

Age-Related Telomere Shortening Occurs in Lens Epithelium from Old Rats and is Slowed by Caloric Restriction

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We have investigated whether the average relative telomere length of lens epithelial cells (LECs) from brown Norway rats decreases with the age of the donor animal, and whether chronic caloric restriction (CR) of the rats delays the telomere shortening. Our previous studies have demonstrated that clonal proliferative potential of rodent LECs as well as the *in vivo* rate of DNA synthesis decreases with age and that this decrease is slowed by chronic lifelong caloric restriction (CR). In order to determine if telomeric shortening might be involved in this loss of proliferative potential, we examined relative telomeric lengths in young, old ad lib fed (AL), and old calorically restricted (CR) brown Norway rats. We used fluorescence *in situ* hybridization with a peptide nucleic acid probe (PNA) complementary to the telomeric repeat sequence to quantitate relative telomere lengths in LECs in lens sections (TELO-FISH). Control experiments demonstrated that the PNA probe binding was restricted almost entirely to the terminal portions of the rat chromosomes with less than 5 % bound at interstitial sites in typical metaphase spreads. The relative telomere lengths of interphase human fibroblast standards, as determined by TELO-FISH, were in good agreement with terminal restriction fragment analyses of the same standards and with literature values for rat cells. The average telomere lengths of interphase nuclei in the old AL rat LECs were found to be 21 % shorter than paired young AL controls ($P < 0.01$ by Wilcoxon signed rank test). The calorically restricted old rats had less telomere erosion (12 %) than the old AL group ($P < 0.05$). Although it is not clear whether such moderate telomeric erosion can limit cell division in rodent LECs, the telomeric shortening correlated well with previous studies demonstrating reduced clonal, replicative potential, and reduced rates of *in vivo* DNA replication in LECs from old rodents and a delay in this attenuation in animals on chronic CR.

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Key words: telomere; aging; lens; rats; fluorescence *in situ* hybridization; peptide nucleic acid; caloric restriction; cataract; confocal microscopy; oxidative damage.

1. Introduction

We have previously reported that rodent lens epithelial cells (LECs) lose clonal proliferative potential *in vitro* with the age of the donor and that the life prolonging effects of chronic caloric restriction (CR) slow the loss of this proliferative potential significantly relative to ad lib fed (AL) controls. Furthermore, studies performed by labelling rodents with BrdU for 2 weeks demonstrated that the rate of DNA replication slows very significantly with age in these same cells *in vivo* and that old CR mice retain youthful rates of DNA replication significantly longer (Li and Wolf, 1997; Li, Yan and Wolf, 1997; Li, Yan, Wolf and Pendergrass, 1998). Similar age-related loss of clonal proliferative potential and its preservation by CR has been reported for many rodent tissues examined (Pendergrass *et al.*, 1995).

CR has also been shown to retard age-related cataractogenesis in several strains of brown eyed mice and in brown Norway rats (Wolf *et al.*, 2000). Furthermore age-related losses of gamma crystallins in the mouse lens are retarded by CR (Leveille *et al.*, 1984). In order to examine the potential molecular

mechanisms of this age and diet related loss of cell division potential in rat LECs and a possible role in cataractogenesis, we have examined the length of telomeres in frozen sections from the lenses of brown Norway rats of different ages on both AL and CR diets. It has been demonstrated that shortening of telomeres is an important mechanism of *in vitro* senescence in many mammalian cell types. The mechanism of DNA replication requires that telomeres will be shortened on the lagging strand following each round of DNA replication unless restored by active telomerase (Olovnikov, 1973; Harley, Futcher and Greider, 1990; also see reviews by Blackburn, 1992; Greider, 1998). Recent studies by Bodnar *et al.* (1998) demonstrate that human fibroblasts and retinal epithelial cells can be immortalized by the addition of recombinant human telomerase (hTERT). Similar findings have been reported by others (Dickson, 2000; Ouellette *et al.*, 2000a,b; Wang, Hannon and Beach, 2000). These findings establish telomere erosion as causing senescence *in vitro* in many human cells types, although alternative senescence mechanisms may exist side by side with telomere shortening (Prowse and Greider, 1995; Blasco, 1997; Farwell *et al.*, 2000). Evidence for telomere shortening is also

