Stochastic Variation in Telomere Shortening Rate Causes Heterogeneity of Human Fibroblast Replicative Life Span* 

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The replicative life span of human fibroblasts is heterogeneous, with a fraction of cells senescing at every population doubling. To find out whether this heterogeneity is due to premature senescence, i.e. driven by a nontelomeric mechanism, fibroblasts with a senescent phenotype were isolated from growing cultures and clones by flow cytometry. These senescent cells had shorter telomeres than their cycling counterparts at all population doubling levels and both in mass cultures and in individual subclones, indicating heterogeneity in the rate of telomere shortening. Ectopic expression of telomerase stabilized telomere length in the majority of cells and rescued them from early senescence, suggesting a causal role of telomere shortening. Under standard cell culture conditions, there was a minor fraction of cells that showed a senescent phenotype and short telomeres despite active telomerase. This fraction increased under chronic mild oxidative stress, which is known to accelerate telomere shortening. It is possible that even high telomerase activity cannot fully compensate for telomere shortening in all cells. The data show that heterogeneity of the human fibroblast replicative life span can be caused by significant stochastic cell-to-cell variation in telomere shortening.

Heterogeneity is a hallmark of aging. Even genetically identical organisms in a controlled homogeneous environment age at different rates and have vastly different life spans. This is also true for aging of human somatic cells in vitro (1). Replicative life spans differ widely between subclones (2), and there is an ever increasing number of senescent cells in dividing cultures (3). Senescence of human fibroblasts is telomere-driven, i.e. induced by the uncapping of telomeres (4). However, it is often assumed that only the life span of the longest surviving clone(s) is governed by telomere shortening, whereas termin- ation of growth in the early senescing clones might be premature, i.e. caused by stress via telomere-independent mechanisms (5). In fact, a senescent phenotype can be generated in response to a variety of stresses that are telomere-independent and inhibit cell growth, for instance, in response to generalized DNA damage (6), oncogene activation (7), histone acetylation (8), or by induction of the p16 pathway through so far not well characterized mechanisms (9).

An alternative to this idea of premature senescence is the proposal that stochastic telomere uncapping can cause heterogeneity in replicative life span (10). The probability of telomere uncapping is dependent on a number of factors, including the presence of active telomerase, the integrity of the single-stranded telomeric G-rich overhang and of the higher order structure of telomeres, the functionality of a number of telomere-binding proteins, and telomere length (for review see Ref. 4). Finally, there is evidence for a substantial interaction between stress and specifically mild oxidative stress and telomeres in cell replicative senescence. Stress accelerates telomere shortening because of a telomere-specific single strand break repair deficiency (11). For most human cells, standard cell culture conditions exert mild oxidative stress (12). Thus, heterogeneity of human cell replicative senescence might be due to either heterogeneous shortening of telomeres in a stress-dependent fashion, to stochastic length-independent uncapping of telomeres, or to premature induction of senescence completely independent of telomeres. To decide between these alternatives would clarify the nature of cellular senescence and will help to resolve the issue as to whether it occurs in vivo as an essential part of the aging process or not (13).

To resolve the role of telomeres for the heterogeneity of replicative senescence, we sorted "prematurely" senescent human fibroblasts from growing cells by fluorescence-activated cell sorting (FACS)† and measured telomere length separately in both populations. The first data revealed that telomeres in sorted prematurely senescent MRC5 fibroblasts were, on average, as short as those at the end of the replicative life span of the cultures (14). Using clonal populations we show now that telomeres shorten faster in those sublineages that senesce early. This faster telomere shortening causes senescence, because the majority of cells can be rescued from early senescence by the expression of telomerase. However, a small fraction of cells still arrest with short telomeres despite active telomerase. This fraction increases under oxidative stress, which accelerates telomere shortening. We conclude that stochastic, stress-induced cell-to-cell variation of telomere shortening is the main cause of the intrinsic heterogeneity in the division potential of human fibroblasts.

