenous proteolytic activities leading to the formation of elastin peptides capable of triggering signals leading to cell cycle progression in an autocrine or paracrine manner (Jung et al., 1998) with complex and profound consequences in vivo. At present, although we cannot exclude that biologically active elastin peptides/fragments may be formed by proteolysis, never the less present data already indicate that the presence of elastin mRNA does not imply that functional and efficient elastic fibers are formed in the extracellular compartment. These findings are in perfect agreement with those demonstrated in the wound healing process (Davidson et al., 1992). Moreover, elastin could be retained within cells possibly due to changes in the expression of chaperones as glycosaminoglycans (Fleischmajer et al., 1972; Pasquali-Ronchetti et al., 1984) or due to inefficient

recycling of the EBP, the elastin binding protein that, acting as a tropoelastin chaperone, allows effective extracellular fiber deposition (Hinek, 1995)

Finally, in agreement with findings from the literature (Kadoya et al. 2005) that fibulin 5 (FBLN5) is markedly decreased in the aged reticular dermis, we have shown that FBLN5 was significantly reduced at mRNA and protein levels in fibroblasts from old donors as well as in in-vitro aged cells. FBLN5 is known to bind tropoelastin, to promote its coacervation (Hirai et al., 2007) and to induce elastic fiber assembly (Nakamura et al., 2002; Yanagisawa et al., 2002). Therefore, reduced FBLN5 expression could contribute to the reduced and less organized deposition of elastin aggregates observed in oHDF already at low CPD. Finally, it has to be mentioned that, in blood vessels, fibulin-5 is required to assure a proper binding of ecSOD to tissues, and to control the redox state (Nguyen et al., 2004). It could be therefore suggested that a similar mechanism may function also at dermal level, and that the age-dependent decreased expression of fibulin-5 and reduced SOD activity may contribute to the altered redox balance.

In conclusion, the present investigation, by directly comparing fibroblasts from young and old donors (ex-vivo ageing model) at early and late CPDs (in-vitro ageing model), demonstrates that a) the great majority of differences, for instance stress response, endoplasmic reticulum and cell membrane compartments and post-translational protein modifications, are mainly observed during in-vitro ageing, independently from the age of donors; b) the redox balance is markedly affected by in-vitro ageing, although impairment depends also on donor's age, suggesting that the in-vitro optimal environment is non capable of restoring this parameter towards a younger phenotype; c) elastin deposition in the extracellular space is reduced in cultures from old donors and is dramatically abolished during in- vitro ageing; d) the high elastin expression is not sufficient to produce elastin deposition in the extracellular matrix, since it seems that in-vitro aged cells loose the ability to extrude tropoelastin in the extracellular compartment; e) fibulin-5 represents a very sensitive ageing marker and could play an important role in EMC homeostasis, controlling also the redox balance.

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LEGEND TO FIGURES

Figure 1. a) Replicative senescence has been evaluated measuring beta-galactosidase positive cells by flow cytometry. A representative experiment reported in the left panel shows the fluorescence intensity of one fibroblast line at different CPD (i.e. CPD 8, 20 and 30). The effect of in-vitro ageing is illustrated in histograms on the right panel. Data are expressed in comparison with values obtained in yHDF at CPD 8 set at one. b) Light microscopy of cultured human dermal fibroblasts from young and old donors at early (CDP8) and late (CPD 30) CPDs. c) F-actin staining with fluorescent-phalloidin was investigated by flow citometry on cultured human dermal fibroblasts from young (Y) and old (O) donors at early (CDP8) and late (CPD 30) CPDs. A representative experiment is reported in the left panel.
Histograms represent mean values of all experiments with different cell lines. Data are expressed as mean values ± SEM. $ $p<0.05$ CPD 30 vs lower CPD 8

Figure 2. Fibroblasts from young (Y) and old (O) donors, at early (CPD8) and late (CPD30) CPDs, were assessed spectrophotometrically, using specific kit assays, for TAS (a) and SOD activity (b) and by flow cytometry, using the fluorescent probes H_2-DCFDA (c) and DH_2 (d), for levels of reactive oxygen species as H_2O_2 and $O_2^{-\bullet}$. Data are expressed as mean values ± SEM.
$ $p<0.05$ CPD 30 vs CDP 8; * $p<0.05$ Y vs O fibroblasts at the same CPD.