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seen in vivo in many human tissues. Age-related shortening has been reported in kidney by [Melk et al., 2000](#), in T and B lymphocytes by [Son et al., 2000](#), and in liver by [Aikata et al., 2000](#). Finally, telomere erosion promotes chromosomal instability and is associated with oncogenesis ([Greider, 1999](#); [Griffith et al., 1999](#); [Katayama et al., 1999](#); [Klingelhutz, 1999](#); [Sprung et al., 1999](#); [Cerni, 2000](#); [Oulton and Harrington, 2000](#); [Smith, Germain and Skelton, 2000](#)). However, telomere shortening is yet to be clearly linked to aging pathologies such as senile cataract in vivo.

Unlike humans most inbred rodents have telomeres that are very long and heterogeneous in length. The evidence for telomere shortening in rodents is less clear than in humans ([Prowse and Greider, 1995](#); [Campisi, 1997](#); [Carman, Afshari and Barrett, 1998](#); [Faragher, 2000](#)). However, recent studies do report age-related telomere shortening in rats in both the kidney ([Jennings et al., 1999](#)) and in the fraction of rat heart myocytes that divide in vivo ([Kajstura et al., 2000](#)). This indicates that telomere shortening may play some role at least as a marker of cell aging in rats although perhaps less of a causal one than in humans. In order to examine telomere shortening directly in lens epithelial cells (LECs) in situ, we have utilized fluorescence in situ hybridization (FISH) of a peptide nucleic acid (PNA) probe to the telomere repeat sequence (TELO-FISH). This PNA-FISH method is more sensitive than the traditional terminal restriction fragment (TRF) method of measuring telomere length ([Lansdorp et al., 1996](#); [Kajstura et al., 2000](#)) and can be used to differentiate telomere losses in small bits of tissue such as individual rat lenses, or in different cell types in the same tissue. These advantages are important in our studies on rat lenses as we are examining small pieces of tissue, and need to differentiate LECs from ciliary body. Examination of LECs in each of nine young and nine old AL, and nine old CR rat lenses, demonstrated that rat LEC telomeres were shortened by 21 % in old AL fed rats relative to young controls ($P < 0.01$) and that CR reduced this loss to 12 % ($P < 0.05$).

2. Materials and Methods

TELO-FISH Hybridization to Telomeric Sequences

For in situ fluorescence hybridization with anti-telomere probe to telomere sequences (TELO-FISH) we used the method of [Lansdorp \(1995\)](#) and [Lansdorp et al. \(1996\)](#) with several modifications. Fourteen μm frozen sections were cut from freshly frozen lenses and dried on probe-on slides (Fischer Scientific, 2000) for 90 min at room temperature (RT). Slides were fixed with fresh 1 % paraformaldehyde for 10 min at RT. The sections were rinsed once with phosphate buffered saline, pH 7.4 (PBS) for 5 min, then treated for 2 min with 1 mg ml⁻¹ pepsin (pH 1.0 at 37°C).

The slides were rinsed with PBS for 5 min then dehydrated with a series of water ethanol mixtures (25, 40, 60 and 95 %) and dried at RT. The slides were then hybridized to the peptide nucleic acid probe [Fluorocin-(CCCTAA)₃, PerSeptive Biosystems, Foster City CA, U.S.A.]. The probe was dissolved in formamide buffer (70 % formamide, 1 % blocking agent, Boehringer Mannheim, Indianapolis, IN, U.S.A. and 10 mM Tris pH 7.2). One hundred μl of PNA hybridization mix containing 2 $\mu\text{g ml}^{-1}$ PNA probe was added to each slide within an adhesive 'Hybriwell' from Midwest Scientific (Valley Park, MO, U.S.A.). The slides were heated at 78°C for 10 min, then placed in the dark at RT for 2 hr to allow hybridization to telomeric regions to proceed. The unbound probe was then rinsed for 15 min, four times with 70 % formamide buffer without a blocking agent. This was followed by rinsing in Tween buffer (0.05 % Tween-20 in PBS), for 5 min, four times with a final rinse in PBS for 5 min, three times. The drained slides were dried with increasing EtOH series as above. The DNA in the cells was then counterstained with 10 $\mu\text{g ml}^{-1}$ 7-AAD (7-aminoactinomycin D), Calbiochem (La Jolla, CA, U.S.A.) or TOTO 3 (1 : 200 dilution, Molecular Probes, Eugene, OR, U.S.A.) for 5 min and coverslipped for viewing. Final dilution's were made in Vectashield (Vector Laboratories, Burlingame CA, U.S.A.).

