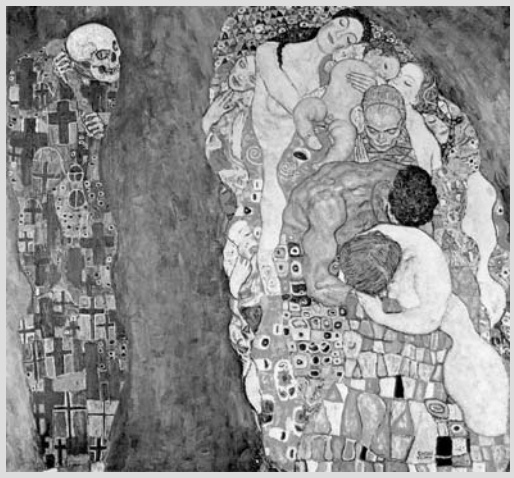
Cellular senescence in vitro and organismal ageing

Maier, A.B.



Chapter 8

Relation between replicative senescence of human fibroblasts and life history characteristics

Andrea B. Maier, Rudi G.J. Westendorp

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Summary

Replicative ageing of fibroblasts *in vitro* has often been used as a model for organismal ageing. The general assumption that the ageing process is mirrored by cellular senescence *in vitro* is based on lower replicative capacity of human fibroblasts from patients with accelerated ageing syndromes or age-related diseases such as diabetes mellitus, and donors of higher chronological age, but these inverse relations have not been reported unequivocally. Therefore, we have performed a formal review on the replicative capacity of fibroblasts from patients suffering from accelerated ageing syndromes, age-related diseases and donor age. Some 13 studies including 79 patients with accelerated ageing syndromes showed replicative capacity of fibroblasts to be consistently lower when compared to fibroblasts obtained from age-matched controls. Some 12 studies reported on a total of 160 patients with various age-related diseases, but no consistent difference in replicative capacity was reported when compared to age-matched controls. Finally, in the period from 1964 to 2006 a total of 23 studies, including some 1115 individuals, reported on the relation between chronological age and replicative capacity of human fibroblasts. Earlier studies preferentially described an inverse relation between replicative capacity and chronological age that was absent in studies including higher numbers of subjects and were published more recently. There was marked heterogeneity between the studies (Egger test: $p = 0.018$) indicating that publication bias is at play. We conclude that, except for premature ageing syndromes, replicative capacity of fibroblasts *in vitro* does not mirror key characteristics of human life histories.

Introduction

It is not considered unreasonable to assume that life history characteristics such as maximal life span should be reflected in properties of isolated cells in culture. However, despite the general assumption that differences in maximal life span result from cellular defects, only few characteristics have yet been identified that could be relevant to life span determination, under which there is maximal replicative capacity of fibroblasts *in vitro*. Under laboratory conditions cells gradually and irreversible lose their ability to proliferate upon subsequent subculturing. This cellular senescence has often been taken as a read out for critical processes that occur during the ageing process of organisms (Hayflick, 1965), a notion that is supported by an accumulation of senescent cells *in vivo* (Dimiri *et al*., 1995). Moreover, replicative capacity of fibroblasts *in vitro* has positively been correlated with life span of species (Hayflick, 1975; Roehme, 1981), whereas a lower replicative capacity is reported when fibroblasts are obtained from patients with accelerated ageing syndromes (Martin *et al*., 1970), or age-related diseases (Goldstein *et al*., 1969a). Earlier studies (Goldstein *et al*., 1969a; Martin *et al*., 1970) also demonstrated an inverse relationship between donor age and replicative capacity of fibroblasts *in vitro* which suggests that progenitor cells in renewable tissues may progressively exhaust their ability to divide and may thus explain for atrophy and tissue dysfunction. However, recent findings have raised questions about the validity of the relation between the replicative capacity of cells in culture and the life span of the organism (Cristofalo *et al*., 1998). Therefore, we set out to perform a formal review on the replicative capacity of fibroblasts *in vitro* obtained from patients with accelerated ageing syndromes, patients who suffer age-related diseases, and donor age.

Material and Methods

Objective

To assess the replicative capacity of human fibroblasts in patients with accelerated ageing syndromes and specific age-related diseases when compared to age-matched controls. To assess the relation between replicative capacity of fibroblasts and the chronological age of the donor.