Figure 3. The expression of tioredoxin (TRX, a); peroxiredoxin 2 (PRDX2, b), co-chaperone FKBP52 (c), chaperones Hsp27 (d), Hsp60 (e), Hsp90 (f), calreticulin (CALR, g), 78kDa glucose regulated protein (GRP78, h), protein disulfide isomerase (PDI, i), caveolin 1 (CAV1, j), annexin II (ANX II, k), galectin 1 (LEG1, l), enolase 1 (ENO1A1, m), elongation factor 1A1 (eEF1A1, n) and receptor for advanced glycosylation end products (RAGE, o) was evaluated by Western blot in fibroblasts from young (Y) and old (O) donors at early (CPD8) and late (CPD30) CPDs. Histograms represent the densitometric analysis of Western blots performed with all cell lines. Expression is normalized to fibroblasts from young donors at CPD8, set as one-fold, and data are expressed as mean values ± SEM. Representative immunoblots are shown in the lower part of each panel.
$ $p<0.05$ CPD 30 vs CDP 8; * $p<0.05$ Y vs O fibroblasts at the same CPD.

Figure 4. Elastin (ELN) mRNA (a) and protein expression (b,c) were evaluated by RT-PCR (a), Western blot (b, with a representative immunoblot shown in the lower part of the panel) and flow cytometry (c) on fibroblasts from young (Y) and old (O) donors at early (CPD8) and late (CPD30) CPDs. In histograms expression is normalized to fibroblasts from young donors at CPD8, set as one-fold, and data are expressed as mean values ± SEM. Lower panels (d-g) illustrate, by confocal microscopy, the anti-elastin immunostaining of fibroblasts from young and old donors at early (CPD8) and late (CPD30) CPDs.

$ $p<0.05$ CPD 30 vs CDP 8.

Figure 5. Fibulin 5 (FBLN 5) mRNA (a) and protein expression (b) were evaluated by RT-PCR (a), Western blot (b, with a representative immunoblot shown in the lower part of the panel) on fibroblasts from young (Y) and old (O) donors at early (CPD8) and late (CPD30) CPDs. In histograms expression is normalized to fibroblasts from young donors at CPD8, set as one-fold, and data are expressed as mean values ± SEM. Lower panels (c-f) illustrate, by confocal microscopy, the anti-fibulin 5 immunostaining of fibroblasts from young and old donors at early (CPD8) and late (CPD30) CPDs.

$ $p<0.05$ CPD 30 vs CDP 8; * $p<0.05$ Y vs O fibroblasts at the same CPD.

Table 1

Characteristics of antibodies used for WB and related experimental conditions.

Immunogen	Short name	Host/ Ab type §	Working dilutions and incubation time & temperature	Catalogue No/ Company*
Annexin II	ANX II	Gp	0.3µg/ml - 12h 4°C	2242/AB
Calreticulin	CALR	Rp	1:2000 - 1h RT	4606/S
Caveolin1	CAV1	Mm	1:1000 - 12h 4°C	17052/AB
Elastin	ELN	Rp	1:500 - 12h 4°C	21607/AB
Elongation factor 1A1	eEF1A1	Rp	1 µg/ml - 1h RT	37969/AB
Enolase 1	ENO1A1	Rp	0.03µg/ml - 1h RT	49343/AB
Fibulin 5	FBLN5	Gp	1:1000 - 12h 4°C	23062/SC
FKBP52	FKBP52	Mm	1 µg/ml - 1h RT	54991/AB
Galectin 1 [1)]	LEG1	Rp	0.2µg/ml - 1h RT	25138/AB
78kDa glucose regulated protein	GRP78	Rp	1µg/ml - 1h RT	21685/AB
Heat shock protein 90	Hsp90	Mm	1µg/ml- 12h 4°C	1429/AB
Heat shock protein 60	Hsp 60	Mm	1:10000 - 1h RT	13532/AB
Heat shock protein 27	Hsp 27	Mm	1:1000 - 1h RT	2790/AB
Peroxiredoxin 2	PRDX 2	Rp	1:10000 - 12h 4°C	15572/AB
Protein disulfide isomerase	PDI	Mm	1:2000 - 1h RT	2792/AB
Receptor for advanced glycosylation end products	RAGE	Rp	1:100 - 30 min 4°C	37647/AB
Thioredoxin [1)]	TRX	Rp	0.5µg/ml - 12h 4°C	16835/AB

[1)] loaded on 12% polyacrylamide gel

§ Mm, mouse monoclonal; Rp, rabbit polyclonal; Gp, goat polyclonal.