Animals

brown Norway rats were used for all studies. These were chosen because they are a long lived hybrid strain of rat that responds well to caloric restriction, and we have data on these rats' rates for cataract formation as well as its delay by CR ([Wolf et al., 2000](#)). The animals were obtained from the National Institute of Aging colony at the Harlan Laboratories (Indianapolis, IN, U.S.A.). The animals were maintained under specific pathogen free conditions and killed by CO₂ inhalation. The ages were: young AL (2–8 months), old AL (32–34 months), old CR (32–34 months). The AL animals were maintained on Harlan 7913 (NIH-31). CR animals were maintained on Harlan diet 7109, which was similar to 7913, but with calories per serving reduced to 60 % of that consumed by the AL rats and also supplemented with vitamins and minerals to the same level as consumed by AL animals.

Human Cell Lines

For the purpose of standardizing our TELO-FISH assay, Geron Corporation (Menlo Park, CA, U.S.A.) provided us with both the 'BJ' normal human foreskin diploid cell line and '6TA cells', a clone of the same cell line immortalized with human telomerase (hTERT), whose telomeric lengths had been determined by the terminal restriction fragment method at

several passage levels (Bodnar et al., 1998; Geron Corp. personal communication).

Preparation of Lenses and Lens Sections for Examination of Telomeres in Interphase Nuclei

Whole eyes were removed from dead rats, then placed corneal side down on sterile gauze and held in place by forceps. An incision was then made across the surface where the optic nerve enters the eye and the sclera was pulled back to expose the lens. The iris was gently teased away with forceps as the lens was removed from the eye. Orientation of the lens was maintained by orienting the equatorial plane where remnants of the ciliary body were visible. The lens, epithelial cell surface down, was then placed in a plastic embedding mold containing embedding matrix (Shandon Lipshaw, Pittsburgh, PA, U.S.A.) and then frozen in 2-methylbutane cooled by dry ice. The frozen blocks were stored at -70°C . Frozen lenses were subserially sectioned on a Reichert–Jung 2800 Frigocut cryostat (Leica Microsystems, Heerbrugg, Switzerland) at a thickness of $14\ \mu\text{m}$ and placed on glass slides to dry. The frozen sections were fixed with 1 % paraformaldehyde for 10 min and passed through graduated ethanol concentrations and dried and covered with vectashield (Vector Laboratories, Burlingame CA, U.S.A.) to protect against oxidation and stored at -20°C until needed for TELO-FISH.

Preparation of Metaphase Lens Epithelial Cells to Test Specificity of Telomere Probe

Following lens extraction the basement membrane, which encapsulates the lens and on which the lens epithelial cells are attached, was removed from the crystalline component. To dissociate the epithelial cells, lens membranes with attached cells were placed in a solution of PBS containing 20 % serum and 0.1 % collagenase/dispase (Roche, Indianapolis, IN, U.S.A.) for 45 min at 37°C with agitation. This solution was decanted, and trypsin stock from Gibco (New York, U.S.A.) added for an additional 15 min. The enzymes were neutralized by washing with PBS containing 10 % fetal bovine serum (FBS) in 1 % Versene (Gibco, New York, U.S.A.) buffer for 5 min. The suspension of cells was centrifuged and the cells were plated into 35 mm collagen IV coated dishes (Becton Dickinson, Bedford, MA, U.S.A.) in 20 % FBS in DMEM for 5 days. Two hr before harvesting $2 \times 10^{-7}\ \text{M}$ colcemid was added to the DMEM media on the cultures. The supernatant was poured off and a hypotonic solution of 0.075 M KCl in distilled water was added drop wise to a final volume of 5 ml for 20 min at 37°C . Ten ml of 3 : 1 absolute methanol : glacial acetic acid fix was then added. After 20 min 1–2 ml of fix was added to each tube. The cells were mixed, spun-down and the supernatant discarded. Ice cold fix was added drop-wise for the first 1–2 ml then faster for the remaining

5–10 ml. The cells were spun down and the supernatant discarded. The pellet was carefully dislodged and another 5–10 ml of fix was added in the same manner. The cells were spun down and resuspended in a further 10 ml of fresh fix and the metaphase suspension was dropped on probe-on slides (Pittsburg, PA, U.S.A.) dried 60 min and stained using the TELO-FISH protocol.

