Cell, Vol. 96, 701–712, March 5, 1999, Copyright ©1999 by Cell Press

Longevity, Stress Response, and Cancer in Aging Telomerase-Deficient Mice

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Summary

Telomere maintenance is thought to play a role in signaling cellular senescence; however, a link with organismal aging processes has not been established. The telomerase null mouse provides an opportunity to understand the effects associated with critical telomere shortening at the organismal level. We studied a variety of physiological processes in an aging cohort of mTR$^{-/-}$ mice. Loss of telomere function did not elicit a full spectrum of classical pathophysiological symptoms of aging. However, age-dependent telomere shortening and accompanying genetic instability were associated with shortened life span as well as a reduced capacity to respond to stresses such as wound healing and hematopoietic ablation. In addition, we found an increased incidence of spontaneous malignancies. These findings demonstrate a critical role for telomere length in the overall fitness, reserve, and well being of the aging organism.

Introduction

Telomeres are essential for chromosomal stability and cell viability in a variety of different species (Greider, 1996). In primary human cells, telomeres shorten with passage in culture, and progressive telomere shortening ultimately limits the replicative capacity of cultured cells (Harley et al., 1990; Allsopp et al., 1992; Wright and Shay, 1992; Counter, 1996; de Lange, 1998). It has been suggested that telomere-associated cellular senescence may contribute to certain age-related disorders, including an increase in cancer incidence, wrinkling and diminished skin elasticity, atherosclerosis, osteoporosis, weight loss, and others (Salk, 1982). Although aged organ systems function adequately to maintain baseline health, short telomeres could be linked more directly to a fundamental feature of aging: a reduced capacity to respond to acute and chronic illness.

Many diverse genes, such as those involved in gene silencing, DNA repair, genomic stability, and growth factor signaling, have emerged as strong determinants of life span in a variety of species (Dorman et al., 1995; Guarente, 1996; Wright et al., 1996; Smeal and Guarente, 1997). Less clear, however, is the relevance of progressive telomere shortening as a potential factor in organismal aging (Johnson et al., 1998). In *Saccharomyces cerevisiae*, although telomere shortening in EST1 mutants causes rapid loss of cell viability (Lundblad and Szostak, 1989), telomere length holds constant over the life span of normal cells, implying that telomere shortening is not a cause of aging in yeast (D'Mello and Jazwinski, 1991; Austriaco and Guarente, 1997; Sinclair et al., 1998). In contrast, the tight relationship between replicative senescence and telomere shortening in cultured human cells has led to the view that telomere length regulation may provide a molecular explanation for diminished reserve and cellular senescence in aged tissues (Cooke and Smith, 1986; Harley et al., 1990; Hastie et al., 1990; Lindsey et al., 1991; Allsopp et al., 1992; Vaziri et al., 1994; Harley, 1997; Frenck et al., 1998). Age-dependent telomere loss may contribute to a reduction in viable cells, altered differentiation functions, and impaired regenerative/proliferative responses, particularly in the settings of stress such as those seen in chronic infections, cirrhosis, chronic skin ulcerations, hypertensive vascular injury, among others (Chang and Harley, 1995; Kitada et al., 1995; Effros et al., 1996). One might anticipate that high turnover organs such as the skin, lymphoid, and gastrointestinal tract would be more adversely affected due to an accelerated loss of telomere repeats (Campisi, 1998).

In humans, telomeres are short, and telomerase activity is low or undetectable in many somatic tissues but present in germ cells, activated leukocytes, and stem cells from a variety of organs (Harley et al., 1994; Wright et al., 1996; Newbold, 1997). However, the degree to which telomerase maintains telomeres in these tissues during aging has not yet been fully explored. The laboratory mouse, an inbred strain of *Mus musculus*, possesses much longer telomeres, and telomerase activity and gene expression of the catalytic subunit of telomerase (TERT) appear to be less stringently regulated in somatic cells (Kipling and Cooke, 1990; Prowse et al., 1993; Prowse and Greider, 1995; Zijlmans et al., 1997;

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Greenberg et al., 1998). Although the mouse is a good model for studying telomerase activity and its regulation, the long telomeres in this species, despite modest attrition with aging (K. L. R. and R. A. D., unpublished data), make it unlikely that telomere length plays a prominent role in normal mouse aging.

A critical evaluation of the effect of telomere shortening on aging in vivo has been hampered by the lack of mammalian-based model systems. We have previously generated telomerase-deficient mice through disruption of the gene encoding the essential RNA component of the telomerase holoenzyme (mTR) (Blasco et al., 1997). These studies established that $mTR^{-/-}$ mice exhibit progressive telomere shortening with each successive generation arising from $mTR^{-/-}$ intercrosses (Blasco et al., 1997; Lee et al. 1998). Sixth generation mice (G6) were infertile, had a decreased proliferative capacity of splenocytes and bone marrow cells in vivo, and had an increased embryonic lethality due to neural tube closure defects (Lee et al., 1998; Herrera et al., 1999). Third generation $mTR^{-/-}$ mice (G3) possess shortened telomeres early in life but, at this point, appear to be phenotypically normal in every respect (Lee et al., 1998). The current study focused primarily on the physiological consequences in an aging population of G3 mice and compared these results to first and sixth generation $mTR^{-/-}$ mice (G1 and G6, respectively) to dissect the contribution of telomere dysfunction to the pathophysiological processes and events associated with aging.

Results

To assess the effect of progressive telomere loss on mice with increased age, we studied a cohort of mice over a 2.5 year period. Specifically, 63 $mTR^{+/+}$, 35 $mTR^{-/-}$ G1, 35 $mTR^{-/-}$ G3, and 36 $mTR^{-/-}$ G6 mice were analyzed for a broad spectrum of phenotypes associated with the aging process. For brevity, we refer to successive generations of telomerase-deficient mice as G1 (for G1 $mTR^{-/-}$), G2 (for G2 $mTR^{-/-}$), and so on.

Hair Graying, Alopecia, and Skin Lesions

A dramatic increase in the incidence of hair graying and alopecia (hair loss) was noted in aging G3 and G6 mice compared to age-matched $mTR^{+/+}$ mice (Figures 1A–1D: 25% in $mTR^{+/+}$, 54% in G3, and 60% in G6, in animals older than 15 months). In addition, hair graying was seen at younger ages: some of the G6 animals showed graying at 6 months of age, while none of the G3 or $mTR^{+/+}$ mice showed alopecia or hair graying at this age. Hair growth is a cyclic process in which the hair follicle progresses through an active growth phase (anagen), an involuting phase (catagen), and a resting phase (telogen) (Courtois et al., 1995). Dorsal skin specimens from 15- to 18-month-old G3 and G6 mice showed equal numbers of hair follicles in the dermis compared to $mTR^{+/+}$ animals (Figures 1E and 1F), but there was a clear increase in miniaturized, involuted, melanin-rich follicles in the superficial dermis (Figure 1F). The number of hair follicles in the growth phase (anagen) relative to the resting phase was 2- to 3-fold lower in G3 and G6 compared to $mTR^{+/+}$ animals (Figures 1E and 1F). These histopathological findings in the aged balding G3 and G6 mice are consistent with a diagnosis of androgenetic alopecia (male pattern baldness; Courtois et al., 1994). In addition to changes in hair number and color, a higher incidence of severe ulcerative skin lesions were seen in G3 and G6 mice 15 months and older compared to $mTR^{+/+}$ animals (Figures 1D, 1G, and 1H). While the frequency of skin lesions in G3 and G6 animals is similar, the onset of skin ulcerations was earlier in life in G6 compared to $mTR^{+/+}$ and G3 animals (average age: 25 months in $mTR^{+/+}$, 26 months in G3, and 15 months in G6). These lesions were located predominantly at anatomical sites that are exposed to chronic mechanical stress, including distal limbs, perineum, snout, and throat area. Histologically, the skin lesions appeared as ulcerations with epidermal hyperplasia, hyperkeratosis, and underlying dermal fibrosis (Figure 1I). Such lesions are similar to those seen after chronic superficial trauma, particularly in debilitated elderly humans.