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Experimental Procedures

Cell Culture—Human embryonic lung fibroblasts MRC5 were obtained from the ECACC. Human foreskin fibroblasts BJ originated from the laboratory of J. R. Smith (Houston, TX). The cells were grown in Dulbecco's modified Eagle's medium (Sigma) plus 10% fetal calf serum (Sigma) under either normoxia (95% air, 5% CO2) or 40% normobaric hyperoxia using a 3-gas cell culture incubator (Zapf Instruments, Sarstedt, Germany). Retroviral particles were generated upon transfection of the vector pLCP-hTERT (Clontech, Palo Alto, CA) containing the gene for the catalytic subunit of the human reverse transcriptase (hTERT) under control of the cytomegalovirus promoter into the packaging line 9NX. MRC5 (PD 33) were infected with the retroviral supernatant, and cells were selected with 0.75 μg/ml puromycin for 10 days. Expression of hTERT was verified by RT-PCR (15) and semiquantitative TRAP-assay (Roche Applied Science). Telomerase activity was similar as in the human prostate cancer cell line DU145. G120-hTERT fibroblasts were grown in Ham's F-10 (Sigma) under an atmosphere of 5% O2, 5% CO2, and 90% N2.

Construction of a p21-EYFP Knock-in Reporter Cell Line by Gene Targeting—A reporter cell line was constructed from FL1, a normal human fibroblast cell strain derived from embryonic lung tissue (16), as described (17). Briefly, the previously described p21 knock-out vector was modified by inserting the EYFP coding region (Clontech) upstream of the neomycin phosphotransferase (Neo) gene. An internal ribosome entry signal was placed between EYFP and Neo to ensure expression of the Neo protein. Early passage FL1 cells were gene targeted by established methods (18). Integration into one of the p21 gene copies was screened by PCR, and the expected genomic structures of the targeted alleles had been determined by Southern hybridization. Expression of p21 protein from the wild-type allele was verified by immunoblotting. Heterozygous p21+/- FL1 cells undergo normal M1 senescence (16), and EYFP knock-in cell strains were verified to likewise do so. One positive clone was retrovirally transfected with hTERT, and telomerase activity was verified by TRAP assay. The resultant cell line G120, used in this investigation, showed intact DNA damage responses following treatment with ionizing radiation or cytostatic drugs, including expression of both p21 and EYFP proteins (17).

FACS Analysis and Sorting—The cells were trypsinized, collected in Dulbecco's modified Eagle's medium plus 10% serum at 4 °C and immediately used for analysis and sorting in a PAS-PCCS flow cytometer and cell sorter (Partec GmbH, Munster, Germany). Autofluorescence of unfixed cells was measured in FL1. Cell size was monitored by forward scatter (FSC). 3-μm calibration beads (Partec) were used for instrument standardization. Populations of senescent fibroblasts were stably detected at higher values of both FSC and FL1 as compared with young, proliferating cultures (see Fig. 1A). The cells were sorted into ice-cold PBS containing 5% FCS for 5 min at 4 °C. The sorting gates were defined to include all cells (ALL gate) or the quartile of MRC5 or BJ cells with the highest (SEN gate) or lowest (PROL gate) values for FL1 and FSC. For sorting of MRC5-hTERT cells, the gates were defined in parallel runs with parental MRC5 under identical instrument settings. Analysis of p21 reporter cells revealed two separate peaks for low and high EYFP fluorescence, and sorting was performed using linear gates in FL1.

Telomere Length—The sorted cells were embedded in 0.65% low melting agarose plugs at a density of 107 cells/ml before treatment with proteinase K (19). DNA was completely digested by Hinfl (60 units/plug; Roche Applied Science) at 37 °C. The plugs were washed in a 1% agarose gel by pulsed field gel electrophoresis (Bio-Rad). The gels were blotted to Hybond N+ membranes (Amer sham Biosciences) and hybridized with the telomeric probe (TtAGGG)4, which was end-labeled with [alpha-32P]dATP. The signals were visualized by phosphorimaging (Storm 820, Molecular Dynamics). To test for unspecific degradation of DNA, the blots were rehybridized with the minisatellite probe (CAC)8 (data not shown). They approach replicative senescence (23, 24). Yellow-green fluorescence, and sorting was performed using linear gates in FL1.

