Cellular stress in vitro and longevity in vivo
Dekker, P.

Chapter 4

Relation between maximum replicative capacity and oxidative stress-induced responses in human skin fibroblasts *in vitro*

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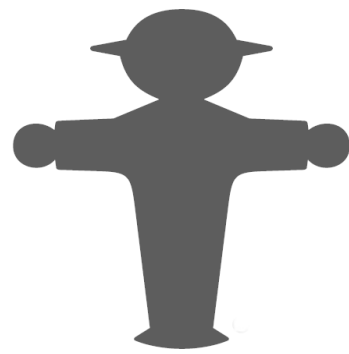
Summary

Cellular senescence, an important factor in aging phenotypes, can be induced by replicative exhaustion or by stress. We investigated the relation between maximum replicative capacity, telomere length, stress-induced cellular senescence and apoptosis/cell death in human primary fibroblast strains obtained from nonagenarians of the Leiden 85-plus Study. Fibroblast strains were cultured until replicative senescence and stressed with rotenone at low passage. Telomere length, Senescence Associated-β-galactosidase (SA-β-gal) activity, Sub-G1 content and Annexin-V/PI positivity were measured in non-stressed and stressed conditions. Fibroblast strains with a higher replicative capacity had longer telomeres (p=0.054). In non-stressed conditions replicative capacity was not associated with β-gal activity (p=0.07) and negatively with Sub-G1 (p=0.008). In rotenone-stressed conditions replicative capacity was negatively associated with β-gal activity (p=0.034) and positively with Sub-G1 (p=0.07). Summarizing, fibroblast strains with a higher maximum replicative capacity have longer telomeres, are less prone to go into stress-induced cellular senescence and more prone to die after stress.

Introduction

Cellular senescence and apoptosis are suggested to be important drivers of aging phenotypes and age related diseases such as diabetes mellitus and coronary heart disease (1). Cellular senescence is characterized by an irreversible state of replication arrest and can be induced by two factors. Firstly, after a finite number of cell population doublings (PDs) cells will cease to divide, a phenomenon also known as the Hayflick limit or Replicative Senescence (RS) (2). Current knowledge indicates that this form of senescence is mostly telomere driven, which means that with every cell division the telomere length shortens until it reaches a critical length after which the cell stops dividing (3). Secondly, cells can become senescent by exposure to stress which is called Stress-Induced-Premature-Senescence (SIPS) (4). Reactive oxygen species (ROS) seem to play a major role in this process because they cause damage to DNA and proteins, resulting in an accelerated accumulation of mutations which eventually causes early senescence (5).

Although cellular senescence can be induced by replicative exhaustion and by stress, the relation between RS and SIPS has remained largely unknown. It has been shown that fibroblasts aged *in vitro*, i.e. having undergone PDs *in vitro*, are more prone to go into stress-induced cellular senescence than fibroblasts that have undergone less PDs (6). If this is indeed the case, fibroblast strains from subjects of the same chronological age but with different maximum replicative capacities may have undergone different numbers of PDs during *in vivo* life history, affecting the response to stress-induced cellular senescence *in vitro*. Factors determining a long-term cell fate decision, undergoing senescence or apoptosis, are not yet fully understood, but there seems to be a clear dependence on cell type and the nature and extent of the damage (7). The transcriptional regulator p53 plays an important role in both senescence and apoptosis and in the interaction between these processes. DNA-damage is one of the important stimuli activating p53, which subsequently regulates the expression and activity of the cell cycle inhibitors p21 (8) and later p16 (9). If the DNA-damage is reversible, p53 activity will decrease after quick repair. However, when repair is slow and/or incomplete, p53 activity is sustained and p16 will also be induced in most cells, which will enter the senescence state. Indeed, earlier we showed that most

stress-induced Senescence Associated β-galactosidase (SA-β-gal) positive fibroblasts are also p16-positive, but not exclusively so (10). Furthermore, p53 plays a major role in replicative senescence induced by telomere erosion (11;12). When telomeres reach a critical length they will resemble broken DNA-strands which cells will recognize as DNA-damage and will respond accordingly, in a p16-independent fashion (8).