Telomere Length Correlates Inversely to the Incidence of Skin Lesion, Alopecia, and Hair Graying

To determine whether telomere shortening played a causal role in the skin phenotypes described above, telomere length analysis was performed on G3 mice of different ages. Quantitative (Q)-FISH is a powerful method to determine telomere length in mice. Using this method, it was shown that telomere length decreases at a rate of 4–5 kb/generation in mTR null mice (Blasco et al., 1997). A newly developed flow cytometry fluorescence in situ hybridization (flow-FISH) method (Hultdin et al., 1998; Rufer et al., 1998) yielded very similar measurements of relative telomere length in successive mTR null MEF cultures (Figure 2A; Greenberg and R. A. D., unpublished data) and showed comparable results for different cell types within a given generation of $mTR^{-/-}$ mice (MEFs and peripheral blood leukocytes, data not shown). Flow-FISH allows the determination of telomere length in a large number of samples and can be performed on primary cells derived from peripheral bleeds. Since young G3 mice are phenotypically normal but progressively experience age-dependent compromise in the skin, flow-FISH telomere length determinations were obtained in leukocytes derived from multiple 1-month-old and >16-month-old G3 mice. A >50% reduction in telomere length was observed in old G3 samples relative to young G3 samples (Figure 2B, $p = 0.035$), a finding consistent with the view that cell proliferation-dependent telomere erosion takes place in highly proliferative organ systems during life.

Next, we addressed whether differences in telomere length correlate with the presence or absence of age-associated cutaneous phenotypes in aged G3 mice. The relative telomere fluorescence signal of disease-free G3 mice was approximately 3-fold higher than in age-matched G3 mice afflicted with ulcerative skin lesions, hair graying, and alopecia (Figure 2C; comparing six animals with ulcerative skin lesions/hair graying or alopecia to five age-matched, disease-free animals, $p = 0.029$). These data suggest that animals with shorter telomeres are more likely to display a disease phenotype

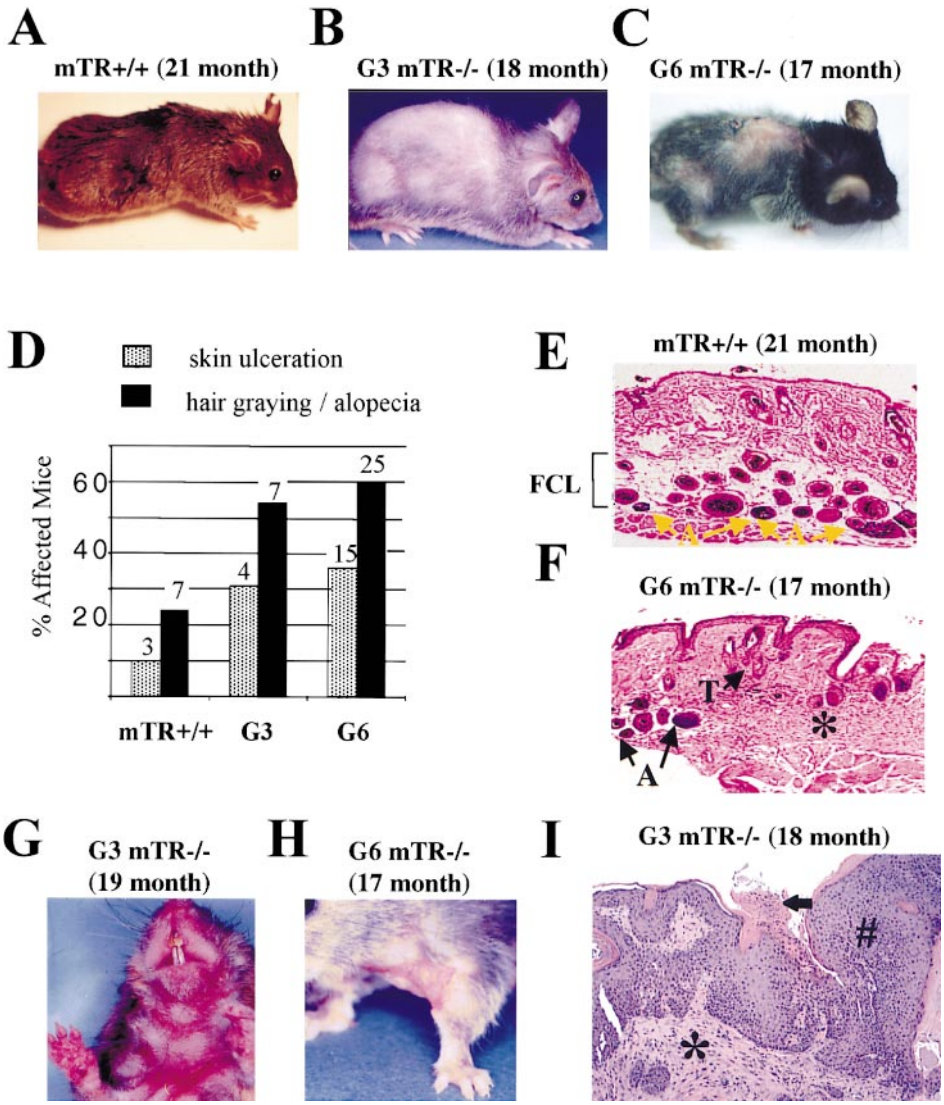

Figure 1. Increased Incidence of Skin Lesions, Alopecia, and Hair Graying in Aging mTR$^{-/-}$ Mice

(A–C) Representative examples of alopecia and hair graying in mTR$^{+/+}$ and mTR$^{-/-}$ mice.

(D) Incidence of ulcerative skin lesions and hair graying/alopecia in aged mTR$^{+/+}$, G3, and G6 mice.

(E and F) Histologic appearance of representative H&E-stained skin sections from a 21-month-old mTR$^{+/+}$ mouse and a 17-month-old G6 mouse. Compared to the mTR$^{+/+}$ skin, in which nearly all hair follicles are in anagen (A, arrow), the G6 skin exhibited an increased frequency of hair follicles in telogen (T, arrows). In G6 skin, the subcutaneous fat cell layer (FCL) was replaced by dense, fibrous tissue (asterisk; 10× objective).

(G–H). Perioral, neck, and hind limb ulcerative skin lesions in G3 and G6 mice.

(I) H&E-stained section of an area of chronic injury in the vicinity of an ulcerative skin lesion in an 18-month-old G3 mouse (40× objective) showing marked epidermal hyperplasia (#), hyperkeratosis (arrow), and a dense dermal fibrosis (*).

in the skin, further suggesting that telomere shortening in vivo contributes to disease susceptibility.

