

Chapter 3

Stress-induced responses of human skin fibroblasts *in vitro* reflect human longevity

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Summary

Unlike various model organisms, cellular responses to stress have not been related to human longevity. We investigated cellular responses to stress in skin fibroblasts that were isolated from young and very old subjects, and from offspring of nonagenarian siblings and their partners, representatives of the general population. Fibroblast strains were exposed to rotenone and hyperglycemia and assessed for Senescence Associated- β -galactosidase (SA- β -gal) activity by flow cytometry. Apoptosis/cell death was measured with the Annexin-V/PI assay and cell-cycle analysis (Sub-G1 content) and growth potential was determined by the colony formation assay. Compared with fibroblast strains from young subjects, baseline SA- β -gal activity was higher in fibroblast strains from old subjects ($p=0.004$) as were stress-induced increases (rotenone: $p<0.001$, hyperglycemia: $p=0.027$). For measures of apoptosis/cell death, fibroblast strains from old subjects showed higher baseline levels (AnnexinV+/PI+ cells: $p=0.040$, Sub-G1: $p=0.014$) and smaller stress-induced increases (Sub-G1: $p=0.018$) than fibroblast strains from young subjects. Numbers and total size of colonies under non-stressed conditions were higher for fibroblast strains from young subjects ($p=0.017$ and $p=0.006$ respectively). Baseline levels of SA- β -gal activity and apoptosis/cell death were not different between fibroblast strains from offspring and partner. For fibroblast strains from offspring, stress-induced greater were smaller for SA- β -gal activity (rotenone: $p=0.064$, hyperglycemia: $p<0.001$) and greater for apoptosis/cell death (Annexin V+/PI- cells: $p=0.041$, AnnexinV+/PI+ cells: $p=0.008$). Numbers and total size of colonies under non-stressed conditions were higher for fibroblast strains from offspring ($p=0.001$ and $p=0.024$ respectively) whereas rotenone-induced decreases were smaller ($p=0.008$ and $p=0.004$ respectively). These data provide strong support for the hypothesis that *in vitro* cellular responses to stress reflect the propensity for human longevity.

Introduction

Organisms are continuously exposed to intrinsic and environmental stressors. Many of these stressors result in the formation of Reactive Oxygen Species (ROS) that are thought to play an important role in aging and age-related diseases (1). Low levels of ROS may be essential to maintain homeostasis, but excess ROS will cause damage to important macromolecules, including DNA, proteins and lipids (2). Organisms with self-renewable tissues can replace damaged cells, rejuvenating tissues to maintain body homeostasis, depending on the balance between cell proliferation and cell death. However, as proliferating cells can transform into cancer cells as a result of genomic damage, several protective mechanisms have evolved to prevent cells from becoming neoplastic (3;4). Faced with damage, a proliferating cell can take several routes, depending on the cell type, and the type and extent of damage that has occurred. First, it can arrest the cell cycle, repair the damage and continue to proliferate. Second, it can permanently stop proliferating and become senescent (5;6). Third, it can be removed in a controlled manner by apoptosis or in an uncontrolled manner by necrosis (7).

Though ROS-induced damage can cause cells to go into apoptosis or senescence, little is known about the relative contributions of these cellular fates in response to stressors and how these change with age. Conversely, mechanisms that determine cellular repair and cell turnover may directly influence the rate of aging. Amongst others, these may include (epi-) genetic factors that determine cellular responses to stressors. So far, evidence for a direct link between cellular stress responses and the rate of aging has come only from model organisms. Between species, fibroblasts from longer living animals (i.e. different genetic background) have a higher resistance to stress *in vitro* (8;9), and within species, fibroblasts from various mutant, long-lived mice were found to be more stress-resistant than their wild-type counterparts (10). Conversely, mutant mice having an enhanced p53 response, associated with excessive induction of apoptosis and senescence, were shown to have a higher cancer resistance but a shortened longevity (11).

In humans, *in vitro* cellular stress responses have not been related to longevity so far. To study how cellular responses to stress may change during aging, we compared stress-induced increases in markers of senescence and apoptosis in fibroblast strains derived from

young and very old subjects. We also studied fibroblast strains from offspring of nonagenarian siblings and their partners with a similar chronological age but who represent the average of the general population. We hypothesized that fibroblasts from old subjects would be more prone to go into senescence and into apoptosis when compared with fibroblasts from young subjects and that fibroblasts from subjects with the propensity for longevity (i.e. biologically younger) would show more resistance to go into senescence and apoptosis compared with fibroblasts of their partners.

Materials and Methods

Study design

The Leiden 85-Plus Study (12) 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. During the period August to November 2006, we also established a biobank from biopsies taken from 27 young subjects (23-29 years old). Given the extreme age difference of almost 65 years between the groups of old and young subjects, a relatively small sample size of 10 subjects per age group was chosen, consisting of 5 fibroblast strains from the 42 nonagenarian females, 5 strains from the 26 nonagenarian males, 7 strains 24 young females and the 3 available strains of young males (Table 2).