* AB, Abcam; S, Sigma; SC, Santa Cruz

Table 2

Characteristics of primers used for qRT-PCR

	Lenght	Position	Tm	%GC	Sequence	Slope	E %	R^2
ELN								
Forward	21	2120-2140	59	52	CAGCTAAATACGGTGCTGCTG	-3.357	98.6	0.973
Reverse	19	2195-2213	59	52	AATCCGAAGCCAGGTCTTG			
FBLN 5								
Forward	21	1263-1283	59	57	CGGCACATACTTCTGCTCCTG	-3.205	105	0.95
Reverse	23	1323-1345	59	48	GCTCACATTCGTTGATGTCTTGG			
CLK2								
Forward	22	1231-1252	59	45	ACCTACAACCTAGAGAAGAAGC	-3.206	105	0.954
Reverse	18	1335-1352	59	61	GGCGAGTGGAGACAATGG			

Figure 1
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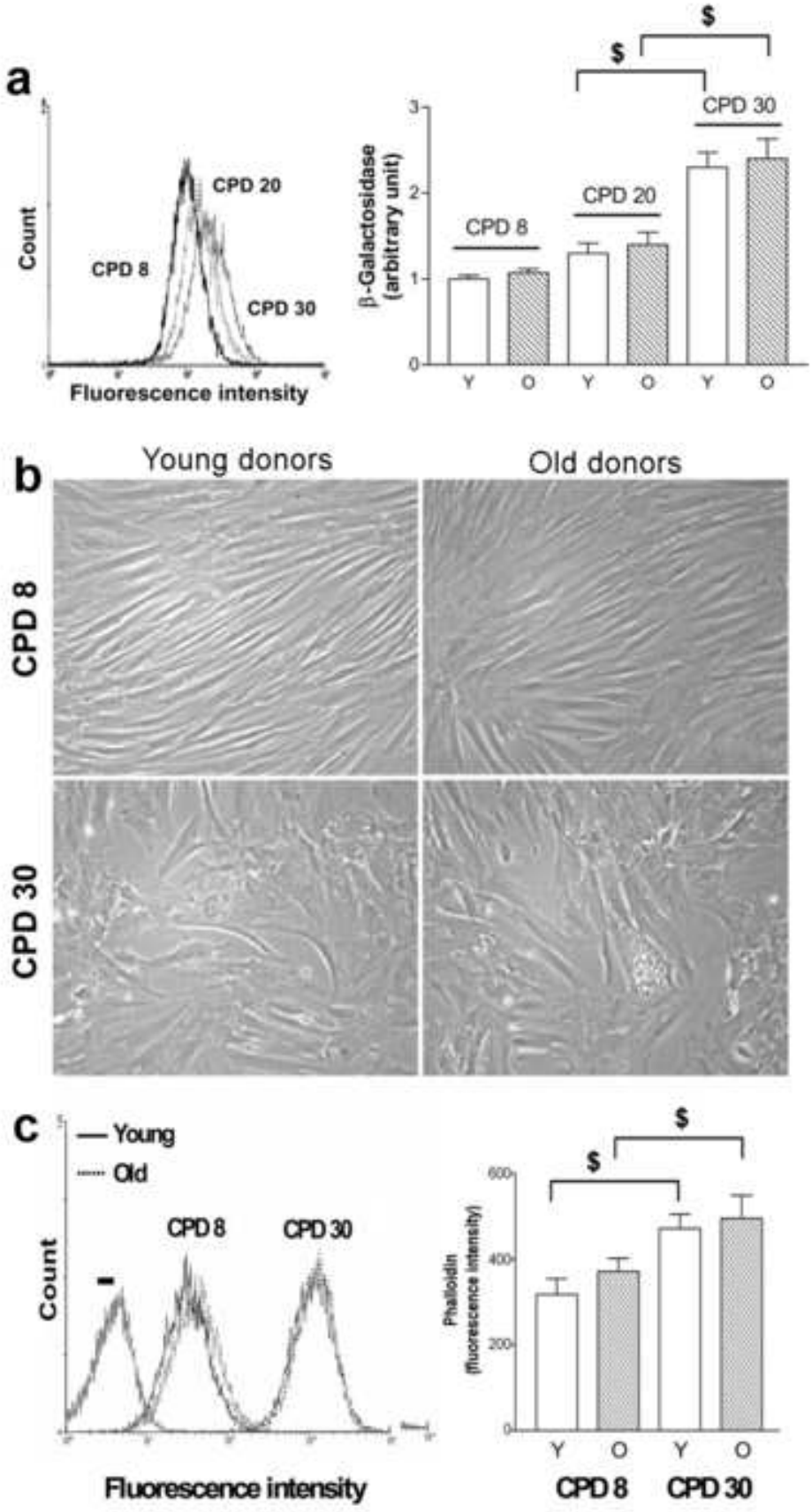