Telomere Shortening Does Not Cause Generalized Premature Aging

To determine whether other phenotypes often associated with aging occur early in the aging G3 animals, we carried out a broad histological survey of many organ systems (see Table 1). Analysis of the cardiovascular system, liver, kidneys, and brain showed no pathological changes typical of aging organisms (data not shown). A comprehensive serum chemistry profile that surveys the functional metabolic and structural integrity of many organ systems was also normal (data not shown). Moreover, radiographic and histologic analyses of the femur failed to demonstrate osteoporosis, histological examination of aged arterial walls did not reveal signs of arteriosclerosis, and cataract formation occurred at similar frequencies in all groups (data not shown). Normal blood glucose levels after fasting and in response to glucose challenge showed that glucose tolerance was normal in these mice (data not shown). With the exception of modest reduction in spleen size, telomere shortening did not result in significant defects in the cellularity and

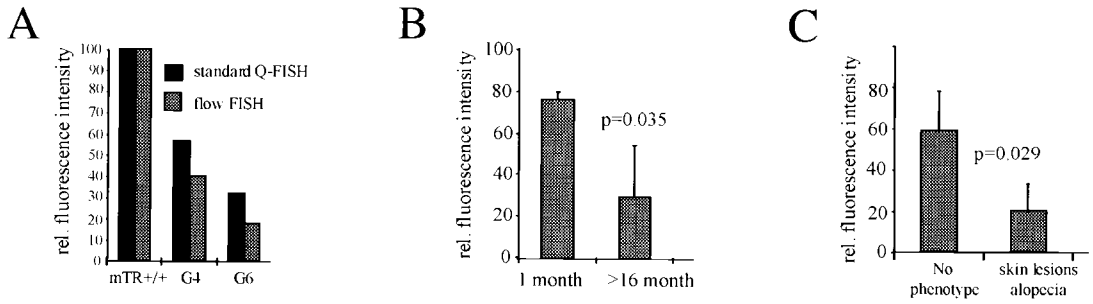

Figure 2. Telomere Length Decreases with Increasing Age and Correlates with the Skin Phenotype

(A) Relative telomere length of mouse embryo fibroblasts (MEF) derived from mTR$^{+/+}$, G4, and G6 mice, comparing data obtained by flow-FISH and Q-FISH analysis (Greenberg and R. A. D., unpublished data). Fluorescence intensities of mTR$^{+/+}$ cells were set at 100%, and the relative intensity of G4 and G6 cells was calculated.

(B) Relative telomere length of peripheral WBC of 1-month-old and >16-month-old G3 mice.

(C) Relative telomere length of age-matched (15- to 24-month-old) G3 animals with and without skin lesions, alopecia, and hair graying.

architecture of hematopoietic organs or in the peripheral blood counts (data not shown). Thus, while telomere shortening with age directly affected the skin, mTR$^{-/-}$ mice did not display other phenotypes classically associated with aging.

Reduced Longevity

Telomeres shorten during the lifetime of aging humans, but there is no direct correlation between the telomere length and the life span of different species (e.g., between mouse and humans) or within species of mice (reviewed in Greider, 1996). Thus, it is not yet clear to what extent telomere shortening might influence aging and longevity of complex organisms. There was no significant difference in the survival of G1 and G3 animals compared to mTR$^{+/+}$ controls (Figure 3). In contrast, a 15% increase in the incidence of spontaneous death was observed in 3- to 12-month-old G4–G6 animals. Further, at older ages, G6 mice showed elevated mortality. The survival curves of >12-month-old G4 and G5 animals paralleled those of mTR$^{+/+}$ and G1–G3 animals, while G6 mice died earlier. The 50% mortality mark occurred at 18 months for G6 mice, while 50% mortality was not reached until 24 months for mTR$^{+/+}$ and G1–G4 mice. As is often the case in natural death in humans, macroscopic and histological analyses at autopsy did not reveal specific causes of death in late generation mTR null mice (data not shown).

Decreased Body Weight

Body weight in mice typically increases in early and late postnatal development up to 1 year of age. Older animals show a terminal decline in body weight, and there is a positive correlation between body weight and life span (Goodrick, 1977; Ingram and Reynolds, 1987). Body weights were recorded at weekly intervals in aging mTR$^{+/+}$, G1, and G6 mice throughout postnatal development. During the first 6 months, body weight curves were superimposable for all groups (Figure 4A). At both 10–14 and 15–18 months of age, the average body weight of the G6 animals was 20%–25% less than early generation mTR$^{-/-}$ and mTR$^{+/+}$ animals (Figure 4A, $p < 0.0001$). Consistent with the decrease in body weight, a diminished fat cell layer between the dermis and the skeletal muscles was seen in the skin sections of old G6 animals (Figure 1F, asterisk), a finding considered to be a typical feature of aged human skin (Zivicnjak et al., 1997). In the gastrointestinal tract, villous atrophy and zonal blunting in the proximal intestine were detected in a subset (3/5) of the aged G6 animals by whole-mount staining and histological sections (Figures 4B–4E, representative samples shown). This disruption of the

Table 1. Summary of Phenotypic Analysis in Aging Mice

	mTR$^{+/+}$	G3 mTR$^{-/-}$	G6 mTR$^{-/-}$
Body weight	Normal	Normal	20%–25% decreased in >6-month-old mice
Diabetes	Normal GTT	Normal GTT	Normal GTT
Osteoporosis	Normal X-ray	Normal X-ray	Normal X-ray
Artherosclerosis	None	None	None
Peripheral RBC & WBC counts	Normal	Normal	Normal
Blood chemistry	Normal profile	Normal profile	Normal profile
Cataract	15%	20%	10%
Male fecundity	12–15 months	6.5 months	Normally infertile, rarely successful in generating offspring
Hair graying and alopecia	25%	54%	60%
Skin histology	Normal	Decrease of hair follicles in anagen, increase in telogen	Decrease of hair follicles in anagen, increase in telogen, loss of subcutaneous fat
Ulcerative skin lesions	10%	31%	37%
Wound healing	Normal	Delayed reepithelilization	Delayed reepithelialization
Cancer incidence	3.3%	13%	19%
Life span (50% mortality mark)	24 months	24 months	18 months

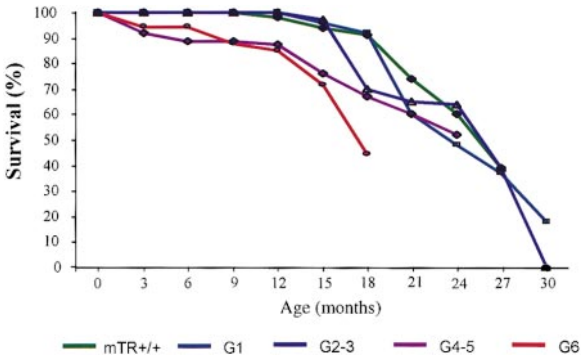

Figure 3. Loss of Telomere Ends Correlates with a Shortened Life Span

Survival curves of mTR$^{+/+}$, G1, G2–3, G4–5, and G6 mice, with percent of surviving animals plotted at 3-month intervals.

normal villous architecture is likely to contribute directly to body weight loss due to decreased nutritional absorption, an observation that has been described for aging rodents (Keelan et al., 1985; Chen et al., 1990).