3. Results

We examined the specificity of the TELO-FISH method by determining that TELO-FISH staining was specifically restricted to the terminal regions of each chromosome. In Fig. 1 a typical rat lens epithelial metaphase spread is shown. More than 95 % of the staining with the PNA anti-telomere probe is restricted to chromosome ends (depicted in computer generated red). The total DNA was stained with TOTO-1 (depicted in computer generated blue). Only a very small amount of interstitial staining is seen (see arrow), as was previously reported for rat chromosomes (Golubovskaya et al., 1999).

To standardize our estimates of telomere length determined by TELO-FISH, we compared the relative

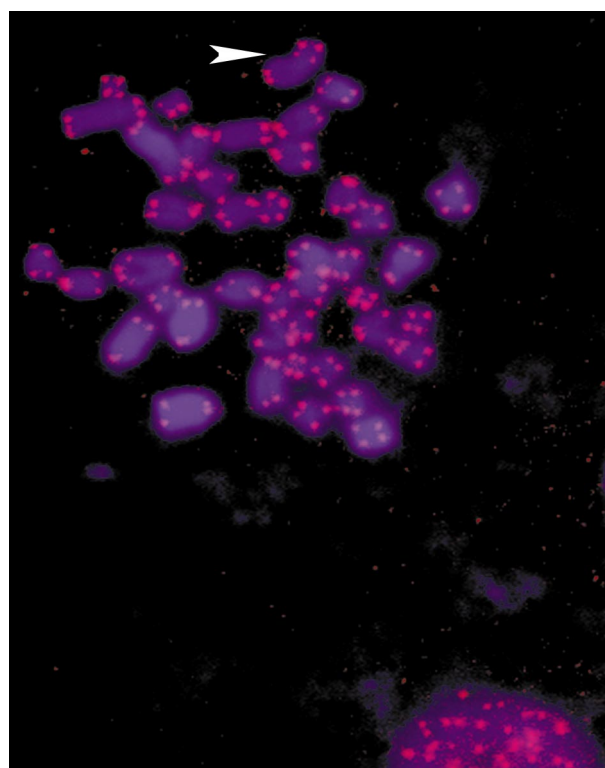


FIG. 1. Example of metaphase telomere staining from rat LECs grown in tissue culture for 5 days from primary explants. Cell preparation and staining with the PNA telomeric probes as well are discussed in Materials and Methods. Telomere staining is depicted in red and DNA staining with TOTO-3 stain is depicted in blue–purple. Data acquisition and computer coloring are by confocal microscopy as described in Materials and Methods. The colors are computer generated.

TABLE I
Comparisons of terminal restriction fragment (TRF) analysis with TELO-FISH of human cell lines and rat LECs

Cell type ^a	Telo/DNA ^b (%)	Telom. lengths from TRF ^c	Telom. lengths from TELO-FISH ^d	PDL ^e	Fraction of in vitro lifespan used ^f (%)
BJ Senescent HDF	11	5.3	4.7	80	94
6ta Immortal HDF	26	11.0	11.0	150	Immortal
82-6 late pass. HDF	15	—	6.5	47	78
LECs from 3 month old rat	91	30–50	38.9	Fresh tissue	0

^aBJ are normal human diploid foreskin fibroblasts (HDF). 6ta cells are the same BJ cells immortalized with telomerase (see text). 82-6 cells are a different line of normal human foreskin fibroblasts. Three month old male brown Norway rat LECs analysed from serially-cut frozen sections.
^bTotal telomeric fluorescence in each nucleus divided by the total DNA fluorescence (see Materials and Methods).
^cTelomere lengths using TRF are expressed in kilobase pairs (Kbp), 6TA and BJ TRF values were obtained from Geron Corp. (personal communication from Geron Corp. and Bodnar et al., 1998). The rat TRF values are from literature estimates (Golubovskaya et al., 1999).
^dTelomere length calculated assuming a linear relationship between TELO-FISH and TRF data.
^ePopulation doublings at time of both TELO-FISH and TRF analyses.
^fPopulation doubling level (PDL) at time of TELO-FISH divided by maximum PDL of subline in our laboratory.