The Leiden Longevity Study (13) was set up to investigate the contribution of genetic factors to healthy longevity by establishing a cohort enriched for familial longevity. From July 2002 to May 2006, 420 families were recruited consisting of 991 long-lived Caucasian siblings together with their 1705 of their offspring and 760 of the partners thereof. There were no selection criteria on health or demographic characteristics. Compared with their partners, the offspring were shown to have a 30% lower mortality rate and a lower prevalence of cardio-

metabolic diseases (13;14). During the period November 2006 and May 2008, a biobank was established from fibroblasts cultivated from skin biopsies from 150 offspring-partner pairs. Because it was expected that the difference in biological age between the offspring and partner groups would be much smaller than the extreme difference in chronological age between the groups of old and young subjects, a relatively large sample size of 80 strains from 40 couples was chosen, consisting of 20 fibroblast strains from the 65 female offspring, 20 strains from the 86 male offspring, as well as 20 strains from the 86 female partners and 20 strains from the 63 male partners (Table 2).

Fibroblast Cultures

Three-mm (Leiden 85-plus Study) and 4-mm skin biopsies (LLS) were taken from the sun unexposed medial side of the upper arm. Fibroblasts were grown in D-MEM:F-12 (1:1) medium supplemented with 10% fetal calf serum (FCS), 1 mM MEM sodium pyruvate, 10 mM HEPES, 2 mM glutamax I, and 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. Different FCS batches were used for fibroblasts from the Leiden 85-plus Study (Gibco, batch no. 40G4932F) and for fibroblasts from the LLS (Bodinco, Alkmaar, the Netherlands, batch no. 162229). This medium will be referred to as standard medium. Fibroblasts were incubated at 37°C with 5% CO₂ and 100% humidity. All cultures that are used in the present study were grown under predefined, highly standardized conditions as published earlier (15) and frozen at low passage.

Experimental setup

Experiments were set up in batches of maximally 10 fibroblast strains which were thawed from frozen stocks on day zero. On day one, the medium was changed and on day four fibroblasts were passaged 1:4 and passaged further in equal numbers on days six and eight to have similar confluences for experiments. On day 11, fibroblasts were seeded for experiments.

To induce stress we used rotenone and hyperglycemia. Rotenone is known to induce ROS at the mitochondrial level (16) and increased ROS can induce senescence (17). Hyperglycemia has also been shown to induce increased ROS at the cellular level (18) as well as premature

senescence in human skin fibroblasts (19). In pilot experiments, fibroblasts were stressed with 0.2 μM and 1 μM rotenone and with 111 mM glucose for three days and seven days. Senescence was measured by SA- β -gal activity and apoptosis by sub-G1 cell debris and by Annexin V/PI assay. Exposure to 1 μM rotenone induced apoptosis after three days and most fibroblasts had died after seven days. Exposure to 0.2 μM rotenone and 111 mM glucose did not induce appreciable apoptosis after three or seven days, but did induce senescence after seven days, more so than after three days. Based on these results, it was decided to measure apoptosis at three days and senescence at seven days after exposure to stress.

Stock solutions of rotenone were prepared in DMSO (both from Sigma, St Louis, MO, USA) at a concentration of 100 μM and stored at -40°C in aliquots. Initial tests showed that DMSO (0.2%) did not affect the results. Hyperglycemic medium was prepared by adding 17 g/L D-glucose (Sigma, St Louis, MO, USA) to standard medium, resulting in a final concentration of 20 g/L (111 mM) glucose. For the seven-day time point, medium was replaced with freshly supplemented medium after three days.

Experiments were repeated for each fibroblast strain and each condition. The experimental conditions and measured parameters are summarized in Table 1.

Table 1. Experimental conditions and measured parameters.

	Stressor	
	3 days	7 days
Sub-G1 (%)	1 μM rotenone	nd
Annexin V+/PI- cells (%)		
Annexin V+/PI+ cells (%)		
FACS SA- β -gal activity (MdfI)	nd	0.2 μM rotenone 111 mM glucose
Colony Formation assay (# colonies & total area in mm^2)		

nd: not done.

Senescence

To determine cellular senescence we used the widely described marker SA- β -gal activity. Earlier we demonstrated the validity and superiority of the flow cytometric method over the cytochemical method measuring SA- β -gal activity in human fibroblasts (20). In short, fibroblasts were seeded at 130 cells/cm² in 75-cm² flasks and were prepared as described by Kurz *et al.* (21). To change the lysosomal pH to pH 6, fibroblasts were incubated with medium containing 100 nM bafilomycin A1 (VWR, Amsterdam, the Netherlands) for one hour and then incubated with 33 μ M of the β -gal substrate C₁₂FDG (Invitrogen, Breda, The Netherlands) in the presence of 100 nM bafilomycin A1. After trypsinisation, fibroblasts were washed once and resuspended in 200 μ l ice cold phosphate buffered saline (PBS) and measured in the FITC-channel. Analysis was performed on the median fluorescence intensity (MdFI) values.