Criteria for considering studies for this review

The articles were identified from a Medline search using mesh terms. The search was focused on research articles and review papers published within 1950 and April 2008. Book chapters and editorials were also scanned. The terms used for the Medline search were: #REPLICATIVE and #SENESCENCE and #AGEING or #PREMATURE or #DIABETES or #COGNITION or #CANCER or #LUNG. Reference lists of articles found were searched for other relevant studies. Language limitations were applied to English, France and German. Studies published in full articles abstracts or letters were eligible for inclusion. The following characteristics of the studies were obtained: year of publication, fibroblast origin, number of included fibroblast strains (cultured up to the onset of replicative senescence), and the definition of replicative senescence *in vitro*.

Replicative capacity and accelerated ageing syndromes / age-related diseases

Studies were included in the review if the replicative capacity of human fibroblast obtained from individuals suffering from a defined disease were compared to age-matched controls. Appropriate matching was defined as less than 20 years difference between the groups. The replicative capacity of patients with a certain disease and controls was expressed as means (SD).

Replicative capacity and chronological ageing

Studies were included in the review, if the relation between chronological age of the donor and *in vitro* replicative capacity of human fibroblasts were studied (as

primary or secondary research question) and, if primary data of individual fibroblast strains were provided (in the text, tables or graphs). Data of fibroblast cultures obtained from subjects that were part of the control group contrasting accelerated ageing syndromes or specific age-related diseases were also included. To be eligible for inclusion in the review, the age range of the subjects should span a minimum of 20 years. If subjects were pooled within groups, the age range within groups had to be 10 years or less, the average age was used for further analysis. The origin of human fibroblasts was restricted to skin, rectum and lung.

Statistical analysis

To assess the difference in replicative capacity between patients and controls, the mean number of population doublings (PDs) of the controls and PDs of patients were subtracted. The SE of the difference was calculated by using the SD of both groups. For studies on the effect of donor age, we assessed the change in replicative capacity per year increase in calendar age using linear regression. Data were summarized using funnel plots; asymmetry indicating publication bias was tested according to Egger (Egger *et al*., 1997).

Results

Replicative capacity and accelerated ageing syndromes

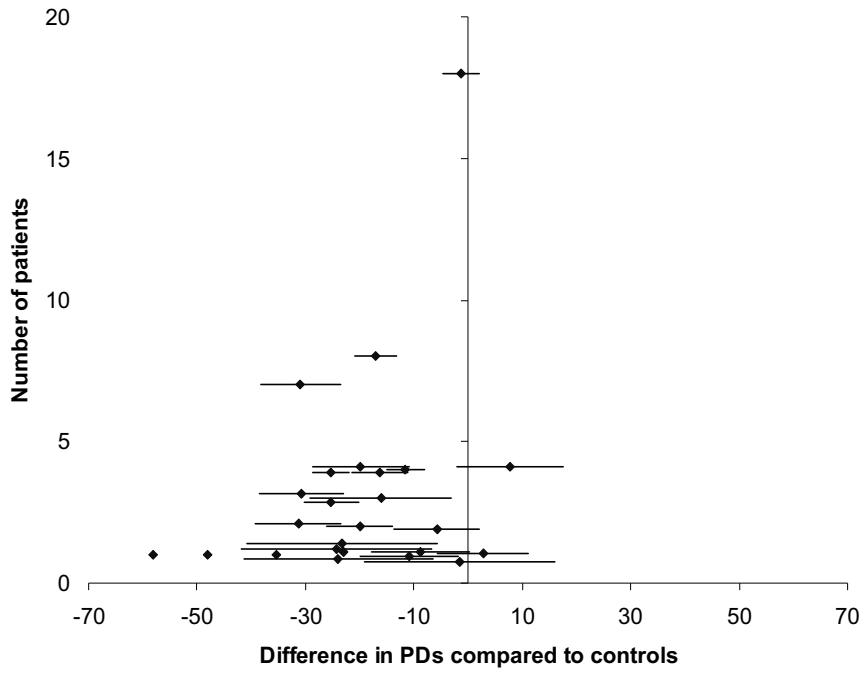
Table 1 summarizes 13 studies, published from 1969 onwards, which were identified as testing replicative capacity of human fibroblast strains of 79 patients suffering from accelerated ageing syndromes compared to age-matched controls. Several studies had included only one patient. Figure 1 shows the mean differences in replicative capacity expressed as PDs of patients with accelerated ageing syndromes versus controls. In almost all studies, fibroblast strains obtained from patients suffering from an accelerated ageing syndrome showed lower replicative capacity as compared to age-matched controls. The decrease in replicative

Table 1. Characteristics of included studies testing the replicative capacity of human fibroblasts *in vitro* from patients with premature ageing syndromes as compared to controls.