Figure 2
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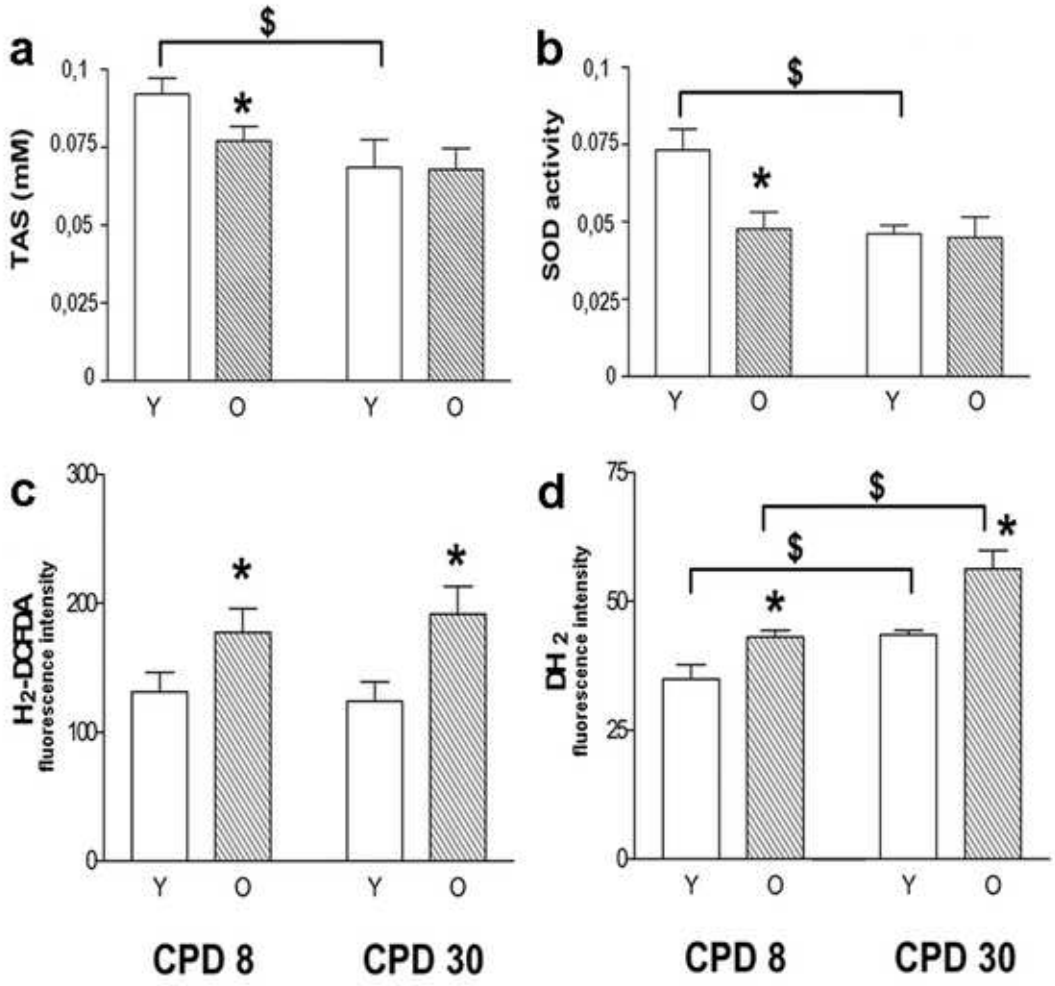