fraction of telomere staining to that predicted by the traditional method of terminal restriction fragment (TRF) analysis on gels (Harley et al., 1990). The TRF analyses were previously determined by Geron Corp. on human fibroblast cell lines (BJ and 6TA) provided to us. Table I compares the values of TELO-FISH staining to TRF analyses of the same lines. The 6TA cell line is a strain of human foreskin cells immortalized by transformation with human telomerase catalytic subunit (hTERT), and the BJ cells are the parental human foreskin derived cells used to produce the immortalized 6TA (Bodnar et al., 1998). The immortal, 6TA cells and senescent BJ cells were reported to have TRF calculated telomere lengths of 11 and 5.56 Kbp, respectively (personal communication, Geron Corp., 2000). Table I compares these TRF values to those determined for the same cell lines at approximately the same passage level by TELO-FISH and the two methods can be seen to give good agreement. Based on the TRF standards, we determined the length of typical rat LECs to be about 39 Kbp by TELO-FISH. This is in reasonable agreement with literature values (Golubovskaya et al., 1999), which report that individual rat telomeres range from 20 to 100 Kbp and on average approximately 30–50 Kbp. These control findings indicate that the TELO-FISH analysis method provided a very sensitive and accurate estimate of average telomere lengths for interphase nuclei from all these cell types.

In determining the relative telomere lengths from TELO-FISH we have used the value of total telomere staining per nucleus divided by the total DNA staining for each nucleus. This corrects for the presence of any partial nuclei or of any polyploid nuclei present in the sections since such telomere staining is reduced in proportion to changes in DNA content in each nucleus. This value of telomere staining/DNA staining is used interchangeably with ‘relative telomere length’ in this manuscript.

Telomere Length Measurements in old AL, Old CR, and Young Rats

The TELO-FISH method was used to determine the relative telomere lengths in frozen lens sections from 27 brown Norway rats. These sections were almost entirely interphase LEC cells taken from the germinative region of the lens. Areas containing ciliary body cells were also present in some sections, but were not analysed. Each telomere analysis preparation consisted of one young AL, one old AL and one old CR rat. Each set of three animals was stained and analysed together to permit paired analyses of the results, with a total of nine such sets examined. The total telomere fluorescence and the total DNA fluorescence was determined in each interphase LEC nucleus in frozen sections as described in Materials and Methods. Fig. 2 shows a sample section of interphase lens epithelial cells from the central region stained in this manner. The telomeres are very heterogeneous in size and are clearly visible in the flat central region LEC nuclei in Fig. 2(A). The DNA staining is shown in purple and the telomeres in greenish blue. The cut here is almost parallel to the surface plane of the lens so two rows of epithelial cells are seen in this example. Fig. 2(B) shows a typical section near the equatorial region and demonstrates a cut almost perpendicular to the LEC cell layer.

In Tables II and III the relative telomere lengths of all nine triplet groups (27 animals) were compared. In Table II the mean values of the relative telomere lengths (telomere fluorescence:DNA fluorescence) are displayed. Individual assays were repeated to increase accuracy (2–4 per triplet group) and are averaged to give one mean value per group.

We have used a nonparametric version of the ‘Paired *t*-test’, the Wilcoxon signed rank test, to test the significance of our hypotheses: (1) that telomeres in LECs shorten during; and (2) that CR slows this age-related process. A one tailed version was used

