# Essential role of mouse telomerase in highly proliferative organs

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**We have investigated the role of the enzyme telomerase in highly proliferative organs in successive generations of mice lacking telomerase RNA. Late-generation animals exhibited defective spermatogenesis, with increased programmed cell death (apoptosis) and decreased proliferation in the testis. The proliferative capacity of haematopoietic cells in the bone marrow and spleen was also compromised. These progressively adverse effects coincided with substantial erosion of telomeres (the termini of eukaryotic chromosomes) and fusion and loss of chromosomes. These findings indicate an essential role for telomerase, and hence telomeres, in the maintenance of genomic integrity and in the long-term viability of high-renewal organ systems.**

Telomeres are guanine-rich, simple repeat sequences that constitute the physical termini of eukaryotic chromosomes[1]. Early work[2,3] indicated that these terminal structures may be important in chromosome function[4]. Synthesis and maintenance of telomeric repeats are mediated by a specialized ribonucleoprotein complex known as telomerase[5,6]. The telomerase holoenzyme consists of an essential RNA template[6,7] and protein components, including one with similarity to reverse transcriptases[8,13].

In the absence of telomerase, the failure of DNA polymerase to fully synthesize DNA termini leads to chromosomal shortening with each round of replication. As most somatic human tissues and primary cells possess low or undetectable telomerase activity, continual organ renewal *in vivo* or passage of cells in culture leads to a steady decline in telomere length[14,15]. Such a reduction in telomere length may play a role in replicative senescence, as constitutive telomerase activity can extend the proliferative capacity of normal human cells[16]. Viral oncoproteins or mutational events can drive senescent cultures through additional rounds of replication with continued telomere loss until 'crisis' ensues—an event highlighted by marked genetic instability and cell death. From this crisis period, immortal cells emerge that have reactivated telomerase and maintain stable telomere structures[17–19].

The reactivation of telomerase during cellular immortalization matches well with what occurs during tumour progression in mice and humans. In particular, a marked upregulation of telomerase activity occurs in >90% of malignant samples compared with normal adjacent tissue[19]. These data led to the speculation that telomerase may be required for the efficient growth of neoplastic cells and that telomerase inhibition could represent a new therapeutic approach for cancer[14,15]. We have recently shown that primary mouse cells lacking the mouse telomerase RNA (mTR) gene, and thus lacking telomerase activity, can still spontaneously become immortalized and generate a fully transformed tumorigenic phenotype after viral oncogenesis[20], indicating that telomerase activity may not be limiting for tumorigenesis in the mouse.

The idea that telomeres play an essential role in normal cellular growth and survival has been substantiated in unicellular eukaryotes. In yeast, deletion of the telomerase RNA gene leads to telomere shortening and, after a generational lag period, to loss of cell viability[21,22]. The lag period presumably comprises cell divisions during which telomeres shorten before they reach a critical length that is the threshold for chromosome instability and cell death. Elimination of a telomeric end from a yeast chromosome causes a RAD9-mediated cell-cycle arrest and chromosomal loss, indicating that telomeres may function to help cells distinguish intact chromosomes from broken DNA molecules and to maintain genomic stability[23,24].

In mammals, an *in vivo* role for telomerase and telomeres in normal cellular growth and long-term maintenance of organ systems has not yet been examined. On the basis of the phenotype of telomerase-null yeast and the great increase in chromosomal aberrations and aneuploidy in $mTR^{-/-}$ cells[20–22], we expected that telomerase deficiency would have its greatest impact on highly proliferative organ systems. Here we demonstrate progressive adverse effects of telomerase deficiency on the reproductive and haematopoietic systems. These effects on cell renewal, coupled with telomere-length reductions and abnormal cytogenetic profiles, establish a role for telomerase in normal cellular homeostasis at the biological and genomic levels.