Author	Year of publication	Premature ageing	Number of patients	controls	Fibroblast type	PD (mean) patients vs controls
Goldstein	1969b	Werner syndrome	1	1	skin	2 vs 25
Martin	1970	Werner syndrome	3	30	skin	7.7 vs 32.9
		Rothmund syndrome	1	34	skin	34 vs 42.8
		Hutchinson-Gilford heterocygote	1	31	skin	37 vs 34.2
		Hutchinson-Gilford homocygote	1	34	skin	32 vs 42.8
Schneider	1972	Down syndrome	4	4	skin	40 vs 51.5
Nikaido	1980	Werner syndrome	8	6	skin	15.7 vs 32.8
		Xeroderma pigmentosum	4	5	skin	42.1 vs 34.3
		Ataxia telangiectasia	2	2	skin	44.5 vs 50.3
Thompson	1983a	Werner syndrome	4	2	skin	25 vs 44.8
Thompson	1983b	Ataxia telangiectasia	3	3	skin	35.9 vs 52
		Cockayne syndrome	1	3	skin	28.1 vs 52
		Bloom syndrome	1	3	skin	27.8 vs 52
		Fanconi anemia	1	3	skin	28.8 vs 52
		Friedrich ataxia	1	3	skin	50.5 vs 52
Carmeliet	1990	Down syndrome	18	11	skin	21.6 vs 23
Colige	1991	Hutchinson-Gilford	1	2	skin	14.5 vs ng
Kill	1994	Werner syndrome	1	1	skin	17 vs 65
Weirich-Schwaiger	1994	Werner syndrome	3	6	skin	25 vs 55.8
		Down syndrome	2	3	skin	45 vs 65
		Cockayne syndrome	4	4	skin	45 vs 61.3
		Ataxia telangiectasia	2	4	skin	30 vs 61.3
Schulz	1996	Werner syndrome	4	3	skin	16.3 vs 41.6
Park	2001	Hutchinson-Gilford homocygote	1	1	skin	7 vs 65
Bridger	2004	Hutchinson-Gilford homocygote	7	3	skin	27.1 vs 58

ng: not given, estimated to 50 PDs

Figure 1. Difference in population doublings (PDs) of human fibroblasts from patients with accelerated ageing syndromes and control subjects. The symbols indicate the mean difference in PDs ± SE between the patient and control series.



capacity was highest in patients suffering from Werner and Hutchinson-Gilford syndrome. The decrease in replicative capacity of fibroblasts obtained from patients with Down syndrome, Xeroderma pigmentosum and Friedreich ataxia was smaller than that in other accelerated ageing syndromes. The largest study on 18 patients with Down syndrome showed no difference when compared to age-matched controls. The Egger test was not significant however, suggesting homogeneity between the studies ($p = 0.86$)**.**

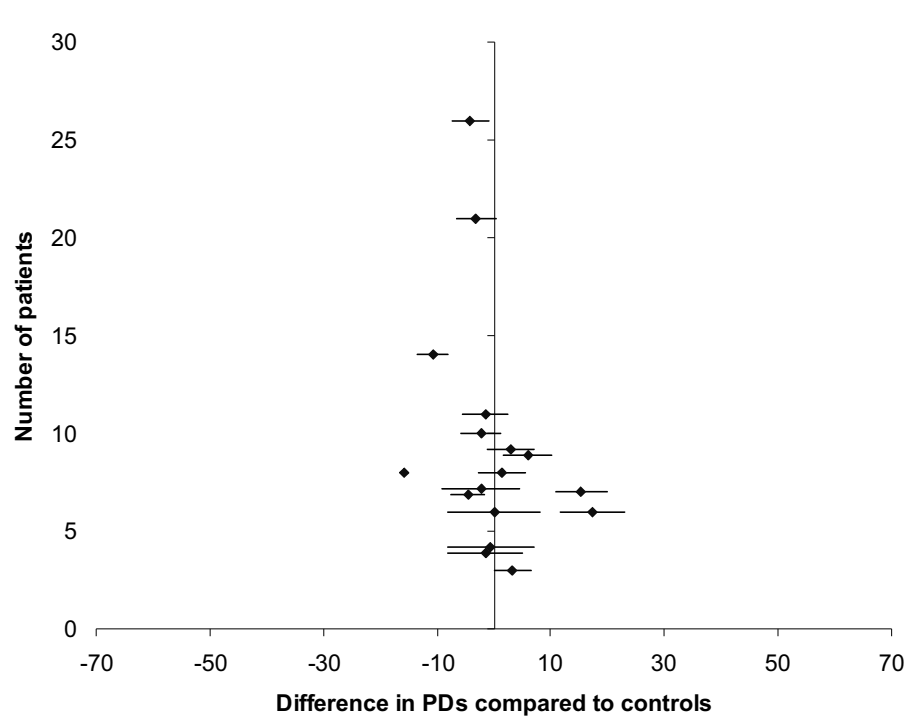
Table 2. Characteristics of included studies testing the replicative capacity of human fibroblasts *in vitro* from patients with age-related diseases as compared to controls.