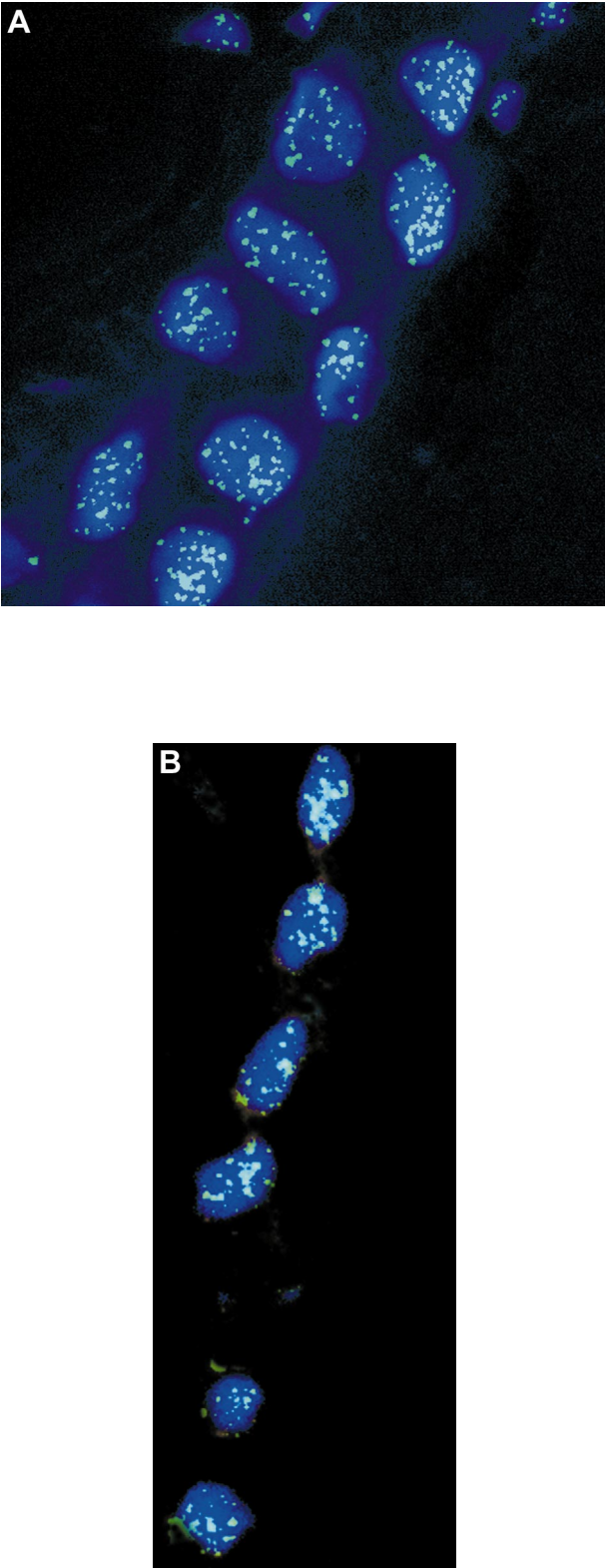


FIG. 2. Sample confocal displays of telomere fluorescence and DNA fluorescence of interphase LECs in 14 μ m frozen sections from: (A) the central region of the lens, and (B) the near equatorial region of the lens. Other experimental details are as described in legend to Fig. 1. The central region is from a tangential cut through the central region of the lens and displays flat epithelial-like cell nuclei with bright green–blue telomeres of varying intensities. The DNA fluorescence from a 7-AA dye is displayed as blue–purple. Multiple confocal sections have been averaged for this display.

TABLE II
Comparison of relative telomere lengths of LECs from young AL, old AL, and old CR rats

	Telo/DNA ratio ^a			Assays ^b
	Young AL	Old AL	Old CR	
Group 1	0.20	0.17	0.19	4
Group 2	0.33	0.26	0.37	3
Group 3	0.20	0.11	0.16	3
Group 4	0.21	0.17	0.20	3
Group 5	0.31	0.24	0.29	4
Group 6	0.30	0.18	0.24	3
Group 7	0.43	0.44	0.39	4
Group 8	0.33	0.24	0.23	2
Group 9	0.34	0.31	0.34	3

A single tailed Wilcoxon signed rank test was used to test our hypotheses that: the mean relative telomere length of old AL group was shorter than the mean of the young AL group, $P < 0.01$; and that the mean telomere length of the old AL group was also shorter than the CR group, $P < 0.05$.

^aThe relative telomere lengths were determined from the ratios of the telomere fluorescence to the DNA fluorescence in each nucleus and expressed as a fraction (see Materials and Methods).

^bNumber of independent assays per group, which were always performed on groups of one young AL (2–8 months), one old AL (26–33 months), and one old CR (26–33 months). The data depicted are the means of two to four separate assays per group in order to assure accuracy.