## Early-generation telomerase-deficient mice

We reported previously a decline in telomere length with each successive generation of the $mTR^{-/-}$ mouse[20]. Here we performed a phenotypic analysis of each generation, and paid particular attention to organ systems with high proliferative activity. Studies of an ageing population of the first-generation ($G_1$) $mTR^{-/-}$ mice (from heterozygous intercrosses) did not reveal adverse effects on the overall clinical condition (lifespan, motor behaviour/activity and weight gain) through 20 months of life (data not shown). Similarly, the haematopoietic organ histology and peripheral white and red blood cell counts, as well as the capacity of haematopoietic progenitor cells to grow and differentiate *in vitro* and of mature immunocytes to respond to mitogenic or infectious stimuli, were not significantly different from those of controls (see below). In addition, other organs with a more moderate level of renewal activity, such as intestine and skin, exhibited normal morphology and rates of proliferation and apoptosis (data not shown). These results indicate that telomerase activity *per se* is not essential in organogenesis or postnatal organ function or maintenance. It is possible that stressful conditions leading to increased cell turnover

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(for example, phlebotomy, chronic infection, skin wounding, petatectomy) might be able to elicit an age-dependent phenotype in old $mTR^{-/-}$ mice. Preliminary analysis indicates that some $G_1$ $mTR^{-/-}$ animals in two independent colonies exhibit erosive dermatitis, which might indicate a defect in dermal stem cells (H.W.L. and R.A.D., and E. H. Herrera, J. M. C. Caballero and M.A.B., unpublished observations). At present the limited sample size does not allow us to assign this phenotype unambiguously to the mTR deficiency, although studies are continuing.

On the basis of previous studies in lower organisms that show a temporal lag between telomere loss and diminished cell viability, we suspected that substantial telomere loss would be required before a compromised clinical condition became apparent. Thus, as described[20], we have produced additional generations ($G_2$, $G_3$, $G_4$, $G_5$ and $G_6$) of $mTR^{-/-}$ mice from successive matings of homozygous-null intercrosses. Avoidance of inbreeding in the later generations was achieved through regular cousin-mating schemes[25] of offspring produced from 20 different $G_1$ $mTR^{-/-}$ matings; these $G_1$ mating pairs were in turn derived from 10 different $mTR^{+/-}$ intercrosses.

## Reproductive system

Reproductive function is highly dependent on proper germ-cell expansion and development. All matings proved to be productive up to the fifth generation. However, a statistically significant decline in litter size became apparent in the $G_4$ intercrosses (Fig. 1a; $G_4$ $P = 0.021$ compared with $G_1$). No offspring were produced from eight different $G_6$ intercrosses. Both sexes appear to be affected, as only one in eight $G_6$ males and one in eight $G_6$ females produced a litter when mated to a wild-type partner.