In order to investigate if there is a relation between RS and SIPS, we tested fibroblast strains with a range of maximum replicative capacities in non-stressed conditions and on their ability to react to oxidative stress. We are in the unique position to have access to a large number of fibroblasts strains which have been cultured to the end of their replicative potential, but which have also been stored frozen at low PDs. We hypothesize that fibroblast strains with a higher replicative capacity will have longer telomeres. Furthermore, if telomere shortening also plays an important role in SIPS, fibroblast strains with a higher replicative capacity will be more resistant to oxidative stress-induced cellular senescence and apoptosis/cell death at low PDs.

Materials and methods

Study design

The Leiden 85-Plus Study (13) is a prospective population-based study in which all inhabitants aged 85 year or older of the city of Leiden, the Netherlands, were invited to take part. Between September 1997 and September 1999, 599 out of 705 eligible subjects (85%) were enrolled. All participants were followed for mortality and 275 subjects survived to the age of 90 years. During the period December 2003 up to May 2004, a biobank was established from fibroblasts cultivated from skin biopsies from 68 of the 275 surviving 90-year-old participants.

Cell strains and maximum replicative capacity

Fibroblast strains were isolated from biopsies of the upper medial arm obtained from 68 participants of the Leiden 85-plus Study at the age of 90 years (14). All fibroblast strains were cultured under highly standardized conditions and serially passaged until the onset of replicative cellular senescence. Maximum replicative capacity was defined as maximum PDs and differed significantly between individual strains (14). Thirty strains with maximum replicative capacity, ranging from 51 to 108 PDs, were randomly selected. One strain with the highest maximum replicative capacity was found to yield results that were not in line with the results for the other strains. Since the highest maximum replicative capacity differed 3.5xSD of the mean of the other strains, this strain was considered to be an outlier and was not included in further analyses.

The relation between known maximum replicative capacity and markers of cellular senescence and apoptosis/cell death was determined at low passage, on average at PD 21 ± 3 ($\pm$SD, range 17-30). Since the fibroblast strains entered phase IIb at PD 52 on average, ranging from PD 26 to 79, these strains were in phase IIa when the experiments were carried out.

Culture conditions

All fibroblasts cultures were grown in D-MEM:F-12 (1:1) medium supplemented with 10% fetal calf serum (FCS) (batch no. 40G4932F), 1 mM MEM sodium pyruvate, 10 mM HEPES, 2 mM glutamax I, antibiotics (100 Units/mL penicillin, 100 µg/mL streptomycin, and 0.25–2.5 µg/mL amphotericin B, all obtained from Gibco, Breda, the Netherlands) incubated at 37°C with 5% CO_2 and 100% humidity. Trypsin (Sigma, St Louis, MO, USA) was used to split cells using a 1:4 ratio each time they reached 80-100% confluence.

Experimental set-up

To test the inducibility of SIPS, cells were thawed from frozen stocks on day zero. On day one, the medium was changed and on day four cells were passaged from 25-cm^2 flasks to 75-cm^2 flasks. Cells were passaged further on days six and eight. To have similar confluences for experiments, cells were counted on day 8 and subcultured in equal numbers.

On day 11, fibroblasts were seeded for the experiment, a remaining part was kept in culture for repeat experiments.

Fibroblasts were stressed using 600 µM rotenone (Sigma, St Louis, MO, USA) for three days. Rotenone is known to induce ROS at the mitochondrial level (15) and increased ROS induce cellular senescence (16). Rotenone-induced increased levels of ROS were confirmed for the experiments described here (data not shown). Because rotenone is light-sensitive, experiments were performed in darkness as much as possible. Samples were prepared three days after exposure to a stressor (day 14). Stock solutions of rotenone were prepared in DMSO at a concentration of 500 µM and stored at -40°C (aliquots). Initial tests sho wed that DMSO (0.2%) did not affect the results. Experiments were repeated for each cell strain and each condition and were performed in batches of maximally four strains simultaneously.