RESULTS

To separate cells with a senescent phenotype from growing fibroblast populations by FACS while maintaining the quality of DNA for telomere length analysis, we used senescence markers that could be discerned in unfixed cells. Senescent fibroblasts are larger than young ones (21), and FACS separation of cells with a senescent phenotype from telomerase-immortalized fibroblast cultures by size, i.e. FSC, has recently been reported (22). Moreover, fibroblasts accumulate lipofuscin as they approach replicative senescence (23, 24). Yellow-green autofluorescence as measured by FACS in fluorescence channel 1 (FL1) is a reliable marker of cellular lipofuscin content (25, 26). In fact, senescent fibroblasts show higher values for FSC and FL1 than young ones (Fig. 1A). In growing populations of...
fibroblasts, there is a fraction of large cells with high levels of cytoplasmic autofluorescence. These cells are negative for KI67, a marker for actively cycling cells (Fig. 2A), and often positive for γ-H2A.X, a component of senescence-associated DNA damage foci (27), and thus, most probably senescent cells.

To separate cells by FACS, we defined the following gates: the one to enrich cells with a senescent phenotype includes the quartile of all cells with the highest FSC and FL1 values (SEN gate), whereas the PROL gate, including the quartile of all cells with the lowest FSC and FL1 values, sorted preferentially proliferating cells (Fig. 2B). A gate encompassing all cells (ALL gate) was used as control. Flow cytometric reanalysis of the sorted fractions confirmed significant enrichment of small cells with low autofluorescence following PROL sorting (Fig. 2C), and of large, autofluorescent cells after SEN sorting (Fig. 2D), respectively. Morphologically, replated PROL-sorted fibroblasts were very similar to young fibroblasts and grew fast to high densities (Fig. 2E), whereas SEN-sorted cells showed hardly any growth but displayed a typical senescent morphology (Fig. 2F). The fraction of cells that stained positive for senescence-associated β-galactosidase activity (28) was nearly 10 times higher in replated SEN-sorted cells as compared with PROL-sorted ones and more than three times higher in comparison with unsorted cells (Fig. 2G). After completion of about half of their replicative life span, 30% of BJ fibroblasts did not incorporate BrdUrd during a 48-h labeling period (Fig. 2H).

Sorting into the PROL gate after BrdUrd labeling increased the fraction of BrdUrd-positive cells to about 90% (Fig. 2I), whereas practically all cells were BrdUrd-negative following SEN sorting (Fig. 2J). We conclude that SEN sorting highly enriches cells with a senescent phenotype.

Telomere lengths in sorted MRC5 and BJ were initially measured by Southern blotting. PROL sorting did not appreciably change telomere length in comparison with sorting through a gate encompassing all cells (ALL gate). However, average telomere length in SEN-sorted MRC5 fibroblasts decreased to values similar to those in senescent cells, mostly because of a higher fraction of short telomeres (Fig. 3, A and B) (14). A similar but smaller shift toward shorter telomeres was obtained for SEN-sorted BJ fibroblasts as compared with ALL-sorted ones (data not shown), which is in accordance with differences in telomere shortening rates that amount to 80–100 bp/population doubling (PD) for MRC5 and less than 20 bp/PD for BJ (29). Next, we wanted to use a more sensitive method to measure telomere length to reduce the numbers of cells required for sorting. Unfortunately, senescent cells cannot be analyzed by metaphase quantitative fluorescent in situ hybridization (FISH), and exact quantification by flow cytometry FISH and interphase quantitative FISH is difficult in human fibroblasts, especially in senescent ones with their large cytoplasmic volumes, because of unspecific probe binding and telomeric associations (30, 31). STELA (32) measures only the length of one single telomere. Thus, we adopted a recently described quantitative real time PCR-based technique (20) to measure telomere template abundance from as few as 10⁴ cells and results in a good linear correlation with Southern blotting data over a wide range of telomere lengths (Fig. 1B). It confirmed the differences in telomere length between SEN- and ALL-sorted MRC5 fibroblasts as seen by Southern blotting. These differences became smaller with increasing PD, mostly because of a decrease in telomere length in ALL-sorted cells as expected (Fig. 3C). Thus, human fibroblasts that develop a senescent phenotype early in mass culture have short telomeres.
To determine whether these differences were caused by pre-existing variability in telomere length or by cell-to-cell variation of the rates of telomere shortening, we subcloned MRC5 cells at PD 23. Out of 20 subclones, four (clones numbered 1–4) still grew vigorously at a PD between 44 and 47. In each of these clones, telomere length was shorter in SEN-sorted cells (Fig. 3D). There was considerable interclonal variation in telomere length that might be due to variation between the founder cells for each clone. Importantly, intraclonal differences between SEN- and ALL-sorted cells were similar to those
found in mass culture (Fig. 3, compare C and D). On the contrary, in a fifth clone that was within less than one PD from senescence at the time of sorting, no difference between SEN- and ALL-sorted telomeres was found (Fig. 3D). Hence, there is intraclonal heterogeneity in the rate of telomere shortening. The differences in shortening rates are most probably underestimated, because at least some of the SEN-sorted progeny will have gone through fewer cell divisions than the growing cells and stopped dividing long before the time of sorting. We conclude that variation of telomere shortening rate occurs within the progeny of a single cell and can cause early telomere loss in a subset of human fibroblasts.