**Male reproductive system.** As the number of spermatocyte

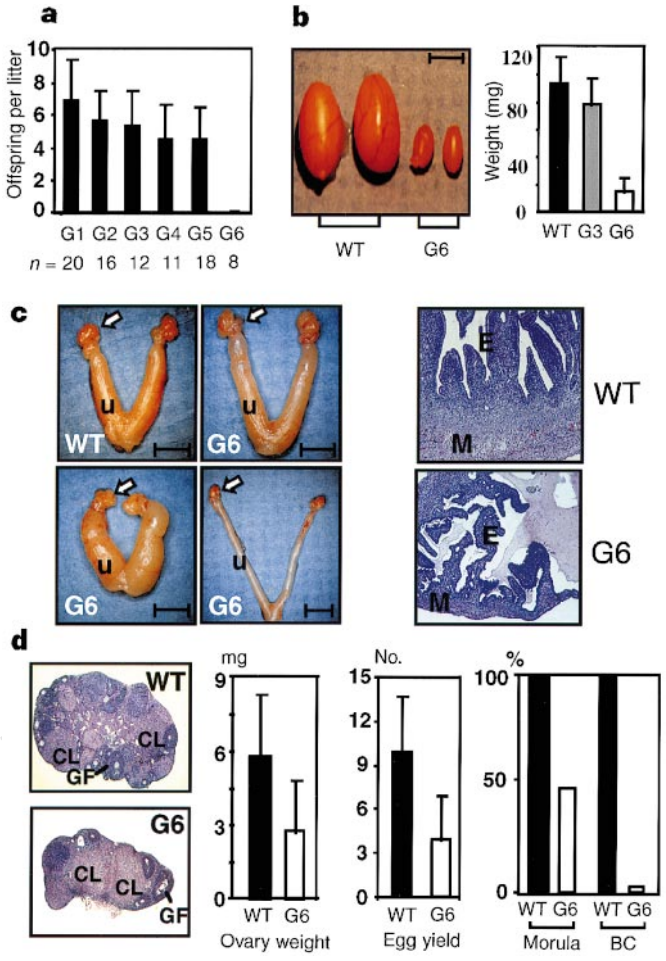



**Figure 1** Effects of lack of telomerase on the mouse reproductive system. **a**, Impaired reproductive function in late-generation mTR-deficient mice. Males aged 6–12 weeks and females aged 4–12 weeks of the same generation of telomerase-deficient mice were mated over a period of 2 months or longer; *n* indicates the number of mating pairs examined. The *P* values of each generation were calculated compared with $G_1$ ($G_2$, $P = 0.063$; $G_3$, $P = 0.070$; $G_4$, $P = 0.021$; and $G_5$, $P = 0.004$). **b**, Testicular atrophy and testes weights. Scale bar, 5 mm. **c**, Uterine horns. Grossly dissected uterine horns (u) and ovaries (arrows) (scale bar, 5 mm) and haematoxylin and eosin (H&E) staining (magnification ×50). **d**, Ovarian structure and function. Oestrus-matched ovaries are shown at ×50 magnification (left). E, endometrium; M, myometrium; GF, Graafian follicle; CL, corpora lutea. The graphs show a weight comparison of ovaries, average egg yields following natural mating with a wild-type stud male, and the percentage of *in vitro* development of pre-implantation embryos. BC, blastocyst.