Apoptosis/cell death

Fibroblasts were seeded at 1000 cells/cm² in 75-cm² flasks. Sample preparation was performed on ice. Aspirated medium and washes were collected to include any floating cells and cell debris indicating cell death in the analysis. Fibroblasts were trypsinized and washed with PBS. The suspension was divided over two tubes, one for Annexin V/propidium iodide (PI) analysis and one for cell cycle analysis.

For the flow cytometric Annexin V/PI analysis, the TACS Annexin V-FITC kit was used (R&D Systems, Abingdon, United Kingdom). Fibroblasts were processed according to the manufacturer's guidelines. Analysis was performed on a LSRII flow cytometer (Becton Dickinson, Franklin Lakes, USA). The Annexin V-FITC signal was measured in the FITC-channel and the PI signal in the PE-Texas Red channel. Events 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.

For the flow cytometric cell cycle analysis, fibroblasts were centrifuged at 1000 rpm for 5 minutes, washed by resuspending in PBS, centrifuged again and resuspended in 200 μ l 70% ethanol. Samples were kept at -40°C at least overnight. After adding 1 mL PBS, fibroblasts were centrifuged at 2000 rpm for 5 minutes and resuspended in 200 μ l PBS containing 50 μ g/mL PI and 20 μ g/mL RNase (Sigma, St Louis, MO, USA). Fibroblasts were stored

overnight at 4°C 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.

Colony formation assay

After having been exposed to the stressors for seven days and allowed to grow in standard medium for another week, medium was removed from the dishes, fibroblasts were washed with PBS and fixed with 0.25% gluteraldehyde for 30 minutes. Fibroblasts were washed with water and stained with 0.6 mg/ml Coomassie in 1:6 methanol/water overnight, washed again with water and air dried. Dishes were then scanned with a high resolution Agfa XY-15 flatbed scanner (Agfa Gevaert, Mortsel, Belgium) and colonies were manually scored with the freely available image analysis software package ImageJ 1.37v.

Statistics

All analyses were performed with the software package SPSS 14.00 (SPSS Inc., Chicago, IL). Differences in cellular characteristics between non-stressed and stressed fibroblast strains were compared by Student's *t*-test and were represented as mean \pm SD. Stress-induced increases were calculated by subtracting the values from non-stressed fibroblasts from those of stressed fibroblasts for each strain separately. Non-stressed levels and stress-induced increases were then compared between fibroblast strains from young and old subjects (Leiden 85-plus Study, SA- β -gal activity: n=20, apoptosis/cell death: n=20) and between fibroblast strains from offspring and partners (LLS, SA- β -gal activity: n=40, apoptosis/cell death: n=80). Stress-induced increases were represented as mean \pm SE.

Since experiments were performed in batches of maximally ten fibroblast strains simultaneously, group differences were also compared using linear mixed-effect models (LMMs) to account for repeat experiments and batch effects. Cumulative frequency plots were made for flow cytometry results of SA- β -gal activity to inspect possible changes in distributions of MdfI values of SA- β -gal activity resulting from exposure to stress and from differences between young and old subjects, offspring and partners.

Results

Table 2 shows characteristics for the randomly selected subset of subjects from whom fibroblast strains were tested. Partners and offspring were of similar age, height and weight. As observed in the whole cohort, the prevalences of cardio-metabolic disease were lower in the offspring group as compared with the partners group (14), perhaps illustrating the lower rate of aging among the long-lived families.

Table 2. Comparison of clinical characteristics in young and old subjects from the Leiden 85-plus Study and in offspring and partners from the Leiden Longevity Study.

	Leiden 85-plus Study		Leiden Longevity Study	
	Young	Old	Offspring	Partners
Demographic data				
N (female)	10 (7)	10 (5)	40 (20)	40 (20)
Age (years, mean±SD)	25.5 (1.8)	90.2 (0.3)	58.2 (7.4)	57.1 (8.5)
Anthropometric data				
Height (cm, mean±SD)	177 (10)	164 (7)	171 (8)	173 (8)
Weight (kg, mean±SD)	69.9 (11.1)	67.5 (9.8)	77 (11)	78 (13)
Current smoking– no.	1/8	3/10	3/40	8/39
Diseases				
Myocardial infarction – no./total known	0/10	3/9	0/35 (0)	1/37
Stroke – no./total known	0/10	1/9	1/37 (2.9)	1/35
Hypertension – no./total known.	0/10	7/9	6/35 (17.1)	10/37
Diabetes mellitus – no./total known.	0/10	1/9	1/34 (2.9)	2/36
Malignancies – no./total known	0/10	0/9	1/33 (3.0)	1/35
Chronic obstructive pulmonary disease – no./total known	0/10	1/10	1/34 (2.9)	1/37
Rheumatoid arthritis – no./total known	0/10	0/10	0/72 (0)	0/72