Telomere length analysis

Telomere length was measured for 19 of 30 randomly chosen fibroblast strains in non-stressed conditions. To measure telomere length, a flow-FISH kit was used (DAKO, Heverlee, Belgium) and fibroblasts were treated according to the manufacturer protocol. In short, fibroblasts were trypsinized, mixed with the reference cell line (line 1301, Banca Biologica e Cell Factory, Genoa, Italy), hybridized without and with Cy3-labeled PNA-probe. After labeling with propidium iodide for DNA-content, samples were measured on a FACS Calibur II flow cytometer (Becton Dickinson, Franklin Lakes, USA). The probe-signal was measured in the FITC-channel and the PI signal in the PE-Texas Red channel. Results were calculated according to the manufacturer's protocol.

Flow cytometric measurement of SA-β-gal activity

Cells were seeded at 1000 cells/cm^2 in 25-cm^2 flasks. Cells were prepared as described recently (10). In short, to change the lysosomal pH to pH 6, cells were incubated with medium containing 100 nM bafilomycin A1 (VWR, Amsterdam, the Netherlands) for one hour. Cells were then incubated with 33 µM of the β-galactosidase substrate C$_{12}$FDG (Invitrogen, Breda, The Netherlands), in the presence of 100 nM bafilomycin. After trypsinisation, cells were washed once and resuspended in 200 µl ice cold PBS. Cells were measured in the

FITC-channel and analysis was performed on the median fluorescence intensity (MdFI) values.

Flow cytometric Annexin V/PI analysis

Cells were seeded at 1000 cells/cm^2 in 75-cm^2 flasks. Sample preparation was performed on ice. Aspirated medium and washes were collected so that any floating cells and cell debris indicating cell death would be included in the analysis. Cells were trypsinized and washed with phosphate buffered saline (PBS). The suspension was divided over two tubes, one for Annexin V/PI analysis and one for cell cycle analysis. For the Annexin V/propidium iodide (PI) analysis, the TACS Annexin V-FITC kit was used (R&D Systems, Abingdon, United Kingdom). Cells were processed according to the manufacturer's guidelines. Analysis was performed on a FACS Calibur II flow cytometer (Becton Dickinson). The Annexin V-FITC signal was measured in the FITC-channel and the PI signal in the PE-Texas Red channel. Cells were gated into quadrants and Annexin V positive/PI negative (Annexin V+/PI-) and Annexin V positive/PI positive (Annexin V+/PI+) fibroblasts were analyzed as percentages of the total cell population.

Flow cytometric cell cycle analysis

After trypsinisation, fibroblasts were centrifuged at 1000 rpm for five minutes, washed by resuspending in PBS, centrifuged again and resuspended in 200 µl 70% ethanol. Samples were kept at -40℃ at least overnight. After adding 1 mL PBS, fibroblasts were centrifuged at 2000 rpm for 5 minutes and resuspended in 200 µl PBS containing 50 µg/mL PI and 20 µg/mL RNAse (Sigma, St Louis, MO, USA). Fibroblasts were stored overnight at 4℃ and measured in the PE-Texas Red channel. In the resulting histograms, Sub-G1 events (dead cells and cell debris) were gated and analyzed as percentages of the total cell population.

Statistics

All analyses were performed with the software package SPSS 16.0.01 (SPSS Inc., Chicago, IL). Fibroblast strains were tested in batches of maximally four strains simultaneously. No intra-experiment replicates were used but experiments were repeated for each strain (in the same batches) one passage later. Values were acquired for non-stressed conditions and for

rotenone-treated conditions of each strain and parameter. The former was then subtracted from the latter to acquire rotenone-induced increases. For parameters of telomere length, cellular senescence and cell death/apoptosis, linear regression analysis was performed between values for the non-stressed condition and maximum replicative capacity and between rotenone-induced increases and maximum replicative capacity, using a linear mixed model taking into account the duplicate experiments as repeated measures and the batches of experiments as random effect. The different batches of strains were run in two rounds of multiple experiments. To be able to better compare the results of these two rounds, the data of the SA-β-gal assay were normalized by z-transforming the data within these rounds of experiments and use these values for the statistical analysis.