Impaired Stress Response in Aged mTR$^{-/-}$ Mice

The limitation of cell replication by telomere shortening could affect the ability of the aging organism to respond to pathological conditions that provoke elevated cell turnover, as for example wounding, chronic hemolysis/bleeding, or infection. To address this possibility more directly, wound healing studies and the recovery potential of the hematopoietic system following blood cell ablation with 5-fluorouracil (5-FU) were monitored in young (1–3 months) and old (15–20 months) mTR$^{+/+}$, G3, and G6 mice.

Wound Healing

Four 3 mm punch biopsies were performed on the skin of the scapulae of mice housed in isolation, and the rate of wound healing was monitored by gross inspection and by serial histological examination. One day after wounding (PWD 1), the wounds of 15- to 18-month-old G3 and G6 mice exhibited delayed coagulum formation and remained opened and wet, while age-matched G1 and mTR$^{+/+}$ mice achieved complete coagulum formation over all wounds (data not shown). The delay in wound closure was most apparent when wounds of PWD 4 and PWD 6 were compared, where day 6 wounds in the old G3 and G6 animals appeared macroscopically equivalent in size to day 4 wounds in age-matched G1 and mTR$^{+/+}$ (Figures 5A and 5B, compare mTR$^{+/+}$ day 4 to G6 day 6; G1 and G3 not shown). Histological sections through the healing wounds revealed a marked delay in wound reepithelialization in the old G3 and G6 mice (Figures 5C and 5D, G3 wounds not shown). Reepithelialization of wounds takes place by migration of keratinocytes from the wound edge toward the center and is known to be impaired in aged mice and humans (Holt et al., 1992; Ashcroft et al., 1997a, 1997b). In the older group of mice 2/16 (13%), wounds in mTR$^{+/+}$ mice displayed incomplete reepithelialization at PWD 4, compared to 4/12 (33%) in G1, 7/10 (70%) in G3, and 12/16 (75%) in G6 mice ($p = 0.001$). On PWD 4, epithelial gap diameters averaged 2097 and 2378 μm in old G3 and G6

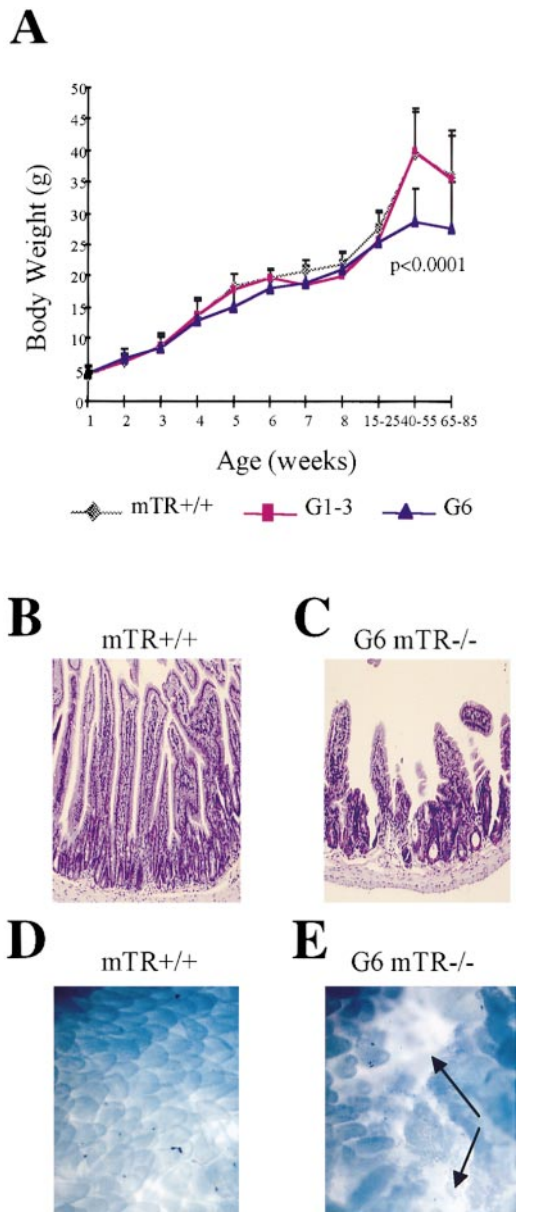

Figure 4. Reduced Body Weight and Villi Atrophy of the Small Intestine in Aged G6 mTR$^{-/-}$ Mice

(A) Body weights in mTR$^{+/+}$ and mTR$^{-/-}$ mice plotted as a function of age. Old G6 animals showed 20%–25% decrease in body weights.

(B and C) H&E-stained cross sections of mTR$^{+/+}$ and G6 duodenum at 18 months of age, showing blunted, markedly atrophic villi (20× objective).

(D and E) Whole-mount preparations of mTR$^{+/+}$ and G6 duodenum stained with methylene blue. Prominent areas of disorganized villi are present in the G6 duodenum (arrows).

animals, respectively. The two epithelial gaps present in old mTR$^{+/+}$ animals possessed a mean gap diameter of 240 μm. On average, the 15- to 18-month-old G3 and G6 mice required an additional 2 days to achieve full reepithelialization (6 versus 4 days for controls). It is interesting to note that, despite comparably short telomeres in old G3 and young G6 mice, impaired wound healing is observed only in aged mTR$^{-/-}$ possessing critically shorter telomeres and accompanying telomere

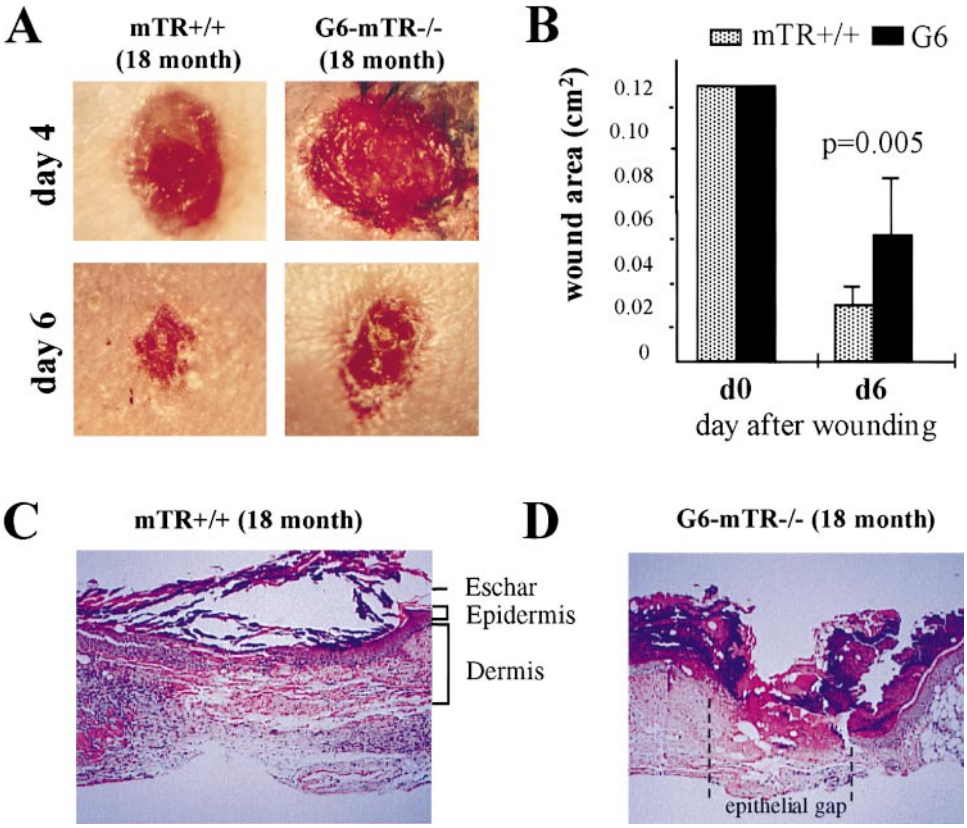

Figure 5. Delayed Wound Healing in Aged mTR$^{-/-}$ Mice

(A) Gross appearance of healing wounds of mTR$^{+/+}$ and G6 mice 4 and 6 days after wounding (2× objective).

