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Letter to the Editor

In vitro acquired cellular senescence and aging-specific phenotype can be distinguished on the basis of specific mRNA expression

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Dear Editor,

The contribution of cellular senescence to the aging of animals is still a controversial issue. Human diploid fibroblasts can be cultured *in vitro* for a finite number of divisions, after which they undergo a metabolic condition called replicative senescence.[1,2] This condition is characterized by the absence of response to mitogenic stimuli and elevated levels of cyclin-dependent kinase inhibitors,[3] which in turn could be responsible for the presence in these cells of dephosphorylated retinoblastoma protein and low E2F transcription factor activity.[4,5] Furthermore, there is convincing experimental evidence that telomere shortening plays a key role in the establishment of the senescent phenotype.[6,7] Cells derived from old individuals share with *in vitro* senescent cells some cellular and molecular phenotypes, but it is not clear whether these phenotypes are completely overlapping. The data currently available show that normal cells from aged donors have a proliferative potential lower than those taken from young individuals[8–12] and, consistently, cells from patients suffering from diseases characterized by a precocious senescence, such as Werner's syndrome, Down's syndrome and Hutchinson–Gilford progeria, show a significant impairment of proliferative potential compared to cells from healthy donors of comparable ages.[13–17] Other results, however, showed that, in a large series of normal subjects of various ages, the maximal population doubling of skin fibroblasts is completely independent from the age of the donor.[18] Furthermore, *in vivo* aged cells, like *in vitro* senescent fibroblasts, show the appearance of the senescence-associated β-galactosidase activity,[19] and the accumulation of cyclin-dependent kinase inhibitors, $p21^{waf1}$ and p16.[3,20,21]

A direct approach to address the possible relationship between cells undergoing *in vitro* replicative senescence and cells taken from old subjects is to compare their gene expression profiles.

In a recent paper, the expression profile of human fibroblasts taken from old subjects has been compared to that of cells taken from young individuals,[22] showing that there are numerous genes expressed at different levels in these two types of cells. We selected some of these genes to analyze whether the differences of their expression observed in fibroblasts taken from old *vs* young individuals could be similarly observed with the appearance of *in vitro* acquired replicative senescence.

The cells used for this study were: (i) IMR-90 human embryo fibroblasts (EF); (ii) two populations of skin fibroblasts taken from young donors of 7 and 9 years (CRL-1474 and CRL-7469), indicated as YF1 and YF2, respectively; (iii) two populations of skin fibroblasts taken antemortem from old individuals of 92 and 96 years of age (AG04064A and AG04059B) indicated as OF1 and OF2, respectively. Proliferation capacity of these cells was examined at different Population Doubling Level (PDL) by measuring BrdU incorporation, during 48 hour incubation in the presence of the nucleoside. EF cell BrdU incorporation is $>90\%$ until 50 PDL, and thereafter decreases, reaching a mean value of 5% at 60 PDL (EF_{60}). YF1 and YF2 cells have a percentage of BrdU incorporation very similar to that of EF cells at 25 PDL (EF_{25}), which remains elevated until 35 PDL. OF1 and OF2 cells maintained in culture up to 20 PDL showed more than 85% of BrdU positive cells, but this percentage decreases after a few more PDL (only 6% of BrdU positive cells after 25 PDL).

We also measured the levels of $p21^{waf1}$ mRNA and the number of SAβ-gal positive cells; $p21^{waf1}$ mRNA is low in EF_{28}, YF1 and YF2 cells, in which SAβ-gal positive cells are quite absent, while EF_{60} and OF cells contain several fold more p21 mRNA than EF_{28} and YF cells and a high percentage of SAβ-gal positive cells ($\geqslant 50\%$). In conclusion these results are in agreement with the observations reported by others, indicating that cells taken from old individuals are similar to the cells that acquired the senescent phenotype *in vitro*: in fact, they have a limited proliferation potential, show increased levels of $p21^{waf1}$ mRNA and most of them are SAβ-gal positive.

To further examine this similarity we analyzed by quantitative real-time PCR the levels of expression of some genes in the various cell populations under examination. Table 1 shows that the two mRNAs encoding prostaglandin endoperoxide synthase (PTGS1) and fibromodulin (Fmod) are significantly increased in OF1 and OF2 cells compared to YF1, in agreement to that reported in the paper of Ly *et al*.[22] The mRNA levels of these genes in YF2 cells are very similar to those present in YF1 cells (data not shown). In particular, we found that $OF1_{20}$ cells compared to YF1 cells, on three different RNA preparations each measured in triplicate, have 16-fold more PTGS1 mRNA and 20-fold more Fmod mRNA. These differences were confirmed when we analyzed the RNA from $OF1_{25}$ cells (slowly- or not-dividing cells, see above), although the differences were less strong. On the contrary, the concentration of PTGS1 mRNA in senescent EF_{60} cells is similar to that observed in EF_{28} cells, while Fmod mRNA shows an opposite change, being decreased by more than sevenfold in EF_{60} compared to EF_{28} cells.