Author	Year of publication	Disease		Number of patients	controls	Fibroblast type	PD (mean) patients vs controls
Goldstein	1969a	Diabetis mellitus	prediabetic	6	11	skin	62.5 vs 62.4
			diabetic	7	11	skin	14.8 vs 62.4
Goldstein	1978	Diabetis mellitus	prediabetic	21	25	skin	48.9 vs 52
			diabetic	26	25	skin	47.8 vs 52
Shapiro	1979	Cystic fibrosis		8	5	skin	28.9 vs 44.8
Azzarone	1981	Breast cancer		7	6	skin	42.1 vs 26.8
Thompson	1983c	Cystic fibrosis		4	3	skin	53.7 vs 54.3
Carmeliet	1990	Familian Alzheimer		11	11	skin	21.5 vs 23
		Sporadic Alzheimer		4	11	skin	21.5 vs 23
Tesco	1993	Familial Alzheimer		3	3	skin	21 vs 17.7
Morocutti	1996	Diabetis mellitus	without nephropathy	10	14	skin	32.6 vs 34.8
			with nephropahty	14	14	skin	24.1 vs 34.8
Pratsinis	2002	M. Cushing		6	6	skin	57 vs 39.6
Holz	2004	Lungemphysema		7	7	lung	13.8 vs 18.4
Getliffe	2006	Colitis ulcerosa		8	9	rectal	22.9 vs 21.5
Maier	2007	Diabetes mellitus		9	47	skin	53 vs 57
		Malignancies		9	48	skin	54 vs 52

Replicative capacity and age-related diseases

Table 2 provides a summary of 12 studies on the replicative capacity of human fibroblasts in 160 subjects with diabetes mellitus, cystic fibrosis, breast cancer, cognitive impairment, Cushing's disease, lung emphysema and colitis ulcerosa as compared to controls. Without exception, studies included small samples of patients. The relation between diabetes mellitus and replicative capacity of fibroblasts has been addressed in four studies. The replicative capacity of fibroblasts in patients suffering from severe diabetes with nephropathy was diminished compared to controls but it was insignificantly decreased in patients with mild to moderate diabetes. A decreased replicative capacity of fibroblasts was found in patients with cystic fibrosis and lung emphysema but a higher replicative

Figure 2. Difference in population doublings (PDs) of human fibroblasts from patients with common age-related diseases and control subjects. The symbols indicate the mean difference in PDs ± SE between the patient and control series.



capacity was observed in patients with breast cancer. For all other diseases there were no significant differences in the replicative capacity of human fibroblasts when compared to controls. When analyzed together, the outcomes of the studies range along zero, indicating that the replicative capacity of fibroblasts of patients with chronic diseases was not different from that of age-matched controls (Figure 2). The Egger test was not significant ($p = 0.073$)**.**