Figure 3

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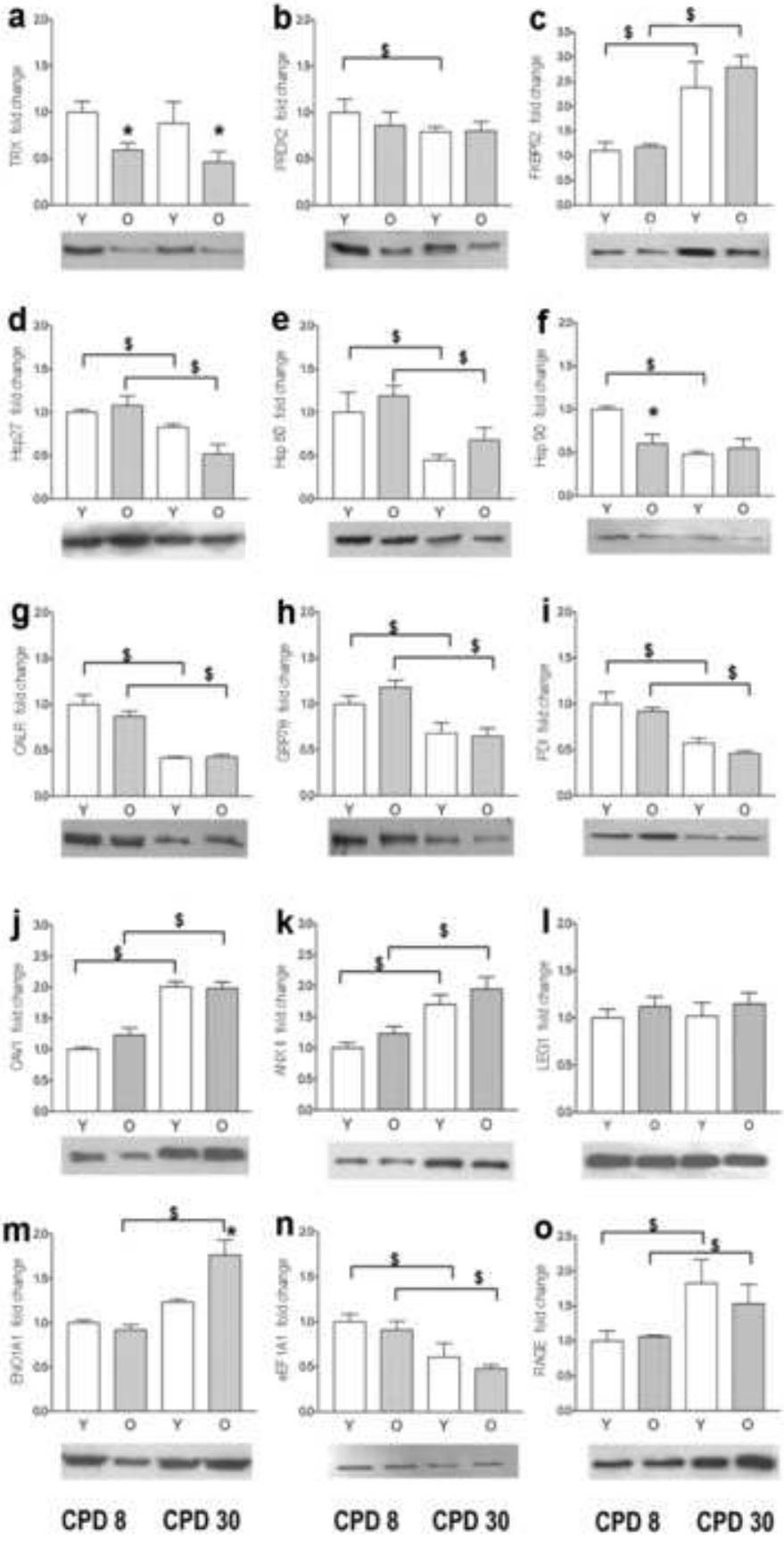