Table 1 Gene expression levels in *in vitro* senescent cells and in cells from old individuals

GENE (Acc. N.)	$OF1_{20}$ vs YF1	OF2 vs YF1	$OF1_{25}$ vs YF1	EF_{60} vs EF_{28}	EF_{28} DEM 2 days	EF_{28} DEM 4 days	EF_{28} DEM 6 days	Oligonucleotides
PTGS1 (M59979)	16.1	12.4	8.0	1.3	1.1	−1.5	−6.3	F.P.: CCAGGAGTACAGCTACGAGCAGT R.P.: AGGATGTGGTGGTCCATGTTC Probe: CTGGTGGATGCCTTCTCTCGCCA
Fmod (U05291)	20.8	19.3	16.6	−7.3	1.3	−2.2	−3.8	F.P.: CTACCTCCAAGGCAATAGGATCA R.P.: TGGCGCTGCGCTTGA Probe: TGCGCCTGGACGGGAACGA
CatC (X87212)	2.1	1.6	−1.1	1.2	−1.5	−1.4	−2.0	F.P.: CCCCAATCCTAAGCCCTCAG R.P.: GGCGTACTTTCCTGCAATAAGG Probe: TTGTGTCTTGTAGCCAGTATGCTCAAGGCTGT
HME (L23808)	5.0	2.8	2.5	2.2	2.6	12.5	8.2	F.P.: ACCCACGTTTTTATAGGACCTACTTC R.P.: GAAGTTCTTGGTAATCAGTTTGGGA Probe: TATTGGAGGTATGATGAAAGGAGACAGATGATGGA
CycA (X51688)	−1.1	1.2	−4.8	−11.5	−11.1	−9.1	−17.0	F.P.: TGGGCACTGCTGCTATGCT R.P.: TTTCTTGGTGTAGGTATCATCTGTAATGT Probe: AAGAAATATACCCCCCAGAAGTAGCAGAGTTTGTG
CycF (Z36714)	−1.1	−1.4	−4.9	−5.4	−4.0	−5.6	−6.3	F.P.: GTGGTCGGTGAGCGGAAG R.P.: TGCAATATGGATGCTTTGTGAGT Probe: CTGCAAGGCCGTGGTTCACGAGA
TS (D00596)	−1.3	1.5	−3.6	−4.7	−1.8	−3.5	−14.0	F.P.: GAGGAGTTGCTGTGGTTTATCAAG R.P.: CCCAGGCTGTCCAAAAAGTC Probe: AAGGGAGTGAAAATCTGGGATGCCAATG
HFH-11A (U74612)	1.1	1.4	−4.3	−6.4	−3.7	−12.5	−21.2	F.P.: AGCCCTTTGCGAGCAGAA R.P.: CCACTGGATGTTGGATAGGCTAT Probe: TGCAGATGGTGAGGCAGCAGGCT
Ucar (U73379)	1.0	−1.6	−5.7	−8.4	−6.6	−12.2	−15.0	F.P.: GGGATTTCTGCCTTCCTGA R.P.: GCATTGTAAGGGTAGCCACTGG Probe: TCAGACAACCTTTTCAAATGGGTAGGGACC

EF cells (for the cell name abbreviations see text), purchased from ATCC at PDL 24, were subcultured weekly in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL), containing 10% foetal bovine serum (Gibco/BRL, New Zealand) in 5% CO_2 at 37°C. The population doubling level (PDL) at each subcultivation was calculated from the cell counts by using the equation: $X = \log_2 N_H/N_I$, where N_I = starting number, N_H = number of harvested cells. OF1 cells were grown in Minimum Essential Medium with Earle's salts (EMEM, Gibco/BRL) with HEPES 26 mM, containing 2× concentration of vitamins, essential and non essential amino acids, and 20% serum (Gibco/BRL New Zealand). OF2 cells were grown in EMEM, with HEPES 26 mM containing 2× concentration of vitamins, essential and non-essential amino acids and 15% serum. OF1 and OF2 cells were purchased from the Coriel Institute for Medical Research, Cadmen, NJ. YF1 cells (from ATCC) were cultured in EMEM, plus 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% foetal bovine serum. YF2 cells (from ATCC) were grown in DMEM containing 10% foetal bovine serum plus 2 mM glutamine. Total RNA was extracted from the cells using a commercially available kit (RNeasy, Qiagen). 1 μg of total RNA was subjected to reverse transcription (M-MLV Reverse Transcriptase, GIBCO/BRL) to synthesize first strand cDNA. The Real Time Quantitative PCR (RTQ-PCR) has been performed as described.[26] In each sample, the level of each transcript was normalized to the amount of c-Abl encoding mRNA whose levels are highly stable in human cells and in various cell lines (personal observation), and which was used as endogenous control for relative quantitation. The efficiency of RTQ-PCR for each of the mRNAs of interest and for c-Abl was assessed experimentally using the dilution method (see ref. [26]), prior to sample analysis. Relative quantitation can be represented by the following equation: $T_0/C_0 = K\cdot(1 + E)^{Ct,EC - Ct,T}$, where T_0 is the initial copy number of target mRNA; C_0 is the initial copy number of c-Abl mRNA; E is the efficiency of amplification; Ct,EC is the threshold cycle for c-Abl; and Ct,T is the threshold cycle for target mRNA.[26] The fold variation of each target mRNA in various samples was derived by the ratios of the relative quantitation performed in the sample and in the control RNA, i.e. $(1 + E)^{(\Delta Ct,S - \Delta Ct,C)}$, where ΔCt,S is the difference Ct,EC – Ct,T obtained in the sample, and ΔCt,C, is the same measure obtained in the control RNA used in the experiment. The primers and probes used are indicated in the table. For the quantitation of c-Abl mRNA (Accession number: X16416) the primers and the probe used are: F.P.: TGGAGATAACACTCTAAGCATAACTAAAGGT; R.P.: GATGTAGTTGCTTGGGACCCA; Probe: CCATTTTTGGTTTGGGCTTCACACCATT. F.P., forward primer; R.P., reverse primer