Replicative capacity and chronological ageing

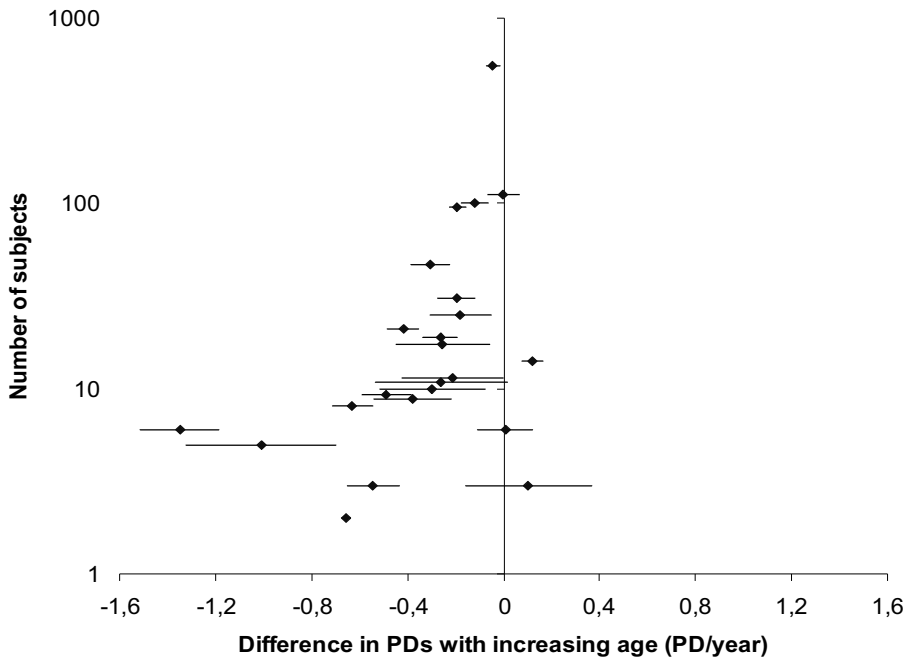
A total of 23 studies, published in the period from 1964 to 2006, including a total of 1115 subjects, were eligible for testing the relationship between replicative capacity of human fibroblasts and chronological age of the donor. The average number of individual strains included in each study was 48.5 (SD 114) ranging from 2 to 552 strains. Table 3 summarises the characteristics of the identified studies. Eighteen studies found an inverse relation between donor age and the replicative capacity of fibroblast strains; two studies found no difference and three studies a positive relation between the chronological age of the donor and the replicative capacity of the strains. As shown in Figure 3, the outcomes of the studies were clearly dependent on the number of subjects included in the study. Overall, the difference in PDs per year increase in donor age minimized with an increase of the study size and became insignificantly different in those studies that included the highest number of strains. The largest study on 552 individuals, published in 2002, did not show any correlation between replicative capacity *in vitro* and donor age. More recent studies proportionally more often reported minimal or absent correlations between replicative capacity and donor age. The Egger test, analysing asymmetry of the funnel plot was significant indicating publication bias ($p = 0.018$). From Figure 3 it can be inferred that smaller studies reporting minimal effects or higher replicative capacities in subjects of older age have preferentially remained unpublished**.**

Table 3. Characteristics of included studies testing the relation between replicative capacity of human fibroblasts *in vitro* and chronological age of the donor.

Author	Year of publication	Number of strains	Fibroblast type	Age range (years)	Definition of senescence	Change in number of PD (SD) per year
Miles	1964	17	skin	fetal-25*	not given	-0.25 (0.19)
Hayflick	1965	21	lung	fetal-87	ceased mitosis	-0.42 (0.07)
Goldstein	1969a	11	skin	21-50	no confluence despite feeding	-0.26 (0.28)
Martin	1970	98	skin	fetal-85	less than 4x10*5 cells after 1 month	-0.19 (0.03)
Dell'Orco	1974	2	skin, lung	fetal, 35	less than 1 PD in 7 days	-0.66
Dell'Orco	1975	5	skin, lung	fetal-35	not given	-1.01 (0.31)
Schneider	1976	47	skin	21-92	no confluence after 28 days	-0.31 (0.08)
Goldstein	1978	25	skin	15-76	no confluence after 28 days	-0.18 (0.13)
Smith	1978	17	skin	27-89	not given	-0.26 (0.07)
Nikaido	1980	9	skin	feb-66	lower cell yields than inoculum	-0.49 (0.1)
Azzorone	1981	6	skin	24-45	not given	-1.35 (0.16)
Schneider	1981	98	skin	29-96	less than 1 PD in 42 days	-0.12 (0.06)
Thompson	1983b	3	skin	aug-65	not given	-0.55 (0.11)
Carmeliet	1990	11	skin	18-65	no growth in 14 days	-0.21 (0.21)
Allsopp	1992	31	skin	0-93	no confluence after 28 days	-0.2 (0.08)
Weireich-Schwaiger	1994	8	skin	25-81	less than 1 PD in 42 days	-0.63 (0.09)
Schulz	1996	3	skin	26-62	not given	0.1 (0.26)
Cristofalo	1998	112	skin	0-92	less than 1 PD in 28 days	-0.002 (0.07)
Tesco	1998	14	skin	7-105	less than 1 PD in 28 days	0.12 (0.05)
Serra	2002	10	skin	28-91	less than 1 PD in 28 days	-0.3 (0.22)
Smith	2002	552	skin	21-96	CSD	-0.04 (0.03)
Chondrogianni	2004	6	skin	18-104	not given	0.01 (0.11)
Getliffe	2006	9	rectum	29-71	less than 1 PD in 28 days	-0.38 (0.16)