Results

Baseline characteristics of the subjects from whom the tested fibroblast strains were derived are summarized in Table 1. The mean maximum replicative capacity was 71±11 (mean±SD) PDs, ranging from 51 to 94. Table 2 shows the rotenone-induced increases in the measured parameters regardless of the maximum replicative capacity of the fibroblast strains. Telomere length was measured for fibroblast strains in non-stressed conditions. A positive relation was found between maximum replicative

Figure 1. Correlation between maximum replicative capacity (PDs) of human fibroblasts and telomere length (% of telomere length of reference cell line 1301). P-value is result of linear mixed model.

capacity and telomere length (p=0.054, Figure 1), which was borderline significant. For non-stressed fibroblasts no significant correlation was found between maximum replicative capacity and SA-β-gal activity (Table 3, Figure 2A), whereas rotenone-stressed fibroblasts showed a significant negative relation (Table 3, Figure 2B).
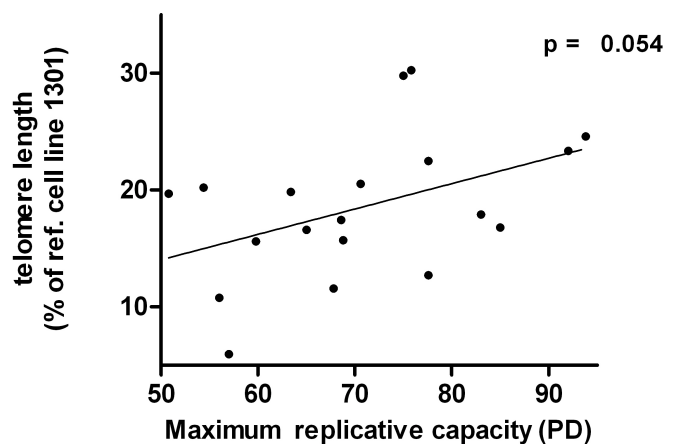
Table 1. Clinical characteristics and fibroblast growth characteristics of subjects from the Leiden 85-plus Study (N=29).

	Leiden 85-plus Study
Demographic and anthropometric data	
N (female)	18
Age (years, mean ± SD)	90.0 (0.6)
Height (cm, mean ± SD)	162 (10)
Weight (kg, mean ± SD)	72.0 (11)
Current smokers – no./total known	2/29
Growth characteristics of fibroblast strains	
PD at which strains were tested (mean ± SD, range)	21 (3), 17-30
Onset phase IIb (PD, mean ± SD, range)*	52 (12), 26-79
Onset phase III, replicative capacity (PD, mean ± SD, range)†	71 (11), 51-94
Growth speed phase IIa (PD per day, mean ± SD, range)*	0.28 (0.04), 0.20-0.36
Growth speed phase IIb (PD per day, mean ± SD, range)*	0.09 (0.04), 0.03-0.19

* according to change-point model (14)

† number of observed PD during serial culturing

PD=population doubling

Table 2. Stress-induced increases in parameters measured in human fibroblasts (N=29 strains). Values are given as mean (SE).

Parameter	Non-treated	0.6 µM rotenone, 3 days	p
β-gal FACS, z-score of MdFI	-0.55 (0.83)	0.52 (0.79)	< 0.001
Sub-G1 (%)	5.4 (0.7)	22.7 (0.7)	< 0.001
Annexin V+/PI- cells, %	1.21 (0.38)	7.15 (0.38)	< 0.001
Annexin V+/PI+ cells, %	3.02 (0.46)	6.67 (0.46)	< 0.001

Data were analyzed with a linear mixed model, taking into account different batches of experiments and repeated measures (duplicate experiments). P-values are results of linear mixed models.