TABLE III
Comparison of relative telomere lengths^a of young AL and old AL animals that have been normalized^b to the old CR animal in each group

	Young AL	Old AL	Old CR	Assays ^c
	2–8 months (%)	26–32 months (%)	26–33 months (%)	
Group 1	91	97	100	4
Group 2	83	74	100	3
Group 3	128	77	100	3
Group 4	128	94	100	3
Group 5	110	87	100	4
Group 6	133	71	100	3
Group 7	112	107	100	4
Group 8	150	109	100	2
Group 9	98	90	100	3
Mean	114 ^d	89.5 ^d		
S.E. ^e	8	5		

^aThe relative telomere lengths were determined from the ratios of the telomere and DNA relative fluorescence by TELO-FISH analysis (see Materials and Methods).

^bThe relative telomere lengths were determined for each separate assay of a triplet (one young AL, one old AL, one old CR), and then normalized to the old CR value in each grouping.

^cTwo–four repetitions of the TELO-FISH analysis were performed on each group of three rat lenses.

^dThe young AL Group is significantly longer than old AL group, $P < 0.01$, by a one tailed Wilcoxon signed rank test.

^eStandard error of the mean.

since our hypotheses are all unidirectional. The results are that: (1) telomeres from the old AL group are significantly shorter than the young AL group with $P < 0.01$; and (2) that telomeres from the old AL group are also shorter than the old CR group, $P < 0.05$.

In Table III the relative telomere lengths are normalized so that old CR individual in each triplet group is set to a relative length of 100 % as a baseline. This normalization procedure allows a calculation of the degree of telomere shortening of the old AL relative to the young AL, and indicates that the old AL mean is 21 % shorter $[(114-89.5\%)/114\%]$ than the young AL mean. In Table III the telomere lengths are normalized to young AL rats = 100 % as a baseline. The old AL telomeres are found to be 12 % shorter $[(94-83\%)/94\%]$ than old CR group consistent with CR induced protection from telomere shortening.

It is of interest that on a scale of 0–5 with 5 representing fully mature cataract, the mean scores by slit-lamp viewing were 0 for the young rats, 2.2 for the old CR and 3.0 for the old AL. This was consistent with previous studies on the effects of aging and chronic CR in brown Norway rats (Wolf et al., 2000).

4. Discussion

It has been demonstrated for the first time that telomeres can be measured in the LECs of individual rats and that they shorten with aging. Chronic life extending CR significantly reduced the rate of age-related telomere erosion. These findings correlate with previous findings that cellular proliferative potential of LECs and of many other tissues in rodents are decreased during aging and that again CR slowed these losses (Pendergrass et al., 1995; Wolf et al., 1995; Li et al., 1997, 1998). They also correlate with the findings that in both rats and mice, cataract appearances are maximum at the time that the cellular replicative potential declines and that CR delays this (Li et al., 1997; Wolf et al., 2000). This suggests a possible relationship between telomeric shortening, loss of replicative potential in LECs, and cataract appearance in rodents. However, evidence for a direct relationship will require animals in multiple age groups and species and a greater understanding of the mechanisms of the cataractogenic and cellular proliferative losses involved.

Rat telomeres are very heterogeneous in length, from 20 to 100 Kbp (Golubovskaya et al., 1999; Jennings et al., 1999), but assuming that rat LEC telomeres start at an average of approximately 39 Kbp as determined in Table I, we can estimate that the LEC telomeres from the old AL rats shortened about 21 % relative to young AL rats (see Tables II and III), and thus $0.21 \times 39 \text{ Kbp} = 8.2 \text{ Kbp}$, and the old CR rats shortened by approximately 12 % or 4.7 Kbp. This modest erosion is less than what is found for human

cell types during aging but consistent with other determinations on aging rat tissues (Jennings et al., 1999; Kajstura et al., 2000).

Age-related and replication related telomeric shortening has been reported in several rodent tissues. Rat kidney telomeres were shown to shorten with age by Jennings et al. (1999), also, in the dividing fraction of rat myocytes in old rats by Kajstura et al. (2000) and recently during serial passage of mouse hematopoietic stem cells (Allsopp, 2001). Colitz, Davidson and McGahon (1999) demonstrated that dog, rat, and murine lens LECs possess telomerase activity, which increases in cataractous lenses. The presence of telomerase, however, does not always prevent telomere shortening (Son et al., 2000). Colitz et al. (1999) also noted that LEC telomeres were not shortened in the LECs from cataractous dogs. However, this group did not examine telomere lengths as a function of age, nor did they report telomere lengths in very old dogs, as we have in rats.