To test whether accelerated telomere erosion accompanied or causes early senescence in these cells, we transfected MRC5 fibroblasts with hTERT, the catalytic subunit of human telomerase. Telomerase activity counteracts telomere shortening and rescues fibroblasts from telomere-dependent senescence at the level of the whole population (33). Telomere length and growth rate in a retrovirally transfected mass culture of MRC5 (MRC5-hTERT) were stabilized for at least 70 PD following transfection (Ref. 27 and data not shown). Only about 5% of MRC5-hTERT fell into the SEN gate, which encompasses 25% of parental MRC5 (Fig. 4, A and B). This difference was not due to lower telomerase activity in SEN-sorted cells. On the contrary, telomerase activity in SEN-sorted cells under normoxia was even higher than in ALL-sorted fibroblasts (Fig. 5B). To test these results using an independent marker for senescence, we next sorted human fibroblasts according to their p21 expression levels. G120 is an hTERT-immortalized LF-1 derived fibroblast strain in which an EYFP reporter was knocked in one copy of the p21 gene (17). p21 is an inhibitor of cyclin-dependent kinases (34). In telomere-driven senescence, DNA damage foci formation at sites of uncapped telomeres activates p53 via phosphorylation of Chk1 and Chk2 effector kinases (27), leading to transcriptional activation of p21. p53-dependent transcriptional up-regulation of p21 is an essential component of the pathway that regulates human cell entry into

![Fig. 3](image-url) **Telomeres are shorter in SEN-sorted fibroblasts.** A, telomere Southern blots from unsorted MRC5 in mass culture. This culture became senescent at a PD of 46. M, λHindIII marker. B, telomere Southern blots from MRC5 (PD31) following sorting into either the SEN gate (SEN), the PROL gate (PROL), or a gate encompassing all cells (ALL). M, λHindIII marker. C, average telomere length as measured by real time PCR (arbitrary units) in MRC5 at the indicated PD, either SEN-sorted (black bars) or ALL-sorted (open bars). The data are the means ± S.E. from three independent experiments/PD. D, telomere lengths in MRC5 subclones were measured as in C. Clone 5 was already senescent at the time of sorting.

![Fig. 4](image-url) **Rescue of fibroblasts from early senescence by hTERT expression is reversed by increased ambient oxygen concentration.** A, scatter plot of parental MRC5 at PD 30, showing the position of the SEN gate. 25% of all cells are within the gate. FSC, size; FL1, green autofluorescence (lipofuscin content). B, scatter plot of MRC5-hTERT at PD 74 obtained using the same settings as in A. The number of cells in the SEN gate is reduced to about 5%. C, scatter plot of MRC5-hTERT at PD 64 after 30 days under 40% ambient oxygen. Same instrumental settings as in A. About 55% of all cells are within the SEN gate.
replicative senescence (16, 35, 36). Thus, p21 reporter fluorescence, under optimal growth conditions and in the absence of DNA damage, is an early marker for human fibroblast replicative senescence. Cell populations with high and low EYFP fluorescence were clearly discernible by flow cytometry. Those cells with high EYFP signal, indicating high p21 promoter activity, had short telomeres (Fig. 5C), thus confirming the results obtained by size/autofluorescence sorting.