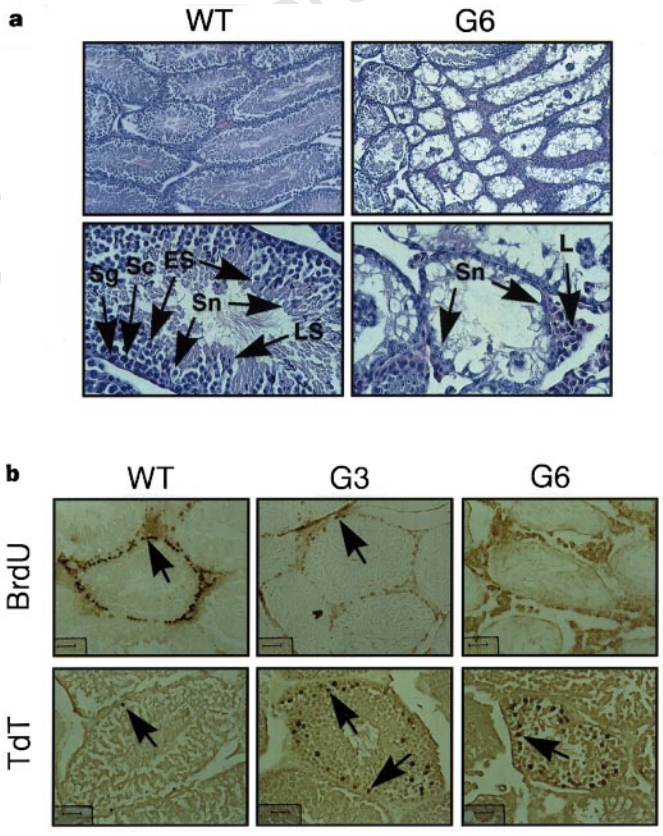


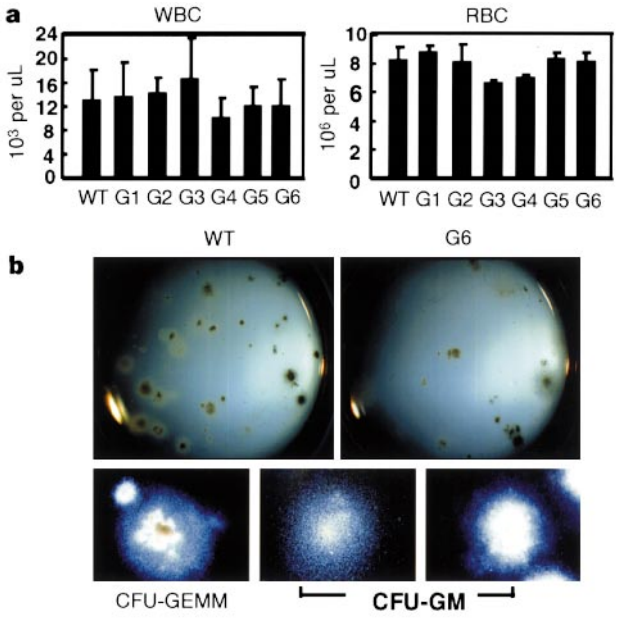

**Figure 2** Proliferation and apoptosis in the male germ-cell compartment. **a**, Histology of WT and $G_6$ testis. Top: sections at low magnification (×50) through WT testes and representative $G_6$ testes show the diminished cellularity in the $G_6$ testes although there are some tubules undergoing nearly normal spermatogenesis (left edge of $G_6$ section). At higher magnification (×200 (bottom)), all stages of spermatogenesis are well represented in WT seminiferous tubules, whereas most $G_6$ tubules lack spermatogenesis yet retain Sertoli cells. In the $G_6$ testes, the apparent local accumulation of Leydig cells in the interstitium is secondary to contraction of the seminiferous tubules and an overall decrease in testis volume. Sn, Sertoli cell; L, Leydig cell; Sg, spermatogonia; Sc, primary spermatocyte; ES, early spermatid; LS, late spermatid. **b**, Comparative BrdU incorporation and TUNEL assays. Relative to WT samples, there was an approximate reduction in BrdU-positive nuclei (arrows) of three- to fivefold for $G_3$ mice and greater than 20- to 100-fold for $G_6$ mice. In the TUNEL (TdT) assay, $G_3$ seminiferous tubules and $G_6$ tubules with adequate cellularity for comparative analysis possessed many TUNEL-stained germ-cell nuclei (arrows), whereas only an occasional apoptotic cell was detected in the WT samples. Magnification ×100.

divisions from zygote to mature sperm has been estimated to be >60, whereas oocytes undergo arrest and await maturation after only 25 cell divisions[26], we suspected that telomerase deficiency would be seen initially and most adversely in the male germ-cell compartment. On gross inspection, the size and weight of $G_6$ testes were found to be reduced by ~80% compared with age-matched wild-type or $G_3$ testes, whereas other organs (such as brain) or accessory reproductive organs (such as seminal vesicles) in $G_6$ animals were proportional to the overall body size (Fig. 1b). At the histological level, testes of wild-type through to $G_4$ mice possessed well-developed seminiferous tubules with a full complement of support cells (Sertoli cells) and germ cells representing all stages of spermatogenesis, similar to what is seen in wild-type age-matched controls (Fig. 2a). By the fifth generation, germ cells became depleted (data not shown) and, by the sixth generation, most seminiferous tubules showed a striking absence of spermatogenesis yet maintained an apparently adequate representation of the less proliferative Sertoli and Leydig cells (Fig. 2a). Thus spermatogenic cells, not support/accessory cells, are particularly affected by telomerase deficiency in the $G_6$ $mTR^{-/-}$ mice. As sexual behaviour and seminal-vesicle development and structure are highly dependent on adequate levels of androgens[27], the observations that male sexual performance (plugging efficiency) and seminal-vesicle size and structure were normal strongly indicate that germ-cell depletion is not a simple consequence of low serum testosterone levels (data not shown).

Progressive hypospermia could result from a checkpoint-arrest response and/or diminished viability (apoptosis) as late-generation germ cells lose telomere sequences and experience genomic instability. To investigate these possibilities, we monitored the rates of proliferation and apoptosis in wild-type, $G_3$, $G_5$ and $G_6$ testes. *In situ* assays of bromodeoxyuridine (BrdU) incorporation revealed abundant S-phase activity in many seminiferous tubules of the wild-type testes (Fig. 2b). In the histologically normal $G_3$ testes, a greater than fivefold decrease in the number of BrdU-positive nuclei was noted (Fig. 2b). This proliferative decline was more pronounced in the $G_5$ and $G_6$ testes, even in tubules with moderate cellularity (Fig. 2b; $G_5$ data not shown). Moreover, a marked increase in apoptosis (measured with TdT-mediated dUTP nick end labelling (TUNEL)) was detected in the $G_3$ and $G_6$ testes compared with wild-type controls (Fig. 2b; compare $G_3$ and $G_6$ with wild-type). Thus, at least two processes are responsible for progressive germ-cell depletion: decreased proliferation and increased apoptosis.