To determine the level of cell death, levels of Sub-G1 cell debris were measured. In non-stressed conditions there was a significant negative correlation between maximum replicative capacity and Sub-G1 content (Table 3, Figure 2C). In rotenone-stressed conditions a positive correlation was found, which was borderline significant (Table 3, Figure 2D). Apoptosis was assessed by the Annexin V/PI assay. Results were tested for correlations between maximum replicative capacity and percentage Annexin V+/PI- (early apoptotic) and Annexin V+/PI+ (late apoptotic or necrotic) fibroblasts. For both Annexin Annexin V+/PI-and Annexin V+/PI+ fibroblasts, no significant correlations were found under non-stressed conditions nor under rotenone-stressed conditions (Table 3).
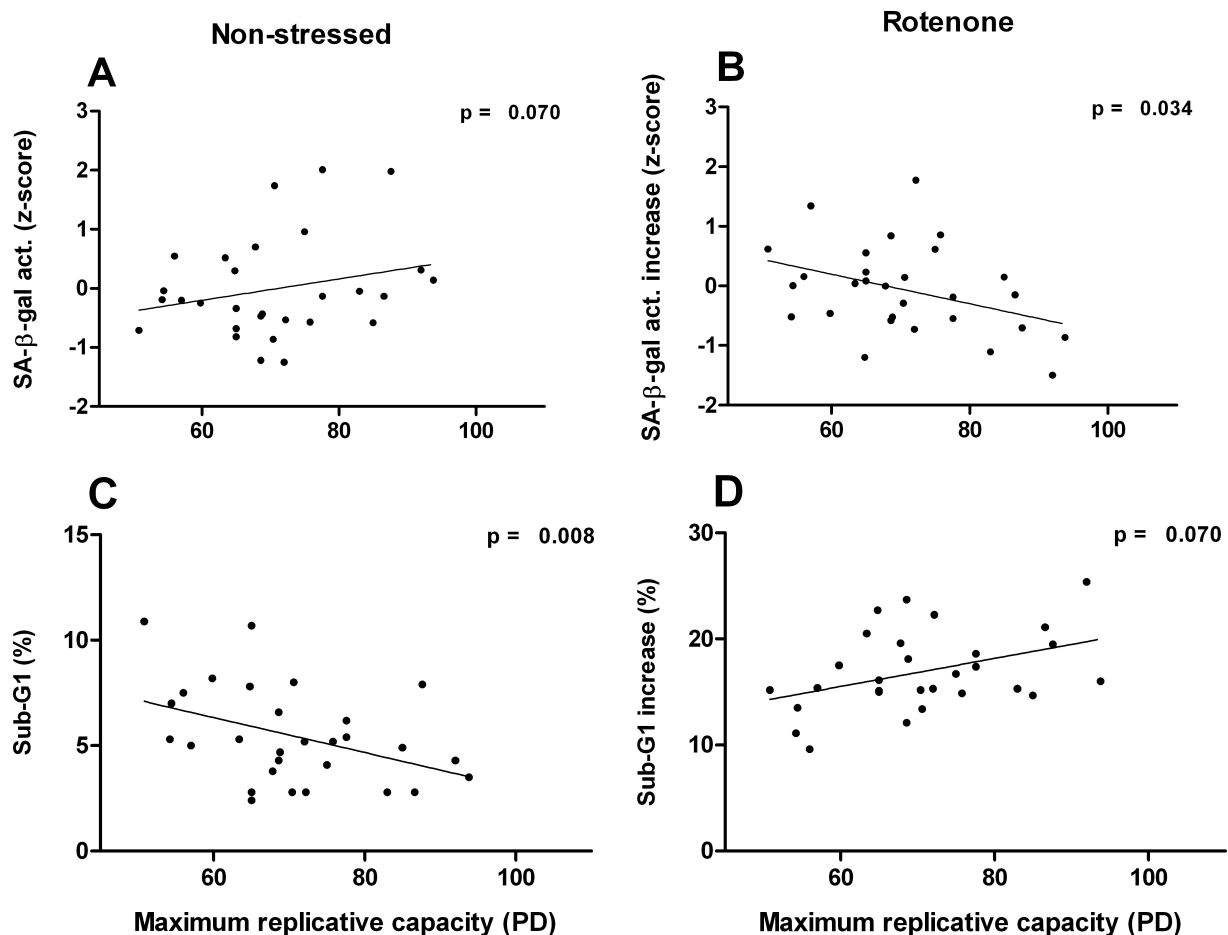