Figure 4
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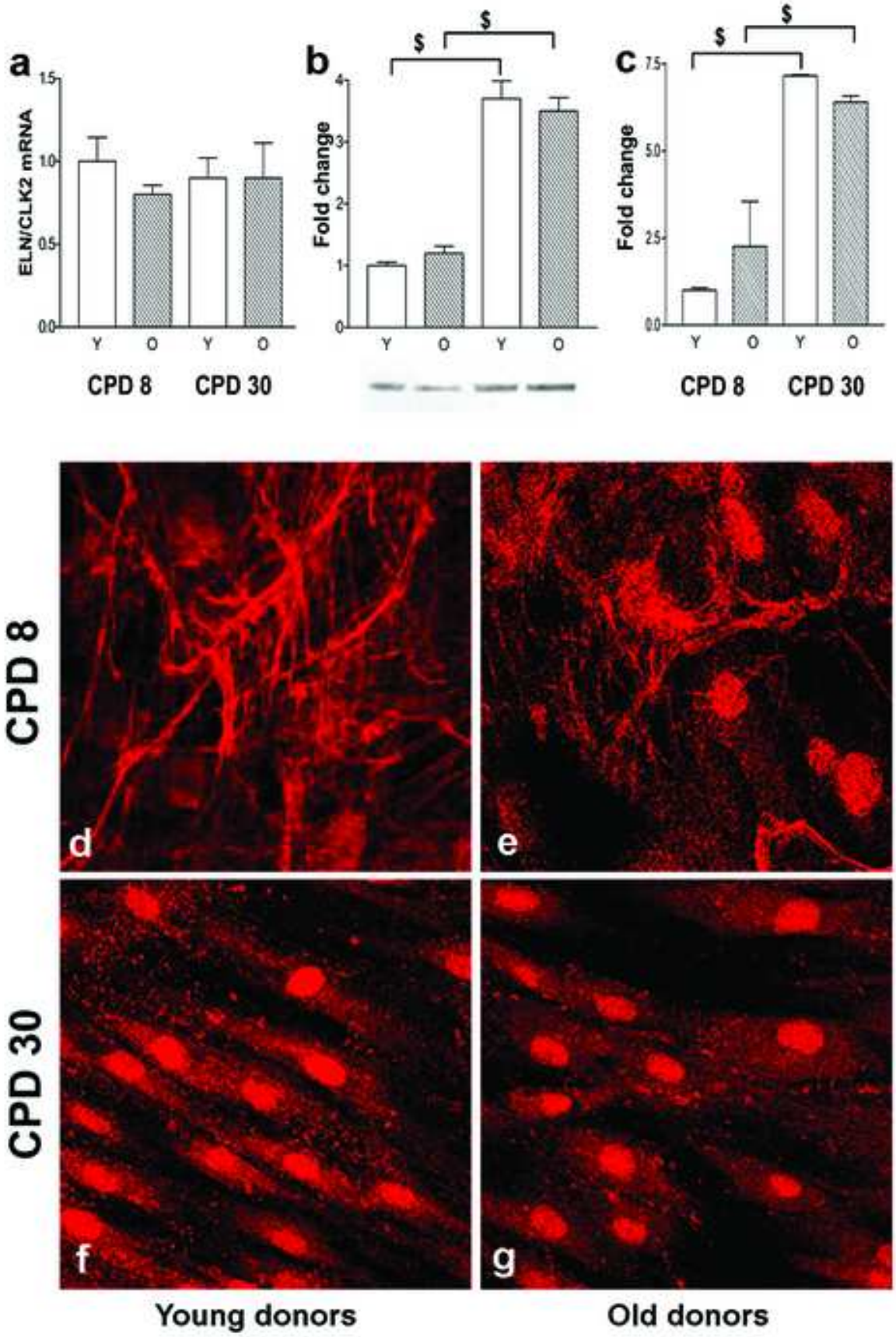