Stress induced changes in markers of senescence, apoptosis/cell death and colony formation

There were significant increases in the distributions of MdFI values (i.e. SA- β -gal activity) in fibroblast strains from young and old subjects after seven days of exposure to stress (non-stressed: 272 ± 90 [mean \pm SD] MdFI, 0.2 μ M rotenone: 544 ± 262 MdFI, 111 mM glucose: 375 ± 261 MdFI, Figure 1A). Similar significant increases were observed in fibroblast strains from offspring and partners (non-stressed: 263 ± 78 MdFI, 0.2 μ M rotenone: 400 ± 112 MdFI, 111 mM glucose: 323 ± 94 MdFI, Figure 2A). For fibroblasts from young and old subjects and from offspring and partners, exposure to 1 μ M rotenone for three days resulted in significant increases of sub-G1 cell debris (young/old, non-stressed: $14.8 \pm 6.4\%$, 1 μ M rotenone $47.3 \pm 5.0\%$; offspring/partners, non-stressed: $18.9 \pm 5.6\%$, 1 μ M rotenone: $44.8 \pm 6.5\%$), Annexin V+/PI- fibroblasts (young/old, non-stressed: $0.13 \pm 0.10\%$, 1 μ M rotenone $7.33 \pm 2.7\%$; offspring/partners, non-stressed: $0.43 \pm 0.31\%$, 1 μ M rotenone: $8.40 \pm 3.06\%$) and Annexin V+/PI+ fibroblasts (young/old, non-stressed: 0.19 ± 0.13 , 1 μ M rotenone $2.77 \pm 0.88\%$; offspring/partners, non-stressed: $0.43 \pm 0.20\%$, 1 μ M rotenone: $1.99 \pm 0.85\%$).

There were significant decreases in colony number for fibroblast strains from young and old subjects after seven days of exposure to stress (non-stressed: 115 ± 78 , 0.2 μ M rotenone: 80 ± 56 , mean \pm SD) and for fibroblast strains from offspring and partners (non-stressed: 164 ± 44 , 0.2 μ M rotenone: 72 ± 43 , 111 mM glucose: 83 ± 47 MdFI, mean \pm SD). All stress induced changes mentioned above were highly significant ($p < 0.0001$) for all parameters measured and showed considerable interindividual variation.

Markers of senescence, apoptosis/cell death and colony formation dependent on differences in chronological and biological age

Under non-stressed conditions, fibroblast strains from old subjects showed significantly higher levels of SA- β -gal activity when compared with fibroblast strains of young subjects (Table 3, Figure 1B). Under non-stressed conditions, there were significantly lower levels of Sub-G1 cell debris for fibroblast strains from young subjects compared with strains from old subjects (Table 3). After exposure to 0.2 μ M rotenone or 111 mM glucose, fibroblast strains from old subjects showed greater increases in SA- β -gal activity than fibroblast strains from young subjects (Table 3, Figure 1C and 1D). After exposure to 1 μ M rotenone, there were