(B) Wound areas at day 0 and 6 post wounding.

(C and D) Midtransverse sections (5 μm) through healing skin wounds at day 4 post wounding (H&E). Near complete reepithelilization was detected in mTR$^{+/+}$ controls, in contrast to the prominent epithelial gap seen in G6 wounds (40× objective).

dysfunction (e.g., chromosomal fusions). Although the mechanistic basis for impaired wound healing is not well understood and thought to involve an interplay of cellular proliferation, growth factor production, and immune system dysfunction, the absence of significant differences in BrdU incorporation (data not shown) indicates that the impact of telomere shortening in wound repair of the aged mTR$^{-/-}$ mice is complex and extends beyond simple proliferation defects.

Response to Blood Cell Depletion

To further examine the capacity to cope with acute stress, peripheral blood cell kinetics and survival following 5-FU-induced ablation of the hematopoietic system were monitored in young and old mTR$^{+/+}$ and mTR$^{-/-}$ mice. mTR$^{+/+}$ mice will typically recover from a low dose of 5-FU (100 mg/kg body weight i.p.), and the ability to regain peripheral blood counts and body weight after 5-FU injection is a measure of the ability of the mice to respond to the induced stress. 5-FU exposure had no significant effect on body weight, WBC, hemoglobin, and the overall clinical status of young animals in all mTR$^{-/-}$ generations (Figures 6A–6C). In contrast, older mice (16–22 months) showed a decline in all peripheral blood cells with the lowest point occuring 6 days after injection (Figure 6B, WBC; Figure 6C, hemoglobin levels; platelets not shown). Similarly, body weight curves showed a marked decline only in the old mice following 5-FU injection. Loss of body weight, which serves as an indicator of gastrointestinal defects and overall fitness, was more pronounced in old G6 compared to old mTR$^{+/+}$, while G3 animals exhibited an intermediate phenotype (average loss of body weight 15% and 10%, respectively). The clinical appearance of the old G3 and G6 animals was markedly compromised, and subcutaneous injections of saline to maintain hydration of the animals had no therapeutic benefit. As a consequence of this generalized morbidity, three out of four old G6 and two out of five old G3 animals died on days 6–11. Notably, the mice that succumbed were those that showed the most profound decrease in WBC counts (specifically neutropenia) and body weight. In contrast, all of the old mTR$^{+/+}$ animals regenerated WBC counts by day 11, regained pretreatment body weights, and survived the treatment protocol. Red blood cells decreased in all treated animals (in part due to repeated phlebotomy for the peripheral blood count determinations); nevertheless, a delay in renewal was observed in old G3 and G6 but not in the mTR$^{+/+}$ or younger cohorts. Whereas three out of four old mTR$^{+/+}$ animals had regained sufficient hemoglobin levels (>10 g/dl) by day 11 after treatment, four out of five G3 and G6 animals

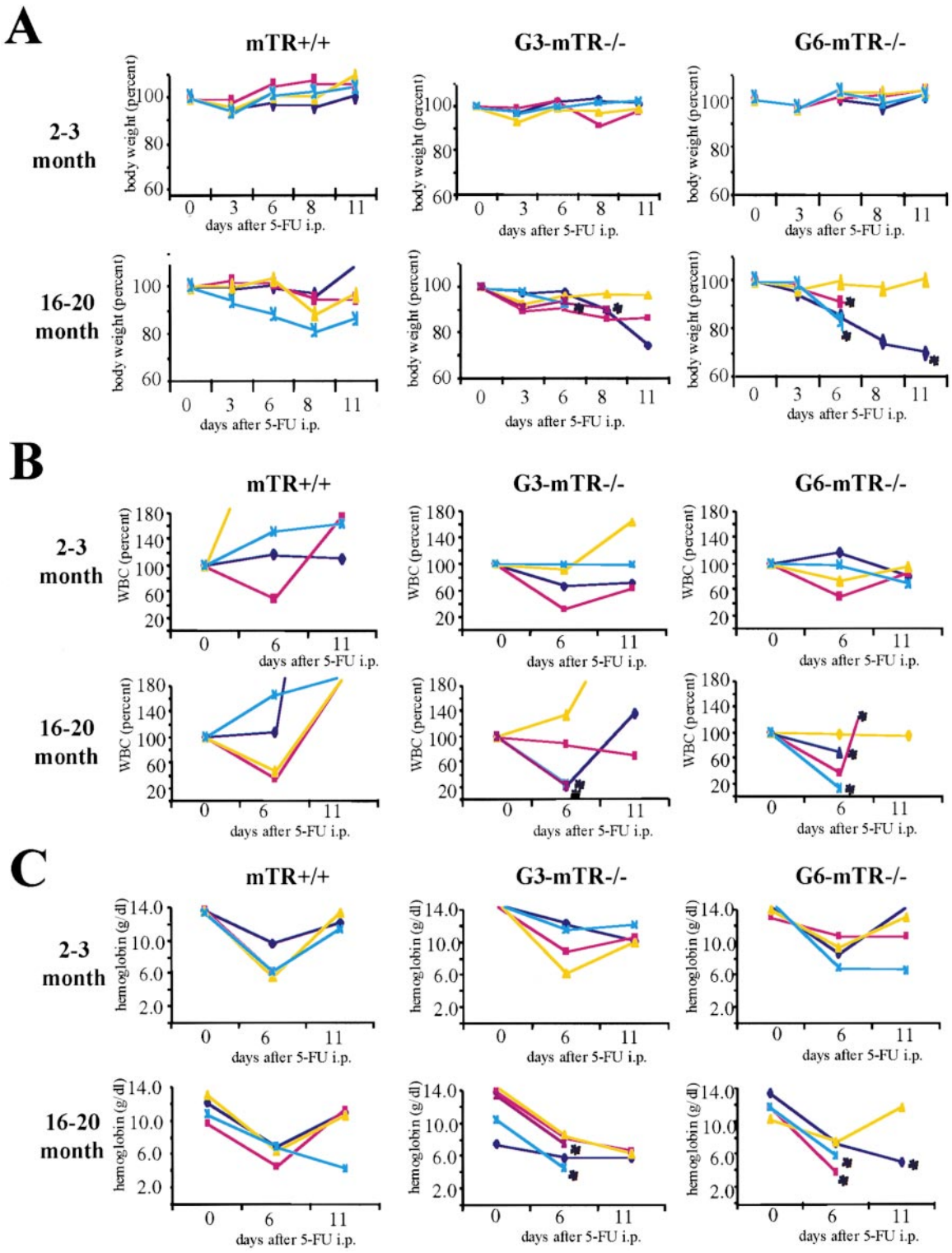

Figure 6. Age and Telomere Shortening Cooperate to Diminish Hematopoietic Reserve

Stress response to 5-FU treatment in young (2–3 months) and old (16–24 months) $mTR^{+/+}$, G3, and G6 mice.