**Female reproductive system.** On gross examination, oestrus-staged $G_6$ uterine horns exhibited minimal to significant reductions in length and diameter compared with age- and stage-matched wild-type controls (Fig. 1c). $G_6$ ovaries also varied considerably in size and, on average, weighed markedly less than wild-type controls (Fig. 1d; wild-type $5.4 \pm 2.5$ mg, $G_6$ $2.6 \pm 2.0$ mg; $P = 0.0212$). Detailed histological analysis of $G_6$ uterine horns showed normal endometrial thickness, integrity and cellularity; however, myometrial thickness was reduced and atrophy of the smooth muscle cells was increased in the smaller and more contracted $G_6$ uterine horns (Fig. 1c). Although histological examination of the ovaries confirmed the reduced size of the $G_6$ ovary, both wild-type and $G_6$ ovaries showed a full spectrum of follicular development including Graafian follicles and corpora lutea (Fig. 1d).

To assess physiological aspects of reproduction, the plugging frequency and egg yields were measured after natural matings of $G_6$ or wild-type females with wild-type males. Under identical temporal and environmental conditions, 11 of 11 wild-type and 8 of 10 $G_6$ females were plugged and produced an average of 9.8 ($\pm$ 3.8) and 3.9 ($\pm$2.9) fertilized eggs per female, respectively (Fig. 1d, $P = 0.0175$). We then examined the capacity of these embryos to progress through early embryogenesis after culturing *in vitro*. Although all (15 of 15) wild-type/wild-type $F_1$ embryos developed to the blastocyst stage (Fig. 1d, $P = 0.0061$), only 46.7% (7 of 15) of $G_7$/wild-type $F_1$ embryos developed to the morula stage and none progressed to the blastocyst stage. To compare reproductive-tract function, wild-type fertilized eggs were implanted into the oviducts of $G_6$ or wild-type pseudopregnant females. Only 2 of 7 $G_6$ females were able to support the full-term development of these embryos compared with 8 of 10 wild-type females. Thus, although the comparable plugging efficiencies indicate normal hormonal regulation and end-organ responsiveness, infertility probably results from several independent factors. These include a decrease in the number of oocytes upon ovulation, poor early developmental progression of $G_7$ wild-type $F_1$ embryos, and possible compromise in uterine structure/function.

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| | | Ratio (KO/WT) | *P* value | *n* |
|---|---|---|---|---|
| **CFU-GM** | G1 | **0.98** ± 0.23 | **0.7572** | 2 |
| | G3 | **1.33** ± 0.49 | **0.1505** | 4 |
| | G6 | **0.57** ± 0.36 | **0.0028** | 8 |
| **CFU-GEMM** | G1 | **0.97** ± 0.34 | **0.8541** | 2 |
| | G3 | **0.76** ± 0.45 | **0.8471** | 4 |
| | G6 | **0.38** ± 0.25 | **0.0011** | 8 |
| **HPP-CFC** | G1 | **0.96** ± 0.13 | **0.4734** | 2 |
| | G3 | **1.08** ± 0.09 | **0.1312** | 4 |
| | G6 | **0.54** ± 0.17 | **0.0005** | 8 |

**Figure 3** Effects of the lack of telomerase on haematopoietic cells. **a**, Peripheral blood counts for red blood cells (RBC) and white blood cells (WBC). Peripheral blood was subjected to the Coulter STK/S counter analysis for the complete blood count. **b**, Haematopoietic colony assays. Formation of bone-marrow-derived colonies was assessed *in vitro* using cells obtained from WT and from $G_6$ femurs. Representative colonies of CFU-GEMM and CFU-GM are shown (magnification ×10). HPP-CFC colonies are scored on the basis of size (see Methods). **c**, Ratios of numbers of knockout (KO) to WT colonies. *P* values were calculated from the comparison between numbers of WT and homozygous-null mice of each generation; *n* is the number of mice that were used for each experiment.