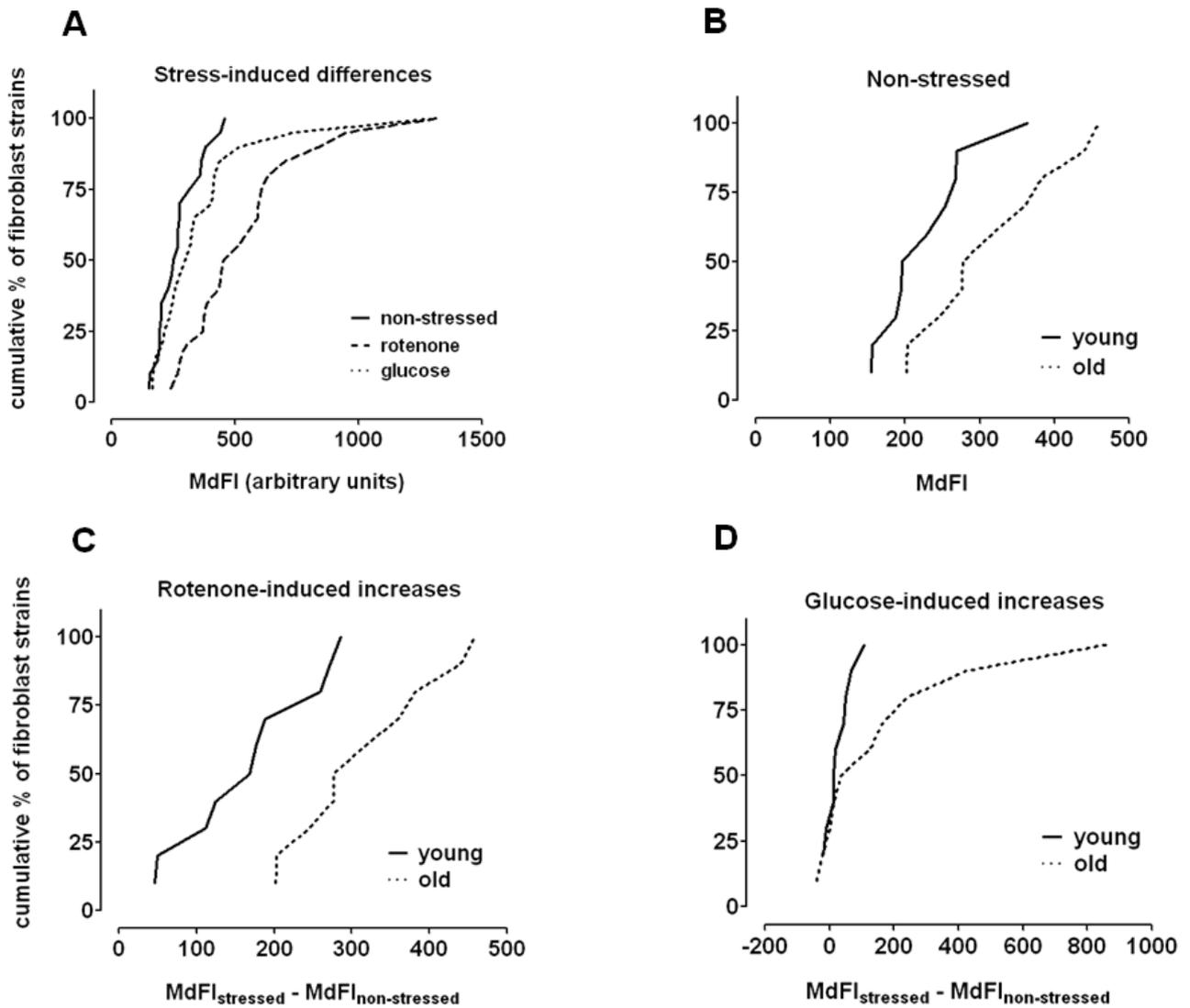


Figure 1 Cumulative frequency plots of SA-β-gal activity in fibroblast strains. A, activity in fibroblast strains under non-stressed conditions, treated with 0.2 μM rotenone and with 111 mM glucose for three days (young and old subjects from the Leiden 85-plus study combined, n=20). B, activity under non-stressed condition in strains from young (n=10) and old (n=10) subjects. C, rotenone-induced increases of activity (stressed minus non-stressed) in young and old subjects. D, hyperglycemia -induced increases of activity in young and old subjects. MdFI: median fluorescence intensity.

Table 3. Markers of senescence and apoptosis/cell death in fibroblast strains from young and old subjects (Leiden 85-plus Study) under non-stressed levels and stress-induced conditions.

	mean (SE)		Δ^a (SE)	P
	Young (n=10)	Old (n=10)		
Non-stressed				
7d				
SA- β -gal FACS (MdFI)	227 (21)	316 (21)	-89 (29)	0.004
3d				
Sub-G1 (%)	12.4 (1.4)	17.2 (1.5)	-4.8 (1.9)	0.014
Annexin V+ / PI- cells (%)	0.14 (0.04)	0.11 (0.04)	+0.03 (0.05)	0.57
Annexin V+ / PI+ cells (%)	0.14 (0.03)	0.23 (0.03)	-0.09 (0.04)	0.040
Stress-induced increase				
0.2 μM rotenone, 7d				
SA- β -gal FACS (MdFI)	+ 168 (36)	+ 377 (36)	-209 (51)	<0.001
111 mM glucose, 7d				
SA- β -gal FACS (MdFI)	+ 26 (47)	+ 180 (47)	-154 (67)	0.027
1 μM rotenone, 3d				
Sub-G1 (%)	+35.8 (2.6)	+29.2 (2.6)	+6.6 (2.6)	0.018
Annexin V+/PI- cells (%)	+7.32 (1.51)	+7.10 (1.51)	+0.22 (1.00)	0.83
Annexin V+/PI+ cells (%)	+2.78 (0.27)	+2.39 (0.27)	+0.39 (0.38)	0.30

Fibroblast strains from young subjects (mean \pm SD age: 25.5 \pm 1.8) were compared with fibroblast strains from very old subjects (mean \pm SD age: 90.3 \pm 2.1). Stress-induced increases: stressed minus non-stressed, MdFI: median fluorescence intensity, a: difference between young and old (young minus old).