Short, dysfunctional telomeres trigger and maintain replicative senescence in normal human fibroblasts via formation of senescence-associated DNA damage foci (27, 37). Phosphorylated H2A.X (γ-H2A.X) is a major component of these foci. To find out whether short telomeres in telomerase-expressing fibroblasts would be able to trigger the same response, we compared γ-H2A.X immunostaining in sorted MRC5-hTERT cells with that in parental MRC5 fibroblasts (Fig. 6). In accordance with earlier data (27), γ-H2A.X foci abundance is low in proliferating (ALL-sorted) cultures of MRC5 and MRC5-hTERT cells, possibly reflecting a frequency of 10–20% senescent cells in these cultures. It is even lower in PROL-sorted cells. Importantly, both SEN-sorted MRC5 and MRC5-hTERT show similarly high frequencies of γ-H2A.X-positive cells (Fig. 6G), suggesting that the short telomeres in both cases can induce the formation of DNA damage foci and are thus functionally uncapped.

Short telomeres in a subset of cells despite high telomerase activity in the TRAP assay could be due to problems with the access of telomerase to telomeres in these cells. Alternatively, telomeres in these cells may shorten so rapidly that even high telomerase activity would be insufficient to maintain telomere length. To resolve this issue, we cultured MRC5-hTERT under conditions that greatly accelerate telomere shortening, i.e., under 40% normobaric hyperoxia. This treatment increased oxidative stress in MRC5 fibroblasts as shown by elevated levels of peroxides, protein carbonyls, and lipofuscin (29). Most importantly, it increased the average rate of telomere shortening about 5–10-fold and severely limited the replicative life span of parental MRC5 (38). However, there is no indication that it could change the telomerase-telomere interaction. MRC5-hTERT cells under 40% hyperoxia showed a slow-down of growth and depletion of cells in the S phase (data not shown) but, in contrast to parental cells, not a complete and irreversible growth arrest. However, at least 50% of cells were now within the SEN gate (Fig. 4C). Sorting confirmed that these cells had short telomeres (Fig. 5D) despite similar telomerase activity for SEN- and ALL-sorted cells (Fig. 5E). We conclude that variation in the primary rate of telomere shortening, especially under conditions of chronic stress, is larger than can be compensated for by telomerase.

**DISCUSSION**

The replicative life span of human primary cells in culture is highly heterogeneous. Within the same population, and even within the same clone, a fraction of cells appears to “drop out” from the cell cycle and eventually to develop a senescent phenotype at every population doubling (3). Cells in culture are exposed to a variety of stresses including oxidative stress, and these stresses can induce senescence prematurely in a telomere-independent, premature fashion. Stress-induced premature senescence and telomere-dependent replicative senes-
ence share not only many phenotypic markers, they are also induced via the same signal transduction pathways involving the DNA damage response kinases ATM and ATR, formation of DNA damage foci, and activation of downstream effector kinases Chk1 and Chk2 (27, 37). Thus, the early senescence of human cells under standard culture conditions might be either telomere-independent and premature (5, 9) or caused by stochastic uncapping of telomeres in a possibly telomere-length-independent fashion (10) or might be due to stress-related heterogeneity in the rate of telomere shortening (11).

Our data show that cells that develop a senescent phenotype early have telomeres that are on average as short as those in senescent populations. Clonal experiments prove that this difference is due to faster telomere shortening in the subclones that senesce early. Stabilization of telomere length by hTERT transfection rescues most cells from early senescence and thus strongly suggests that the observed telomere shortening causes early senescence in the majority of cells.

However, active telomerase does not rescue all cells from senescence. There is a small fraction of cells that senesce despite active telomerase. These cells display, on average, short telomeres as well as nuclear foci containing γH2AX, which is a marker for senescence-associated DNA damage foci, formed in response to short, functionally uncapped telomeres (27, 37). Although it is tempting to speculate that short telomeres might be able to trigger senescence even in a minor fraction of telomerase-positive cells, it should be noted that the evidence for that is far only correlative. It is possible that senescence in hTERT-expressing fibroblasts, albeit that it occurs with short telomeres, is triggered by some other mechanism. Oxidative stress, in addition to accelerating telomere shortening, might also activate additional factors that could be important for telomere-independent senescence. For instance, up-regulation of p16 provides an additional, telomere-independent barrier to proliferation in human keratinocytes and at least some fibroblast strains (39). Whether p16 is activated by oxidative stress or not is not completely clear (9, 40). Besides, it was recently claimed that diminution of the single-stranded overhang rather than shortening of telomeres per se could be the main determinant of senescence (41). Free telomeric G-rich single-stranded overhangs induce a growth arrest with many features of senescence (42, 43). Moreover, highly regulated low level expression of telomerase, which in itself is not sufficient to significantly slow down telomere shortening, appears to retard telomere uncapping even in normal human fibroblasts (44), and this could be due to stabilization of telomeric overhangs. Thus, our data suggest that telomere length-dependent uncapping can occur in the fraction of telomerase-positive cells that shows fast telomere shortening but cannot formally rule out roles for stress-induced premature senescence or length-independent telomere uncapping in this minor fraction of cells.