## Haematopoietic system

Most haematopoietic cells have a lifespan considerably shorter than that of other somatic cells, and these cells are continually replen-

ished by a self-renewing populations of stem cells[28]. A histological survey of adult bone marrow, spleen and thymus revealed normal organ size, architecture and cellularity through to the sixth generation (data not shown). A complete peripheral blood count and profile of sex- and age-matched mice did not show any differences in all genotypes through successive generations of telomerase deficiency (Fig. 3a). Moreover, no differences were observed in the percentages of lymphocytes, neutrophils, basophils, eosinophils and monocytes. Fluorescence-activated cell sorting (FACS) analysis of splenocytes showed identical cell-type profiles as measured by antibodies against T cells (antibodies against CD4, D8, and CD3), B cells (anti-B220), lymphoid cells (anti-CD45), macrophages (anti-F4/80), and erythrocytes (anti-TER-119) (data not shown). Similarly, FACS analyses of bone-marrow cells showed identical profiles using antibodies against D45, F4/80, TER-119 and CD34 (stem cell) (data not shown). To test the immune response of telomerase-deficient mice, six wild-type and six $G_5$ naive mice were infected intravenously with a sublethal innoculum of *Listeria monocytogenes*. The two groups showed similar survival and recovery rates. Upon rechallenge with a dose that would be lethal to naive mice, all mice survived, thus demonstrating that gross function of the immune response and memory is not affected. Mature haematopoietic organ structure and function seem to be well compensated in *mTR*-deficient mice.

As relatively few haematopoietic stem cells (HSCs) sustain long-term haematopoiesis, reductions in HSC numbers or growth/survival potential might not be reflected in peripheral blood count measurements. Thus we used *in vitro* haematopoietic colony-forming unit (CFU) assays to determine whether the early progenitor-cell compartment was compromised. Bone-marrow cell suspensions derived from $G_1$ and $G_3$ $mTR^{-/-}$ mice were comparable to WT suspensions in their ability to generate CFU-granulocyte, monocyte (CFU-GM), CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) and high-proliferative-potential colony-forming cell (HPP-CFC) colonies (Fig. 3c). In contrast, multiple CFU assays comparing wild-type, $G_1$, $G_3$ and $G_6$ progenitor-cell profiles showed a statistically significant decrease in the total number of CFU-GM ($P = 0.0028$), CFU-GEMM ($P = 0.0011$) and HPP-CFC ($P = 0.0005$) colonies in the $G_6$ samples (Fig. 3b, c). These results indicate that the long-term renewal of haematopoietic stem cells is compromised upon telomere loss. Nevertheless, the stochastic growth behaviour of these cells may allow the generation of a reserve pool that is still capable of populating the peripheral haematopoietic compartment.

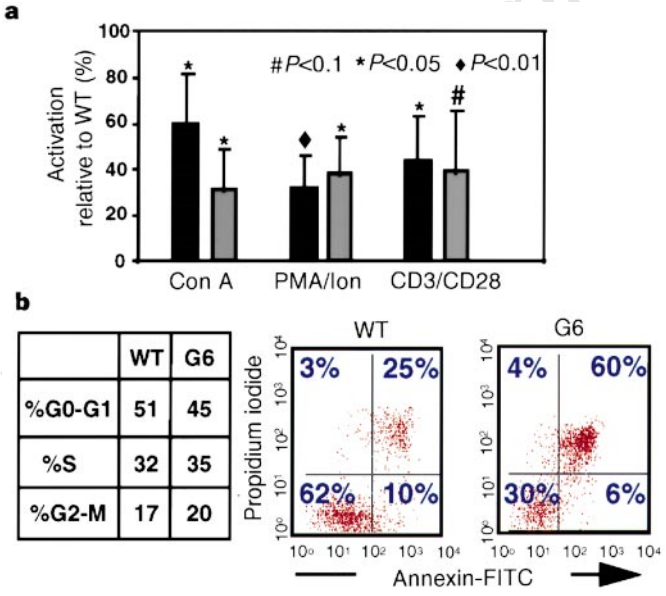


**Figure 4** Response of lymphocytes to lack of telomerase. **a**, Splenocyte mitogenic response. Splenocytes from WT, $G_5$ and $G_6$ homozygous-null animals were mitogenically activated and their uptake of $^3$H-labelled thymidine was measured. The *y*-axis represents the percentage of fold-activation of mTR-deficient splenocytes by indicated mitogens (*x*-axis) compared with activation of WT splenocytes. Mitogenic activation of WT splenocytes was set at 100%. Error bars indicate standard deviations from quadruplicated samples of four $G_5$ mice and three $G_6$ mice. Black bars show the data from $G_5$ mice and grey bars indicate data from $G_6$ mice. **b**, Cell-cycle profile and annexin/PI-stained FACS profiles. Cell-cycle profiles are shown at the left. Percentages of the early and late stages of apoptotic cells are shown at the right. There were approximately 30% more $G_6$ apoptotic splenocytes (annexin-positive) than WT apoptotic splenocytes 48 h after activation by PMA/Ion.