significantly greater increases in levels of Sub-G1 cell debris for fibroblast strains from young subjects compared with fibroblast strains from old subjects (Table 3). Under non-stressed conditions, no differences in tested parameters were found between fibroblast strains from offspring and fibroblast strains from partners (Table 4, Figure 2B). After both rotenone- and hyperglycemia-treatment, fibroblast strains from offspring showed smaller increases in SA- β -gal activity than fibroblast strains from partners (Table 4, Figure 2C and 2D), being statistically significant for hyperglycemia-stressed fibroblasts. When stress-induced increases in apoptotic parameters were compared between offspring and partners, increases were

consistently greater in fibroblast strains from offspring (Table 4), being statistically significant for Annexin V+/PI- fibroblasts and Annexin V+/PI+ fibroblasts. Under non-stressed conditions, significantly more colonies were formed in fibroblast strains from young subjects compared with strains from old subjects (Table 5).

Table 4. Markers of senescence and apoptosis/cell death in fibroblast strains from offspring and their partners (Leiden Longevity Study) under non-stressed and stress-induced conditions.

	$n_{\text{offspr}}/$ n_{partner}	mean (SE)		Δ^a (SE)	p
		Offspring	Partner		
Non-stressed					
7d					
SA- β -gal FACS (MdFI)	20/20	269 (23)	266 (23)	3 (16)	0.85
3d					
Sub-G1 (%)	40/39	17.4 (0.8)	16.9 (0.8)	+ 0.5 (1.0)	0.64
Annexin V+/PI- cells (%)	40/40	0.43 (0.05)	0.41 (0.05)	+ 0.02 (0.07)	0.68
Annexin V+ PI+ cells (%)	40/40	0.42 (0.03)	0.43 (0.03)	+ 0.01 (0.03)	0.89
Stress-induced increase					
0.2 μM rotenone, 7d					
SA- β -gal FACS (MdFI)	20/20	+ 121 (19)	+ 156 (19)	- 35 (19)	0.064
111 mM glucose, 7d					
SA- β -gal FACS (MdFI)	20/20	+ 30 (13)	+ 88 (13)	- 55 (15)	<0.001
1 μM rotenone, 3d					
Sub-G1 (%)	40/39	+ 26.1 (1.2)	+ 23.8 (1.2)	+ 2.3 (1.3)	0.115
Annexin V+/PI- cells (%)	40/40	+ 8.69 (0.59)	+ 7.77 (0.59)	+ 0.92 (0.45)	0.041
Annexin V+/PI+ cells (%)	40/40	+ 1.83 (0.15)	+ 1.51 (0.15)	+ 0.32 (0.12)	0.008

Fibroblast strains from offspring (mean \pm SD age: 58.2 \pm 7.4years) were compared with fibroblast strains from partners (mean \pm SD age: 57.1 \pm 8.5 years). Stress-induced increases: stressed minus non-stressed, MdFI: median fluorescence intensity, a: difference between young and old (offspring minus partner).