Another experimental system that has been used to induce replicative senescence *in vitro* is based on the exposure of fibroblasts to low doses of oxidants.[23–25] EF_{28} cells, grown in the continuous presence of 100 μM diethylmaleate (DEM), a glutathione depleting agent, stop growing after about 6 days, as demonstrated by their BrdU incorporation (about 5%). Accordingly, p21 mRNA levels significantly increased and many cells became SAβ-gal positive (>45%) (data not shown). In EF_{28} cells, after 6 days of mild oxidative stress, PTGS1 and Fmod mRNA levels are both decreased about six- and four-fold, respectively, compared to untreated cells and this modification is again opposite to that observed in OF cells compared to both YF populations. Therefore, the behaviour of PTGS1 and Fmod mRNAs demonstrate that the molecular phenotypes of *in vitro* induced senescence and *in vivo* acquired aging are distinguishable.

Two other genes analyzed are cathepsin C (CatC) and metalloproteinase (HME). As shown in Table 1, the first is expressed at similar levels in both EF_{28} and EF_{60} cells and in EF_{28} cells exposed to low concentrations of DEM, and no significant difference is seen comparing both OF1 and OF2 cells with the counterparts taken from young donors or with the no longer dividing $OF1_{25}$ cells. HME mRNA was

always increased, both in OF cells compared to YF, in EF_{28} vs EF_{60} and in EF_{28} cells following mild oxidative stress. Therefore, HME mRNA modifications appear to be similar to those observed for $p21^{waf1}$: they cannot distinguish between *in vitro* acquired senescence and *in vivo* cellular aging.

Five other genes showed a behaviour completely different from those described above. Table 1 shows that the mRNAs encoding cyclin A (CycA), cyclin F (CycF), thymidylate synthase (TS), hepatocyte nuclear factor-3/fork head homolog 11A (HFH-11A) and cyclin-sensitive ubiquitin carrier protein (Ucar) are many fold decreased in EF_{60} cells compared to EF_{28}. Similarly, EF_{28} cells exposed to low doses of DEM displayed a significant decrease of these mRNAs after 2 days of treatment, except for TS mRNA whose level decreases starting from day 4. It is worth noting that the extent of the decreases observed in the cells treated for 6 days with DEM are, in many cases, several fold higher that those observed in EF_{60} cells.

Surprisingly, OF1 and OF2 cells compared to YF1 and YF2 cells did not show any significant change in the expression of these mRNAs (Table 1, columns 1,2). On the contrary, OF1 cells, grown until they have reached 25 PDL ($OF1_{25}$) and no longer incorporating BrdU, show a significant decrease of the mRNA levels of all the five genes (Table 1, column 3). Therefore, the levels of these five genes appear to be modified only in cells which have ceased to grow, regardless their origin.

In summary, these experiments demonstrate that it is possible to distinguish the molecular phenotype of human fibroblasts which have acquired the senescent phenotype *in vitro*, either by repeated *in vitro* passages or by mild oxidant treatment, with that of fibroblasts taken from old individuals (i.e. *in vivo* acquired senescence). Two examples of the differences between *in vitro* and *in vivo* acquired senescence are the genes encoding prostaglandin endoperoxidase synthase and fibromodulin. Their opposite behaviours in the two senescent phenotypes support the hypothesis that other genes could also be regulated in a similar fashion.

The existence of a gene expression profile characteristic of *in vitro* senescent cells, regardless of their origin, is suggested by the changes observed in the expression of some other genes, including cyclin A, cyclin F, thymidylate synthase, hepatocyte nuclear factor-3/fork head homolog 11A and cyclin-sensitive ubiquitin carrier protein. All these genes encode proteins that play a role in cell cycle progression, and it is reasonable that in a cell, no longer dividing but still surviving, many of the genes involved in S-, G2- and M-phases of the cell cycle are downregulated.

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