Figure 2. Correlation between maximum replicative capacity and markers of cellular senescence (SA- β-gal activity; A and B) and cell damage (sub-G1 cell debris; C and D) under non-stressed and rotenone-stressed conditions. Due to batch differences SA-β-gal values were z-tranformed.

Table 3. Relation between maximum replicative capacity, telomere length and markers of stress induced cellular senescence (SA-β-gal activity), cell damage (Sub-G1 cell debris) and apoptosis (AnnexinV/PI) under non-stressed and rotenone-stressed conditions in human fibroblasts (N=29 strains).

	Change/PD (SE)	p
Non-stressed		
Telomere length, % of ref. cell line 1301	0.193 (0.095)	0.054
SA-β-gal activity, z-score of MdFI	0.022 (0.011)	0.070
Sub-G1, %	-0.080 (0.029)	**0.008**
Annexin V+ / PI- cells, %	-0.003 (0.007)	0.69
Annexin V+ / PI+ cells, %	-0.005 (0.019)	0.81
600nM Rotenone, 3d		
SA-β-gal activity, z-score of MdFI	-0.024 (0.011)	**0.034**
Sub-G1, %	0.121 (0.066)	0.070
Annexin V+/PI- cells, %	0.044 (0.041)	0.29
Annexin V+/PI+ cells, %	-0.031 (0.031)	0.32

Data were analyzed with a linear mixed model, taking into account different batches of experiments and repeated measures (duplicate experiments). P-values are results of linear mixed models.

Discussion

The aim of this study was to investigate whether observed variations in maximum replicative capacities of fibroblast strains derived from the oldest old correlate with telomere length and inducibility of stress-induced parameters of cell death and cellular senescence *in vitro*. We found that fibroblast strains with a higher maximum replicative capacity have longer telomeres, are less prone to go into stress-induced cellular senescence and more prone to die after exposure to stress.

Telomere shortening and senescence

There is much variation in maximum replicative capacity of human fibroblast *in vitro* (14). This could be due to interindividual differences in number of PDs obtained during *in vivo* life history and/or differences in intrinsic replicative capacity and telomere length, which is supported by the observation that telomere length varies already considerable very early in life (17). Telomeres clearly play an important role in cellular senescence since *in vitro,* every cell lacking the enzyme telomerase can undergo a limited number of cell divisions before becoming senescent. Even so, murine cells *in vitro* senesce after a few PDs despite expressing telomerase (7). Hence it is questionable whether data that have been used for establishing the maximum replicative capacity of cells *in vitro* are a reliable reflection of the real maximum replicative capacity *in vivo*.

However, it has been described that telomere length is a good indicator for the maximum replicative capacity of fibroblasts (17). Indeed, we found that fibroblasts with a high maximum replicative capacity had long telomeres, although the correlation was borderline significant due to the small samples size tested. Thus, fibroblasts already having shorter telomeres (i.e. lower maximum replicative capacity) might enter the senescent state faster than fibroblasts with longer telomeres (i.e. higher maximum replicative capacity).

Oxidative stress accelerates the rate of telomere shortening, mainly because of insufficient repair mechanisms (18;19). We did not measure telomere length after induction of acute stress, but it is unlikely that after three days of stress telomeres would have shortened measurably within a cell, since shortening of telomeres is the result of cell division.