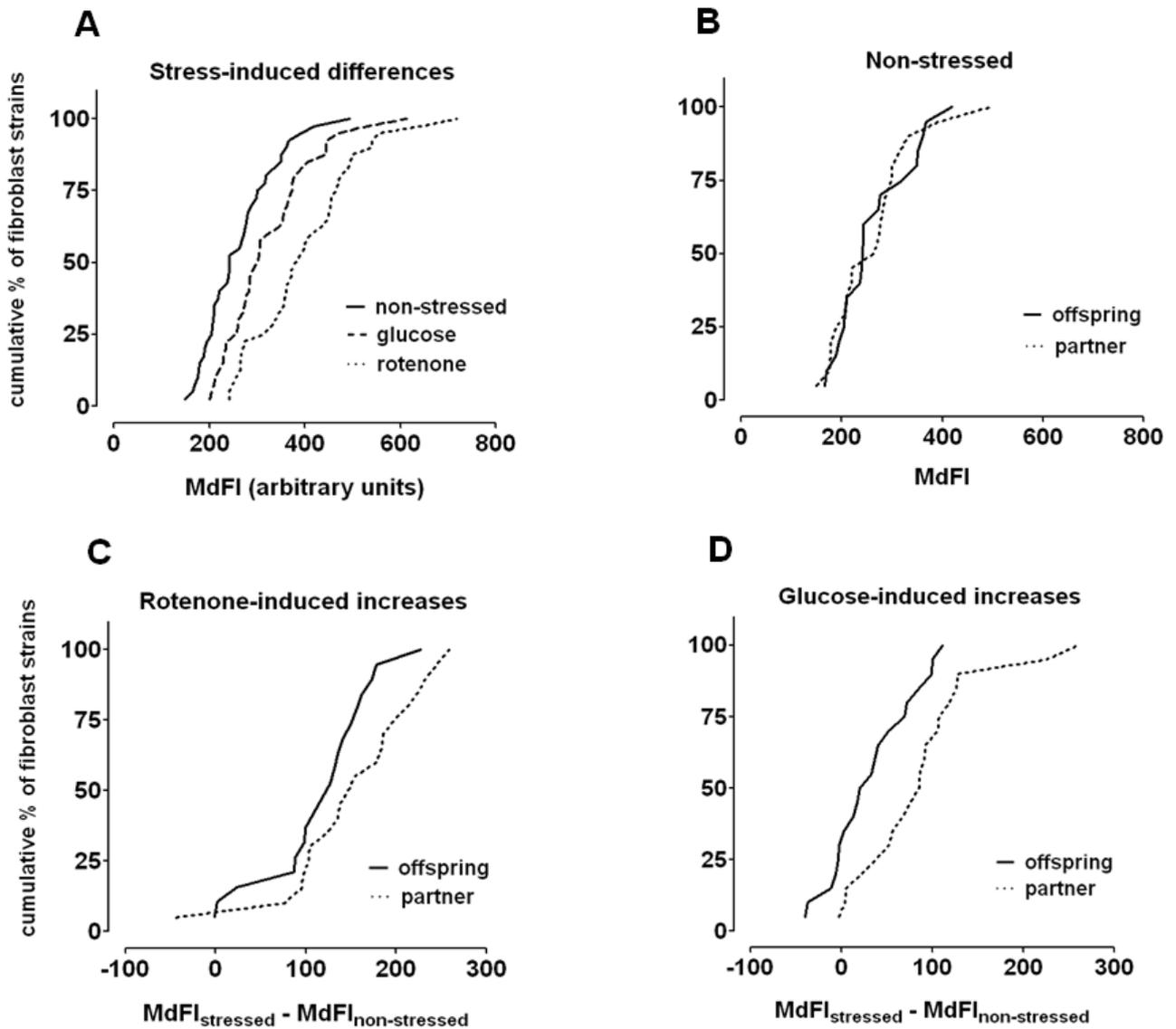


Figure 2. Cumulative frequency plots of SA-β-gal activity in fibroblast strains. A, activity in fibroblast strains under non-stressed conditions, treated with 0.2 μM rotenone and with 111 mM glucose for three days (offspring and partners from the Leiden Longevity Study combined, n=40). B, activity under non-stressed conditions in strains from offspring (n=20) and partners (n=20). C, rotenone-induced increases of activity (stressed minus non-stressed) in offspring and partners. D, hyperglycemia-induced increases of activity in offspring and partners. MdFI: median fluorescence intensity

Total colony size was also greater. After rotenone treatment, although not significant, a higher fraction of surviving colonies and a higher fraction of total colony size were observed for strains from young subjects compared with old subjects. Considering the contrast in biological age, the number and total size of colonies were significantly higher for fibroblast strains from offspring when compared with partners under non-stressed conditions (Table 5). After treatment with rotenone or hyperglycemia, significantly higher fractions of surviving colonies and bigger total colony sizes were observed in strains from offspring when compared with partners (Table 5).

Table 5. Differences in non-stressed levels and stress-induced decreases in growth potential observed with the colony formation assay dependent on differences in chronological (Leiden 85-plus Study) and biological age (Leiden Longevity Study).

	Leiden 85-plus Study		p	Leiden Longevity Study		P
	Young (n=10)	Old (n=9)		Offspring (n=37)	Partner (n=37)	
Number of colonies						
Non-stressed, 7d (# colonies/plate)	142 (16)	96 (16)	0.017	171 (7)	149 (7)	0.001
Surviving fraction (%)						
0.2 µM rotenone, 7d	66 (5)	56 (5)	0.12	44 (3)	37 (3)	0.008
111 mM glucose, 7d	nd	nd		50 (3)	45 (3)	0.056
Total colony size						
Non-stressed, 7d (Total colony size in mm ² /plate)	1535 (167)	918 (167)	0.006	1980 (95)	1734 (95)	0.024
Fraction remaining (%)						
0.2 µM rotenone, 7d	59 (5)	50 (5)	0.21	40 (2)	33 (2)	0.004
111 mM glucose, 7d	nd	nd		44 (3)	40 (3)	0.16

'Fraction remaining': total area of colonies per plate under stressed conditions divided by total area of colonies per plate under non-stresses conditions. nd: not done.

Discussion

In this study, we addressed the questions whether *in vitro* cellular responses to stress change with chronological age and whether *in vitro* cellular responses to stress depend on the propensity for longevity. To address these questions, we used fibroblast strains derived from young and old subjects, and from middle-aged offspring from nonagenarian siblings and their partners. Subjects of the last two groups were of similar chronological age and had equal distributions of men and women, thus any observed difference in cellular stress responses might be attributable to the biological age of the subjects, i.e. the propensity for longevity in the offspring.