(A) Body weight, (B) peripheral WBC, and (C) hemoglobin levels (g/dl) plotted against days after 5-FU treatment; all parameters showed a dramatic reduction 6 days after treatment in old but not in young animals, with eventual recovery in $mTR^{+/+}$ animals. Asterisks indicate animal death: 0/4 in $mTR^{+/+}$, 2/5 in G3, and 3/4 in G6 mice.

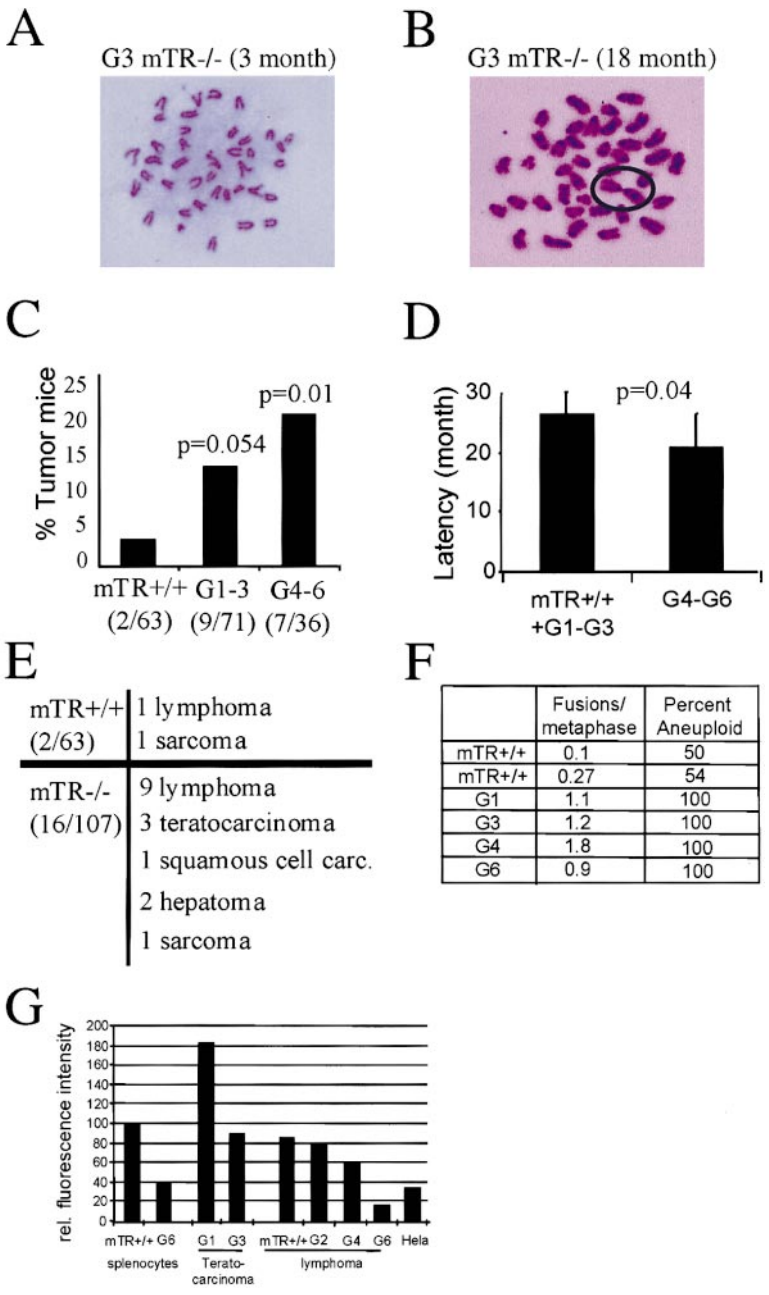

Figure 7. Genomic Instability and an Increased Incidence of Spontaneous Cancer in Aging mTR$^{-/-}$ Mice

(A and B) Representative metaphase spreads of lymphocytes. Circled area in (B) denotes chromosomal p-arm fusion in old G3 mouse.

(C) Incidence of macroscopically visible and clinically apparent spontaneous cancers.

(D) Age of onset of spontaneous cancers.

(E) Histological classification of spontaneous tumors.

(F) Cytogenetic analysis of representative spontaneously arising tumors.

(G) Telomere length in splenocytes of 18-month-old WT and G6 mice, and in teratocarcinomas and lymphomas derived from WT and mTR$^{-/-}$ mice. A human telomerase positive cervical carcinoma cell line (HeLa) is included for comparison of telomere lengths.

still showed critically low hemoglobin levels (<7 g/dl) (Figure 6C). Together these results demonstrate a diminished capacity of the aged telomerase-deficient mice to respond to a stress known to challenge the regenerative potential of the hematopoietic and gastrointestinal systems.

Increased Cytogenetic Abnormalities in Aged Late Generation mTR$^{-/-}$ Mice

Loss of telomere function in the mTR null mouse is associated with an increase in the frequency of chromosomal fusions and aneuploidy (Blasco et al., 1997; Lee et al., 1998). Since the studies described above demonstrated telomere loss as a function of age, it was possible that an increase in genomic instability with age could contribute to the increase in phenotypic abnormalities. To examine this possibility, metaphase spreads were prepared from mitogen-stimulated peripheral white blood cells and splenic lymphocytes of young and old mTR$^{+/+}$ and G3 mice. No chromosomal fusions were observed in >30 metaphases derived from 2- to 3-month-old mTR$^{+/+}$ (data not shown) and G3 mice (Figure 7A). In >15-month-old G3 animals, an increased frequency of chromosomal fusions occurred (Figure 7B). While fusions in mTR$^{+/+}$ animals were rare and seen only in animals older than 24 months of age (frequency: 0.14 fusions per metaphase), chromosomal fusions were seen at a frequency of 0.45 fusions per metaphase in G3 mice older than 15 months. The increased incidence in chromosomal fusions with increasing age in the

$mTR^{-/-}$ mice is consistent with the observation that $mTR^{-/-}$ cell lines exhibit 50-fold more chromosomal fusions than $mTR^{+/+}$ cell lines under similar passage conditions (Hande et al., 1999).