## Lymphocyte mitogenic response

Telomerase activity is low or undetectable in normal human B and T cells and increases dramatically after *in vitro* mitogenic stimulation[29,30]. This transient increase in telomerase activity indicates that somatic activation of telomerase may delay replicative senescence and facilitate mitogen-induced clonal expansion[31]. To determine the effects of telomerase deficiency on the ability of mature lymphocytes to proliferate, we exposed splenocytes, isolated from wild-type through to $G_6$ $mTR^{-/-}$ mice, mitogens that mainly activate T cells (phorbol myristate acetate (PMA) plus ionomycin (Ion), or anti-CD3/CD28 antibodies, or a more general T-cell-mitogenic activator (Concanavalin A (Con A)). For the three mitogenic stimuli, splenocytes derived from wild-type or $G_1$ through to $G_4$ $mTR^{-/-}$ mice showed almost identical degrees of uptake of $^3$H-labelled thymidine, although a decrease first became apparent in an occasional $G_4$ mouse (data not shown). In contrast, $G_5$ (four mice) and $G_6$ (three mice) stimulated $mTR^{-/-}$ splenocytes exhibited a statistically significant decrease in incorporation of $^3$H-labelled thymidine compared with wild-type (seven mice) controls (Fig. 4a). Incorporation of $^3$H-labelled thymidine in $G_6$ $mTR^{-/-}$ splenocytes was ~32% with Con A, ~38% with PMA/Ion, and ~39% with anti-CD3/CD28 antibodies relative to wild-type controls (Fig. 4a).

To determine the basis for the decreased incorporation of $^3$H-labelled thymidine, we assayed the cell-cycle profile and the percentage of apoptosis after PMA/Ion stimulation of wild-type and $G_6$ splenocytes. Although wild-type and $G_6$ cell-cycle profiles were nearly identical after stimulation, a marked increase in the percentage of late apoptotic cells (annexin-positive and propidium iodide (PI)-positive) was observed in the $G_6$ splenocytes; this increase was inversely proportional to the decrease in incorporation of $^3$H-labelled thymidine (Fig. 4b). The normal mitogenic response of earlier generation $mTR^{-/-}$ splenocytes and the pronounced and progressive effect on the later generations indicates that, although telomerase does not play a direct role in splenic lymphocyte activation and expansion, telomere shortening and chromosomal aberrations in late generations may lead to induction of apoptosis, possibly through either DNA-damage-response pathways or significant chromosome loss or rearrangement after mitogenic stimulation.

## Chromosome analysis

To examine whether the impaired mitogenic (pro-apoptotic) response of cultured lymphocytes could be related to significant telomere shortening and genomic instability, PMA/Ion- or Con A-activated splenocytes from two wild-type and six $G_6$ $mTR^{-/-}$ mice were treated with colcemid and karyotyped by G-banding. The percentage of activated cells with an abnormal chromosome count (aneuploidy) was 16.7% (7 of 42) for $mTR^{+/+}$ and 37.7% (53 of 144) for $G_6$ samples (Table 1a). In contrast, control metaphase spreads of

**Table 1 Cytogenic analysis**

| **a** Number of chromosomes per metaphase | Number of metaphases | |
|---|---|---|
| | WT | $G_6$ |
| >40 | 1 | 12 |
| 40 (normal) | 35 | 91 |
| 39 | 1 | 26 |
| 38 | 1 | 7 |
| 37 | 1 | 4 |
| 36 | 1 | 0 |
| 35 | 1 | 1 |
| 34 | 0 | 1 |
| 33 | 0 | 3 |
| 32 | 1 | 0 |
| 31 | 0 | 2 |
| Total number of metaphases examined | 42 | 155 |
| Aneuploidy (%) | 16.7 | 37.7 |

| **b** Sample number | Chromosome involved in fusions | |
|---|---|---|
| | WT | $G_6$ |
| 1 | t(1;1) | t(3;3) |
| 2 | t(X;#) | t(3;3) |
| 3 | | t(3;3) |
| 4 | | t(3;13) |
| 5 | | t(3;14) |
| 6 | | t(13;13) |
| 7 | | t(14;14) |

**a**, Metaphase spreads derived from activated WT and $G_6$ splenocytes were examined for abnormal chromosome number (aneuploidy). Percentages of aneuploidy were calculated by the number of metaphases carrying abnormal chromosome number (not 40) divided by the total number of metaphases examined. **b**, Chromosome numbers that participated in the fusions were identified by G-banding and listed. All fusions seemed to be the Robertsonian type and the short (p) arms were involved in all cases. #, not identifiable.