The main findings of our studies are as follows. First, under non-stressed conditions, we observed that, compared with fibroblast strains obtained from the very old, fibroblast strains from young subjects show lower levels of senescence, as observed with SA- β -gal activity, higher growth potential, and also lower levels of cell debris. In line with our findings, it has been shown in primates that the proportion of senescent skin fibroblasts increases with age *in vivo*, supporting the biological relevance of cellular senescence for this cell type (22-25). Both findings are consistent with the notion that cellular senescence and apoptosis increase with chronological age and play an important role in age-related pathology (7;26;27).

Second, after induction of stress, either by rotenone or hyperglycemia, fibroblast strains from old subjects showed a higher increase in levels of senescence when compared with fibroblast strains from young subjects as observed with SA- β -gal activity and lower growth potential as measured with the colony formation assay. These results are in line with the findings of Ressler *et al.* who showed *in vivo* that with increasing age, an increasing number of human skin cells (dermal and epidermal) become positive for p16, a protein involved in the regulation of stress induced premature senescence (25).

Third, after stress, fibroblast strains from offspring of long-lived families showed lower increases in levels of senescence compared with fibroblasts from partners as observed with SA- β -gal activity and growth potential. Such a stress-resistant phenotype of fibroblasts has been reported for long-lived dwarf mice when compared with wild type mice (10), but, to the best of our knowledge, not for humans with different aging trajectories.

Rotenone was chosen as senescence-inducing agent because it acts on mitochondrial complex I, leading to increased levels of intracellular Reactive Oxygen Species (ROS) (16), which would mimic the physiological process of ROS induced damage that is hypothesized to underlie the aging process. When used in lower concentrations, we have found earlier that rotenone induces senescence as measured by colocalisation of increased p16 expression and increased SA- β -gal activity (20). As a positive control, we have also observed colocalisation of increased p16 expression coinciding with increased SA- β -gal activity in high passage fibroblasts (20), as was previously observed by Itahana *et al.* (28). Various cellular stressors have also been reported by others to induce SA- β -gal activity coinciding with increased p16 expression (29-35). In addition it was shown that p16 inhibition by means of antisense p16 resulted in delayed expression of SA- β -gal activity (36).

Glucose was chosen as an alternative stressor as it has been implicated to interfere in aging processes for a relatively long period of time. Many of the age-related disorders and diseases occur earlier in life in diabetics than in healthy people (37) and in various model organisms, glucose handling (insulin/IGF-signaling) has been shown to affect longevity (38). Furthermore, hyperglycemia has been shown to induce increased ROS at the cellular level (18) as well as premature senescence in human skin fibroblasts (19). Recently it was shown within the Leiden Longevity Study that the prevalence of diabetes was higher in partners compared with offspring (4.4% vs. 7.6%, $p=0.004$) and similar percentages are present in the randomly chosen set of subjects from whom fibroblast strains were tested (14).

Contrary to our hypothesis we found arguments for stress-induced apoptosis to be more present in fibroblast strains from fibroblast strains from offspring compared their partners. The fact that fibroblast strains from biologically younger subjects are more prone to go into apoptosis than to become senescent, could be advantageous to the organism to prevent damaged cells from becoming neoplastic. Although both apoptosis and senescence will prevent damaged cells from becoming neoplastic, apoptosis is thought to lead to greater loss of cells that would need to be replaced by new cells from a pool of mitotically active cells or stem cells. In aging organisms, stem cells lose the ability to replace cells lost by apoptosis, partially explaining age-related decline of cellularity in tissues (39). Though the number of fibroblasts in the dermis is markedly reduced during aging (40;41), skin fibroblasts from very

old subjects still show a high replicative capacity *in vitro* (15), making it unlikely that loss of cellularity in skin can be solely explained by exhaustion of replicative capacity. There is recent evidence that loss of cellularity in skin is more likely the result of the accumulation of senescent cells, which are capable to change the micro-environment. For instance, senescent cells have a secretory phenotype that promotes the invasiveness of premalignant epithelial cells (42).

An important strength of our study is the large number of fibroblast strains obtained from subjects of various chronological and biological ages, collected and stored in a highly standardized manner. Another strength is the fact that we studied stress-induced senescence rather than replicative senescence, since it has been shown that the maximum number of population doublings does not reflect human life history trajectories (43). This suggests that replicative senescence *in vitro* may be significantly influenced by cell culture conditions and that stress-induced senescence might better differentiate between cellular phenotypes. During the lifetime of an organism, its cells will be subjected to endogenous and exogenous chronic stressors, leading to aging of cells and consequently the organism. For these reasons we used fibroblasts at subconfluent levels and mimicked natural chronic stress by exposing fibroblasts to low concentrations of stressors up to one week.

In conclusion we report stress-induced responses of human cells *in vitro* reflect the propensity for human longevity of the subjects from whom these were derived.

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