Progressive Increase in the Rate of Spontaneous Tumor Formation in Successive Generations of $mTR^{-/-}$ Mice

Telomere loss and resultant chromosomal fusions can be consequences of cell growth and selection during tumorigenesis but may also play a role in increased cancer incidence in aging populations as speculated previously (Hastie et al., 1990; de Lange, 1995). The increase in chromosomal fusions in successive generations of young mTR null mice (G4 onward) and the age-dependent increase in chromosomal fusions in peripheral blood leukocytes of non–tumor-bearing aged G3 mice provided an opportunity to assess the role of loss of telomere function in cancer incidence. We monitored an aging population of $mTR^{+/+}$ and successive generations of $mTR^{-/-}$ mice for visible tumors. The tumors from both $mTR^{+/+}$ and $mTR^{-/-}$ mice were confirmed malignant by histological analysis. There was a 4- to 6-fold increase in the incidence of spontaneous cancers in $mTR^{-/-}$ animals (Figure 7C, $p = 0.01$, compare $mTR^{+/+}$ to G4–G6). Although the absolute number of tumors was low, it is noteworthy that the average age at tumor formation was younger in G4–G6 mice compared to $mTR^{+/+}$ and G1–G3 mice (20.5 versus 26.3 months, respectively, $p = 0.04$; Figure 7D). In addition, the cancer rate in later generations (G4–G6) is higher than in G1–G3, even though many G6 mice have yet to reach an equivalently old age, during which the highest cancer rates were observed for $mTR^{+/+}$ and G1–G3 animals. (Thus, the cancer incidence reported here is likely to be an underestimate for the G4–G6 cohort.) The majority of tumor types originate from highly proliferative cell types (i.e., teratocarcinomas [germ cells], lymphomas [leukocytes], and squamous cell carcinomas [keratinocytes]). These cell types are likely to sustain the highest degree of telomere shortening with increasing age (Figure 7E). Together, these findings are consistent with the hypothesis that loss of telomere function and the resulting genetic instability that ensues facilitate the development of certain cancers. In line with this hypothesis, cytogenetic analysis of the tumors showed a 3- to 18-fold increase in the number of chromosomal fusions per metaphase and a 2-fold increase in the incidence of aneuploidy in $mTR^{-/-}$ tumors compared to $mTR^{+/+}$ tumors (Figure 7F). Although only two $mTR^{+/+}$ tumors were examined in this study, the low incidence of chromosomal fusions detected in these tumors was also observed in $mTR^{+/+}$ tumors derived from the $Ink4a^{-/-}$ mice (Greenberg and R. A. D., unpublished data). Flow-FISH analysis revealed shorter telomere ends in late generation $mTR^{-/-}$ tumors compared to $mTR^{+/+}$ tumors and early generation $mTR^{-/-}$ tumors (Figure 7G), indicating that loss of telomere function might contribute to tumorigenesis in these animals.

Discussion

Telomere and chromosome stability is necessary for cell viability. Previous studies showed that the $mTR^{-/-}$ mice exhibit progressive telomere shortening and loss of proliferative cell types with each progressive generation (Blasco et al., 1997; Lee et al., 1998). Here we find that the ability of the $mTR^{-/-}$ mice to respond to various physiological stresses is compromised and declines with increasing age of the mouse. The $mTR^{-/-}$ mouse may be a good model for the age-associated decline in certain organ systems, particularly with regard to a hallmark feature of aging—a reduced capacity to tolerate acute stress. In addition, the increased tumor formation in the aging $mTR^{-/-}$ colony suggests that loss of telomere function may initiate genetic instability.

Telomere Length, Cellular Senescence, and Life Span

The correlation of telomere length and cellular senescence in human cells suggested that telomeres may somehow signal entry into cellular senescence in tissue culture (Harley et al., 1990; Allsopp et al., 1992). Recent experiments have provided significant support for this model (Bodnar et al., 1998; Vaziri and Benchimol, 1998; reviewed in Greider, 1998). The role of both cellular senescence and telomere length in aging is less clear. Many of the phenotypic consequences of in vitro cellular senescence are seen in aging tissue in vivo (Stanulis-Praeger, 1987; Campisi, 1997). However, given that life span determination likely has multiple inputs, it is not yet clear what role cellular senescence plays in determining organismal life span. While telomere length correlates well with cellular senescence, there is no evidence for a clear correlation at the organismal level with human life span. Further, mouse telomeres are significantly longer than human telomeres, and species differences in telomere length in the mouse do not correlate with life span (Greider, 1996). Thus, to the extent that there is a similar mechanism of life span determination between these two species, it is unlikely that telomere length is the driving determinant. Although some aspects of the $mTR^{-/-}$ mouse mimic age-associated disease, many signs and symptoms classically associated with premature aging mouse models were not evident in $mTR^{-/-}$ mice. In particular, there was no evidence of increased cataract formation, osteoporosis, glucose intolerance, or vascular disease. However, the decreased stress response seen in the $mTR^{-/-}$ mouse provides a good model to study the role of the age-related decline in stress response seen in humans.

Impaired Telomere Function and Stress Response

The aged, late generation $mTR^{-/-}$ mice showed diminished response to stress in several different settings. This decreased stress response could be a direct result of the decline in proliferative capacity of specific cell types or could indicate a more global organismal response to telomere shortening. Although studies from yeast show that telomere shortening only manifests itself after many cell divisions, the work presented here suggests that under some circumstances in mice, phenotypes can be observed that are not directly related to cell division. The inability of B and T cells from $mTR^{-/-}$ animals to respond to mitogenic stimulation is apparent in the first cell cycle after a mitogen is provided (Lee et

al., 1998). Since this phenotype can already be seen in G3 animals, and yet G4–G6 animals have viable B and T cells, the lack of response to mitogens cannot simply be due to reaching a critically short telomere length or a limit to cell division capacity. In a similar fashion, although the decreased response to stress described in this paper might be due in part to a diminished cell renewal capacity, it is also possible that short telomeres may directly impact upon more differentiated functions in certain tissues (e.g., cytokine production, signaling, etc.).

As individuals age, although baseline organ function remains adequate to sustain a healthy state, aged organs exhibit a markedly diminished capacity to cope with diverse acute and chronic stresses (Jazwinski, 1996). Impaired lymphocyte responsiveness in acute infections or diminished epithelial repair leading to chronic skin ulcers is often seen in aging individuals (Jurivich et al., 1997). Similarly, increased mortality and morbidity are evident following physiological stresses associated with chemotherapy or general surgery (Haeney, 1994). Although the mechanisms for how short telomeres block cell division and trigger apoptosis without ongoing cell division are not yet known, the mTR$^{-/-}$ mouse provides an excellent model to study the possible role of telomere length in these cellular stress responses at the organismal level. This mouse model may thus shed light on this very important aspect of aging. Specifically, since the stress responses observed in old mTR$^{+/+}$ and G1 mice were similar to those seen in young G6 mice, the aged late generation mTR$^{-/-}$ mouse may provide an in vivo system to model age-related decline of gastrointestinal, hematopoietic, and cutaneous systems of humans.

Genetic Instability and Increased Cancer Incidence in Aging mTR$^{-/-}$ Animals

One of the functions of the telomere is to protect chromosomal ends from fusions and other rearrangements. Previous studies employing human fibroblasts have established a correlation between a progressive decline in telomere length during passage and an increased incidence of telomere fusions (Counter et al., 1992; Vaziri et al., 1993). Similarly, a reduction in telomere length in successive generations of mTR null mice is associated with Robertsonian end-to-end fusions (Blasco et al., 1997; Lee et al., 1998). The fact that telomeres are shortened in a variety of human cancers in which chromosomal abnormalities are also commonly present has fueled speculation that telomere erosion might be a risk factor for the genesis of some tumors (Pathak et al., 1994; de Lange, 1995).