* fetal, infant, child adult were estimated to 0, 0.5, 8, 25 years resspectively, CSD = colony survival distribution

Figure 3. Decrease in population doublings (PDs) per calendar age dependent on the number of subjects included in each study. The symbols indicate the change in the number of PDs ± SD per year increase in donor age.



Discussion

The main results presented in this formal review are threefold. First, reports on the replicative capacity of fibroblasts from patients with accelerated ageing syndromes were consistently lower when compared to strains from age-matched controls. Second, no consistent difference in replicative capacity was found in fibroblasts from patients with age-related diseases when compared to age-matched controls. Third, the earlier described inverse relation between replicative capacity and chronological age was absent in studies including higher numbers of subjects and were published more recently, indicating publication bias.

Replicative capacity and accelerated ageing syndromes

Deficiencies of specific DNA damage recognition and repair pathways resulting from defined monogenetic diseases undoubtedly contribute to the loss of cellular function, including a lower replicative capacity of human fibroblasts in culture. The mechanisms leading to a diminished replicative capacity in patients with accelerated ageing syndromes have extensively been studied. Cells from patients with Werner syndrome (Chen *et al*., 2003; Szekely *et al*., 2005; Chang *et al*., 2004) are characterised by chromosomal instability (Bartam *et al*., 1976) and the cell cycle is changed towards a senescent phenotype (Faragher *et al*., 1993; Franchitto *et al*., 2003). The premature death of cells from patients with Hutchinson-Gilford progeria syndrome is caused by toxic accumulation of progerin at the nuclear envelope (Goldman *et al*., 2004). The cellular machinery in patients with Fanconi anemia patients is disturbed by a high burden of radical oxygen species (Adelfalk *et al*., 2001), in Bloom's syndrome by hyper-recombination of double strand breaks (Chaganti *et al*., 1974), and in ataxia telangiectasia by p53 instability and disruption of genomic and telomere integrity (Lavin *et al*., 1997; Shiloh, 2003). Symptoms in patients with Cockayne syndrome and Xeroderma pigmentosa are caused by deficient nucleotide excision repair (NER) (Cleaver, 2005; Troelstra *et al*., 1992). Both syndromes were not associated with a significant decrease in replicative capacity of fibroblasts. Likewise, replicative capacity was not decreased in cells from patients with Rothmund–Thomson syndrome; however, it has been shown that the ability of cells to recover from oxidative stress and DNA damaged is severely impaired (Wang *et al*., 2003; Werner *et al*., 2006). Fibroblasts obtained from large series of patients suffering from Down syndrome did not differ in replicative capacity when compared to controls and contrasts with the first report on the topic (Schneider and Epstein, 1972).

Replicative capacity and age-related diseases

Three decades ago, the system of culturing human fibroblasts has been indicated to serve as an excellent model to investigate the cellular and molecular basis of