It should also be noted that our results are not in accordance with a recent study in which cells with a senescent phenotype were sorted from hTERT-immortalized fibroblast mass cultures using size alone as sorting criterion (22). Using flow cytometry FISH, these authors were not able to detect any difference in telomere length between the sorted fibroblast populations. As mentioned above, this might well be due to limitations of the flow cytometry FISH technique if applied to human fibroblasts with relatively short telomeres and large differences in size between the sorted populations. Real time PCR quantification of telomere length would not be hampered by these differences, and we obtained consistent results with two completely independent sorting regimens.

In conclusion, our data show that MRC5 fibroblasts in early senescence have on average short telomeres and that rescue of telomere shortening by hTERT expression also rescues most, but not all, cells from early senescence. Thus, stochastic variation of the telomere shortening rate even between cells from a single clone appears to be the major cause of heterogeneity in the replicative life span for most MRC5 human fibroblasts.

Levels of oxidative stress and antioxidant defense can greatly modify the rate of average telomere shortening (11, 19, 38, 45–47). There is evidence for increased oxidative stress in the early senescent cells because lipofuscin content, which was used here to identify these cells, is in itself a cumulative marker for cellular oxidative stress (23, 24). Thus, oxidative stress is a possible cause for the stochastic variation in the telomere shortening rate.

This conclusion is confirmed by the results obtained with hTERT-overexpressing fibroblasts. Mild continuous oxidative stress significantly increases the fraction of cells with a senescent phenotype and short telomeres. The most obvious explanation for this observation is that even forced telomerase expression resulting in high activity is not sufficient to compensate telomere shortening in all cells and that the fraction of cells with telomere shortening faster than can be compensated by telomerase increases under stress. This might help to explain why human fibroblast strains with low antioxidant defense capacity like WI38 (38) were sometimes not successfully immortalized by hTERT transfection under ambient oxygen partial pressure (47).

Evidence for the existence of variation in telomere length in an apparently stochastic manner has recently been found at the level of a single telomere (32). Measuring the length of individual Xp/Yp telomeres in MRC5 clones, it was shown that most telomeres shortened gradually with increasing PD. Besides, however, ultrashort (<0.5 kb) telomeres occurred at each allele of the Xp/Yp telomeres with a low frequency (probably less than one percent of the cells) (32). Assuming that all other telomeres behave similarly, these data suggest high probabilities that at least one telomere per cell might be ultrashort, probabilities that are very similar to the actual frequencies of senescent cells in the population. These data are also in agreement with estimates of strand break frequencies in all MRC5 telomeres and the resultant average telomere shortening rates (19, 48). It is not clear yet whether the frequencies of ultrashort telomeres with PD. However, together with the fact that the average Xp/Yp allele telomere length at senescence was widely different from clone to clone (32), the data are in accord with the idea that in the majority of cases it is not the gradual, “clockwise” overall shortening of telomeres that causes senescence but rather the effect of large stochastic events on possibly very few telomeres. Crossing experiments with telomerase knock-out mice have indicated that few critically short telomeres can arrest cell growth (49). Thus, it is possible that the average telomere shortening rates measured here are largely determined by massive losses on few individual telomeres and reflect primarily the probability of such events to occur.

In conclusion, our data suggest that at the level of a single cell lineage, telomeres are not regular “biological clocks,” but senescence is induced largely by stochastic telomere loss events. In other words, stress-induced senescence and telomere-dependent replicative senescence are not necessarily mutually exclusive. Rather, there exists a continuum in which telomere shortening is driven to a large extent by stress and, possibly, other stochastic events. Telomere shortening by intrinsic mechanisms (i.e. the end replication problem) sets an outer limit to the replicative life span, which might only seldom be reached. Stochastic damage to telomeres is sufficient to explain why the human fibroblast replicative life span is heterogeneous.
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