It has been shown that high passage fibroblasts *in vitro* (i.e. more PDs) show more stress-induced cellular senescence when compared with low passage fibroblasts of the same strain (6). It could be argued that a fibroblast strain with a higher maximum replicative capacitiy *in vitro*, underwent less PDs *in vivo* (and as such has longer telomeres), and would thus be less inclined to go into stress-induced cellular senescence at low passage *in vitro*. Our results seem to be consistent with the data of other workers (6) since we found a significant negative correlation between maximum replicative capacity and stress-induced cellular senescence as measured by SA-β-gal activity, suggesting that fibroblasts with a higher maximum replicative capacity would indeed be less prone to go into cellular senescence.

Since there is debate about the validity of SA-β-gal as a marker of cellular senescence, we earlier performed a proof-of-principle experiment to ascertain to what extent our SA-β-gal results are consistent with the more conventional marker of cellular senescence p16 (10). From this we concluded that the increase in SA-β-gal activity after rotenone treatment does indeed reflect senescence.

Apoptosis and senescence

Cell death, and more specifically apoptosis, is an important factor in tissue dysfunction during the aging process (7;20). Possibly, apoptosis contributes even more to this process than cellular senescence because dysfunctional cells are removed altogether. In healthy tissues these cells are replaced with new ones from a pool of proliferative cells to prevent tissue atrophy. It has already been described that, in contrast to cellular senescence, *in vitro* aged fibroblasts are increasingly resistant to oxidative stress-induced apoptosis (7;21). This would be consistent with the fact that we found a positive correlation between maximum replicative capacity and amounts of stress-induced sub-G1 cell debris. Furthermore, under non-stressed conditions there was a negative correlation between maximum replicative capacity and the amount of sub-G1 cell debris. This is especially striking since earlier we described that fibroblasts derived from chronologically young subjects, when compared with fibroblasts from chronologically old subjects, also show less sub-G1 cell debris under non-stressed conditions and more stress-induced sub-G1 cell debris (22). This would suggest that fibroblast strains with a high maximum replicative capacity but derived from old subjects are more comparable to fibroblasts strains from young subjects than from old subjects (with average maximum replicative capacity), i.e. are biologically younger. The sub-G1 results were not corroborated by the Annexin V/PI results, for which no significant relations were found. Possibly, we missed the early phase of the apoptotic response and were only able to measure the end result (sub-G1 cell debris).

Culture conditions

To determine stress resistance in fibroblasts, strains were selected on their maximum replicative capacity *in vitro* (14). Not much is known about the process of culturing itself but it is suggested to be an important stressor by which fibroblasts undergo cellular senescence

following the pathway of SIPS (23). An important factor in the process of culturing is the concentration of oxygen (pO_2) to which fibroblasts are exposed. The pO_2 in most tissues is much lower than the atmospheric pO_2 which is often used for cell culture. These hyperoxic conditions are a significant stress for cells. Murine fibroblasts go into senescence after only a few PDs at 20% oxygen but proliferate much longer at 5% oxygen (24). Although less dramatic, a similar effect can be observed for human fibroblasts, which grow considerably slower in 20% oxygen when compared with 5% oxygen (25). Additionally, the density at which fibroblasts are grown determines to what extent hyperoxic conditions affect growth speed (25). Furthermore, when fibroblasts are explanted from skin biopsies, they are removed from their normal environment in which they were exposed to lower pO_2, different levels and types of growth factors and many other factors (e.g. cytokines, metabolites).

Summarizing, fibroblast strains with a higher maximum replicative capacity seemed to have longer telomeres, are less prone to go into stress-induced cellular senescence and more sensitive to stress-induced stimuli, as measured by levels of sub-G1 cell debris. These results suggest that replicative cellular senescence and stress-induced premature cellular senescence share interacting cellular pathways, which should be determined within individual cell strains in further studies.

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