diabetes mellitus (Goldstein *et al*., 1969a, 1978). In more recently conducted studies, the replicative capacity of fibroblasts was only significantly impaired in patients with longstanding diabetes and severe complications (Morcocutti *et al*., 1996), a finding that may be explained to the systemic effects of reactive oxygen species that have been implicated in the pathogenesis of diabetic complications (Morocutti *et al*., 1997). These findings suggest that the duration and the severity of diabetes is of importance when recognizing cellular deficits in human fibroblasts. Furthermore, some cell types may be more susceptible to cellular disease related changes than others, e.g. chronic hyperglycaemia causes higher numbers of single strand DNA breaks in endothelial cells but not in fibroblasts (Lorenzi *et al*., 1986), and replicative capacity, assessed by renal telomere length, might be more pronounced in renal cells than in skin fibroblasts (Famulski and Halloran, 2005). It has been hypothesized that fibroblasts from patients with inflammatory diseases such as colitis ulcerosa and lung emphysema show a lower replicative capacity due to oxidative stress which leads to an accelerated telomere shortening (von Zglinicki, 2002). However, as not all studies have shown a lower replicative capacity of fibroblasts when sampled from patients with degenerative age-related diseases, this cell type may just not reflect the systemic damage that has occurred during the disease course.

Replicative capacity and chronological ageing

The earliest reports on fibroblast cultures from donors of different age-groups provided arguments for an age-limited replicative capacity. The negative regression of growth potential on age was interpreted as a dysfunction of the cells of origin. It was generally assumed that cells *in vivo* progressively exhausted their replicative capacity during ageing. Replicative senescence of fibroblasts has since then been used as an *in vitro* model of ageing. However, this interpretation has been plagued by miscellaneous outcomes on the relation between replicative capacity and age (Hayflick and Moorhead, 1961; Hoehn *et al*., 1978; Maier *et al*., 2007). It has been argued that the diverse outcomes in more recently conducted studies were distorted

by the health status of the donors (Cristofalo *et al*., 1998). However, as indicated above, the influence of age-related diseases on the replicative capacity of fibroblasts is not consistent and therefore it is unlikely that the health status explains the diverse outcomes.

Replicative capacity of fibroblasts from nonagenarians and centenarians who arrived close to the maximum lifespan are well preserved and not lower than that of fibroblasts obtained at younger age (Grassili *et al*., 1996; Maier *et al*., 2007). These latter findings do not refute that cellular senescence *in vivo* contributes to organismal ageing (Campisi, 1998; Faragher and Kipling, 1998; Hornsby, 2002) but indicated that there is little basis for directly linking cellular replicative capacity *in vitro* with length of life. The well-preserved replicative capacity of elderly donors could be explained in two ways. On the one hand, mitotic activity of human fibroblasts during adulthood *in vivo* may be extremely low leading to an insignificant reduction of the remaining replicative capacity *in vitro*. However, this assumption is rather unlikely due to maintenance and regeneration of tissues during lifetime. On the other hand, fibroblast mixed mass cultures may not be representative for *in vivo* characteristics of the entire organ, i.e. the skin.

The variability of life span of individuals is thought to arise from the stochastic nature of cellular and molecular mechanism controlling development and ageing. Many of the cells carry their own repertoire of ‘acquired errors of metabolism’ (Kirkwood *et al*., 2005). The loss of proliferative potential *in vivo*, indicated by an accumulation of β-galactosidase, p53 and p16 positive cells (Pendergrass *et al*., 1999; Dimri *et al*., 1995; Herbig *et al*., 2006; Ressler *et al*., 2006) occurs as a mosaic in which both long- and short-lived cells may lie in close proximity, amongst others due to differences in mitotic history. *In vitro*, cultures undergo senescence in a stochastic manner with a half-life of only ~8 doublings from starting the culture (Rubin, 2002). By analysing fibroblast clones, these exhibit a wide variety of replicative capacity from the earliest passages onward, including many with very few doublings (Merz and Ross, 1969; Cristofalo and Sharpf, 1973;

Merz and Ross, 1973; Martin *et al*., 1974; Smith and Hayflick, 1974; Absher and Absher, 1976; Bell *et al*., 1978; Rabinovitch, 1983). The fraction of clones from early passages that reach 50 PDs is 0.05-0.1, the probability having 3 such clones is 10^{-4} to 10^{-3} (Smith and Hayflick, 1974). The overall replicative capacity might decline with age but the rare fibroblasts having extended replicative potential continue to be present at old age, which do not necessarily reflect the properties of the overall population. The replicative capacity *in vitro* reflects therefore only the expansive propagation of the longest surviving clone (McCarron *et al*., 1987), which in general show to have comparable *in vitro* characteristics when sampled from young or old individuals.

In conclusion, except for premature ageing syndromes, replicative capacity of fibroblast *in vitro* does not mirror key characteristics of human life histories.

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