We found a correlation between an age- and generation-dependent increase in cytogenetic abnormalities (consistent with telomere dysfunction, e.g., end-to-end fusions) and an increase in the incidence of spontaneous cancers. This link is supported by the earlier age of onset of tumors in the later generations and by the spectrum of cancer types and their emergence from telomere-dependent organs (lymphoid, testes, and skin; testis not shown). Particularly striking is the high incidence of teratocarcinomas in the mTR$^{-/-}$ mice in light of the extremely low incidence of such tumors in our large mTR$^{+/+}$ mouse colony over many years. Such a correlation is consistent with the hypothesis that telomere erosion may initiate genetic instability that leads to cancer. At face value, the increased tumor incidence seems to contradict the decrease in tumor formation expected from the loss of cell viability associated with telomere dysfunction. It also appears confusing in light of the frequent upregulation of telomerase postcrisis (Harley and Sherwood, 1997; Shay, 1997) or the ability of telomerase to facilitate transit through crisis (Counter et al., 1992; de Lange, 1994; Weinberg, 1998). Moreover, we have documented a significant decrease in tumor incidence in mice doubly null for mTR$^{-/-}$ (G4 onward) and the INK4a tumor suppressor gene (Greenberg and R. A. D., unpublished data). The difference in these studies may be that in early divisions loss of telomere function can initiate genetic instability, while at later points in tumor progression the absence of telomerase inhibits long-term growth. Thus, while telomerase inhibition may be a valid approach in the treatment of established tumors, age-dependent telomere shortening may be an important risk factor for cancer in settings of bypass of the mortality I checkpoint. Together, these studies underscore the complex relationship between telomeres, tumorigenesis, and aging and highlight the need to conduct relevant studies in intact organisms against the backdrop of defined cancer-relevant mutations.

Experimental Procedures

mTR Null Mouse and Mating Scheme

Production of the mTR null mouse has been described previously (Blasco et al., 1997). Generation 1 (G1) knockout animals and mTR$^{+/+}$ control animals were derived from heterozygous intercrosses (mixed genetic background). Mating of G1-mTR$^{-/-}$ animals to each other generated G2 animals. Following this mating scheme, mTR$^{-/-}$ animals up to the sixth generation (G6) were produced. All animals were kept in the same room and fed ad libidum. Routine serologies confirmed that all mice remained pathogen free over the entire study period.

Alopecia and Hair Graying

Hair graying was quantitated at different parts of the animal's body by estimating the percentage of gray hairs, and the results were classified as either mild ($<$10%), medium (10%–30%), or severe ($>$30%) hair graying. Alopecia was quantitated by counting hairs in one square centimeter areas of the animal's back (Courtois et al., 1995). Quantification of hair follicles in the telogen or anagen phase of hair growth was performed on hematoxylin and eosin (H&E) stained cross sections of the animal's dorsal skin.

Skin Analysis and Wound Healing Studies

Four 3 mm full thickness punch biopsies extending through the epidermis and dermis to the panniculus carnosus were made overlying the scapulas using circular skin bioptomies. Wounds were created in four different animals per group (2- to 3-month and 15- to 18-month-old mTR$^{+/+}$, G1, G3, and G6) after chemical depilation. Wound sizes were measured with a 2$\times$ objective. At 4 and 6 days after wounding, 5 μm midtransverse paraffin sections were stained with H&E. Epithelial gap diameters were determined by optical micrometer measurements using calibrated 10$\times$ and 20$\times$ objectives at post wounding day 4.

5-Fluorouracil Study

Two-month and 16- to 20-month-old mTR$^{+/+}$, G3, and G6 animals were treated with an i.p. bolus injection of 5-fluorouracil (5-FU) at a dose of 100 mg/kg body weight. Peripheral blood was obtained via tail bleeds before injection and on days 6 and 11 after injection. Bleeding was terminated upon withdraw of 150 μl blood volume. Peripheral blood counts and white blood cell differentials were analyzed by an automated CBC analyzer and by microscopic examination.

Gastrointestinal Tract and Overall Organ Assessments

Whole-mount staining of the intestine was performed on 1 cm^2 preparations as described previously (Pretlow et al., 1991). For histology, the organs were fixed in 10% formalin, and 5 μm paraffin sections were stained with H&E. Serum analysis for LDH, bilirubin, AST, ALT, total protein, albumin, alkaline phosphatase, GGT, and cholesterol, uric acid, creatinine, calcium, and serum electrolytes was analyzed using a DAX analyzer. Glucose tolerance tests (2 mg glucose/g body, i.p.) were performed after a 16 hr fast on $mTR^{+/+}$, G3, and G6 animals. Bone density radiographs were taken on dissected mouse femurs of 2- to 3-month-old and 18- to 24-month-old $mTR^{+/+}$, G3, and G6 animals (three different animals per group) using AD Mammo Fine film (Fuji).

Telomere Length Analysis

Telomere length analysis was performed on peripheral white blood cells by flow-cytometry-FISH as recently described (Hultdin et al., 1998; Rufer et al., 1998). Peripheral blood cells were obtained after tail bleeds (500 μl) and red cell lysis with ammonium chloride. Day to day variations in the linearity of the flow cytometer were controlled by the use of FITC-labeled fluorescence beads (Flow Cytometry Standards Corporation, San Juan, Puerto Rico).

Metaphase Spreads

Splenocytes and peripheral blood cells were stimulated with mitogens (5 μg ConA/ml + LPS [100 μg/ml]) for 24 hr followed by incubation in 0.1 g/ml colcemid for 60 min. Tumor cells were treated for 1–4 hr with colcemid, depending on the growth characteristics. After spinning at 1000 rpm, the cells were incubated in 75 mmol KCl for 15 min, spun again, and fixed in methanol:glacial acetic acid (3:1, v:v). Metaphases were dropped from 1 m onto frosted microscope slides positioned at a 45° angle. Giemsa staining was performed according to standard protocols. Quantification of fusions and photographs were taken with 60× and 100× objectives.

Cancer Incidence

Aging mice were inspected weekly for macroscopically visible tumors or palpable abdominal tumors. Tumor-bearing mice were anesthezised, and tumor biopsies were taken for histology and metaphase preparations. All cancer diagnoses were confirmed histologically. Animals that were found dead were dissected and inspected for macroscopically visible tumors.

Statistics

Unpaired t test and Fisher's exact test were used for calculation of p values.

Acknowledgments

We thank Steven Artandi, Lynda Chin, Ronan O'Hagan, and Nicole Schreiber-Agus for critical reading of the manuscript, and B. Furman, K. E. Cedeno-Baier, L. Husted, and S. Rao for excellent technical assistance. The work was supported by grants from the National Institutes of Health and American Heart Association grant-in-aid to R. A. D.; R. A. D. is an American Cancer Society Research Professor. Support from the Dana Farber Cancer Institute Cancer Core grant to R. A. D. is acknowledged. K. L. R. is supported by the Deutsche Forschungsgemeinschaft (Ru 745/1-1), and C. G. is supported by the National Institutes of Health. M. B. is supported by the Ministry of Education and Culture of Spain and by the Department of Immunology and Oncology (CSIC-Pharmacia and Upjohn).

Received December 29, 1998; revised January 28, 1999.

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