CR does not merely conserve a total fixed number of replications by slowing cell division in the LECs early in life but, rather, appears to extend the total number of cell doublings LECs can undergo (Li et al., 1997, 1998). We and others have proposed that this is accomplished by some protective effect of CR, possibly against oxidative stress (Reed et al., 1996; Wolf and Pendergrass, 1999; and see review by Merry, 2000). Li et al. (1998) demonstrated that CR mice LECs are extraordinarily resistant to oxidative stress. Oxidative stress has also been reported to accelerate telomere shortening dramatically in human fibroblasts (von Zglinicki, 1998; von Zglinicki, Pilger and Sitte, 2000a; Serra et al., 2000). Thus, any protection from oxidative damage afforded by CR or in young animals relative to old AL animals might quite reasonably be expected to delay shortening of telomeres, protect cell replication and possibly prolong life-span according to the telomere hypothesis. Such protection could be in the form of superior antioxidant systems, better DNA structural resistance enhanced DNA repair, or any combination of these.

Extensive shortening of rat telomeres may not be necessary to increase the probability of cellular senescence. Recent reports indicate that failure of the 'capping' mechanism as telomeres shorten may actually trigger exit from the cell cycle or apoptosis rather than telomere length per se (Blackburn, 2000). Oxidative or other damage to the telomeric DNA appears to act synergistically increasing the probability of capping failure and cell senescence even with only moderate shortening (Blackburn, 2000; von Zglinicki et al., 2000a; von Zglinicki, 2000b). Possibly a combination of only partial shortening of these long heterogeneous telomeres and oxidative damage to the telomeric DNA in rats and mice leads to premature exit from the cell cycle in LECs, and CR slows the rate of such damage. Alternatively, some

other process must be inferred to cause senescence in rodent cells than that in higher mammals.

Recent studies by Tang et al. (2001) and Mathon et al. (2001) indicate that some types of mouse and rat cells may be propagated indefinitely in special serum free culture conditions without loss of proliferative potential or overt transformation. These findings do not speak to the accumulation of age-related damage to rodent cells that may occur during in vivo aging, and in fact their studies indicate that damage is accruing even in vitro although the cells are compensating. Shay and Wright (2001) point out that some form of subtle damage does occur to cell cycle check points during cell passaging. Evidence for this is that Mathon et al. (2001) and Tang et al. (2001) report that the checkpoint factors: p19^{ARF}, p16^{INK4a}, p18^{INK4a}, p21, p27, and p57 build up with passage level in vitro even under their optimum serum free conditions. These factors do not result in arrest or senescence in their studies because there are compensatory increases in positive growth factors such as Cdk2, Cdk4, and the cyclins D1, D3, and E in the special serum free medium conditions to counter the negative factors, and sufficient telomerase to prevent drastic shortening of telomeres. We propose that during in vivo aging of rodent LECs, such an evolution of counteracting positive and negative factors does not occur to the extent that it does in serum free tissue culture, nor is telomerase able to repair efficiently all telomere damage. Thus age-related damage (including possibly oxidative damage to telomeric DNA) triggers sufficient check point activity to reduce cell turnover rate and proliferative potential with age in LECs and other rodent tissues as previously described (Pendergrass et al., 1995; Wolf et al., 1995; Li et al., 1997).

In summation, we have shown that significant telomere shortening does occur in aged rat LECs and that CR provides a partial protection against this shortening. Telomere shortening in old animals correlates in time with an age-related reduction of LEC replication rate in vivo and clonal proliferative potential in vitro (Li et al., 1997) and with a reduced resistance to oxidative challenge (Li et al., 1998), as well as the late life occurrence of rat and mouse cataracts (Wolf et al., 2000). Animals on CR are partially but significantly protected in all of these instances. The possible relationships of cause and effect for these phenomena are suggestive for oxidative damage, telomere shortening and loss of replicative capacity contributing to age-related cataract formation, in that order. However, this evidence is presently only correlative.

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