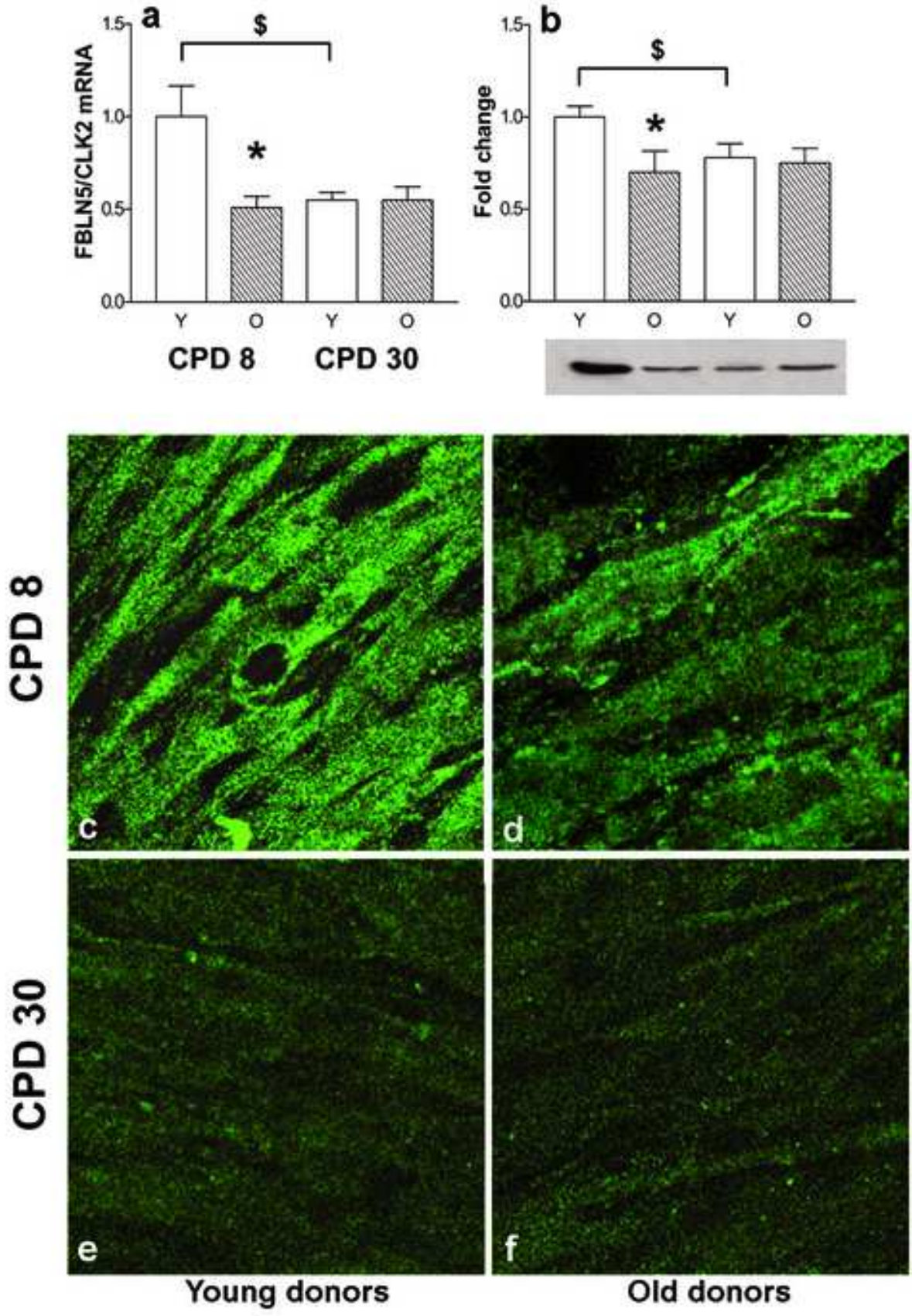
Figure 5
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