$mTR^{+/+}$ mouse embryonic fibroblast (MEF) cells showed no (0 of 17) aneuploidy (data not shown); the high baseline aneuploidy in activated $mTR^{+/+}$ splenocytes is not understood. When splenocyte metaphase spreads were scored for abnormal chromosome structures, 3.6% (2 of 55) of wild-type and 21.1% (40 of 190) of $G_6$ samples had chromosomes with abnormal G-banding patterns. We found end-to-end fusions that seem to be of the Robertsonian type[32,33].

To identify the specific chromosomes involved in the fusions, we conducted chromosomal G-banding on randomly selected $G_6$ metaphases and on the sole wild-type metaphases exhibiting fusion. In seven informative cases for the activated $G_6$ splenocytes, chromosomes 3, 13 and 14 were involved in the fusion events. This differed from the fusions in the two wild-type samples, where chromosomes 1 and X were involved (Table 1b). Although the frequent involvement of chromosome 3 in the $G_6$ samples (5 out of 7 fusions; Table 1b) is intriguing and could relate to the unusually short telomeres of its short (p) arm[34], the biological significance of this is unknown. Studies of human tumours and ageing cells did not show correlations between telomere length and frequency of involvement in fusion events for a given chromosome[35].

Finally, analysis of telomere length in several tissues by Southern blotting did not show any significant differences between the different generations or among tissues of the same generation (data not shown). In contrast, fluorescence *in situ* hybridization (FISH) using a probe for telomere repeat DNA generated fewer than four end signals in only a few chromosomes (~7.5%) in the wild-type metaphase spreads, whereas far more chromosomes in $G_6$ $mTR^{-/-}$ cells exhibited fewer than four signals (~58.75%) (data not shown). As all Robertsonian fusions in the $mTR^{-/-}$ sample failed to generate a signal at their junctions, these fusion events seem to be caused by the loss of telomere function at these chromosomes.

## Discussion

In dividing cells, telomerase is essential in telomere maintenance and, in its absence, significant erosion of telomeres is thought to signal replicative senescence and eventually lead to a crisis period highlighted by genetic instability and loss of cell viability. The presence of telomerase activity in germ cells and highly proliferative stem-cell compartments, and its upregulation in activated T lymphocytes, indicate that telomerase may be required for the proliferation and long-term viability of such cells. Here we demonstrate that telomerase deficiency leads to a depletion of male germ cells, diminished haematopoietic colony formation, and impaired mitogen-induced proliferation of primary splenocytes.

In the testes, the significant decline in BrdU incorporation and increase in TUNEL-positive nuclei suggests that telomere shortening leads to both checkpoint and apoptotic responses *in vivo*. In T cells, mitogenic stimulation of cells with short telomeres led to cytogenetic abnormalities and increased apoptosis. These results indicate that telomerase is essential for the long-term maintenance of diverse cell types with high proliferation profiles, and suggest that alterations in telomere structure dramatically affect the number, growth potential and/or survival of renewing cell populations. Marked aneuploidy and Robertsonian fusions were observed with high frequency in activated splenocytes derived from $G_6$ $mTR^{-/-}$ mice, a finding similar to that reported for late passage $mTR^{-/-}$ MEF culture[20]. These chromosomal anomalies were associated with a striking absence in telomere-repeat signal on a pronounced number of chromosome ends, further substantiating the concept that telomeres are vital in maintaining chromosomal integrity and genomic stability in normal cells *in vivo*.

The delay of several generations in emergence of a phenotype parallels the lag period observed in telomerase-deficient yeast cells, in which viability was compromised only after many cell divisions. The long latency in the appearance of defects in the mouse may simply relate to the long telomere lengths of the laboratory mouse strain *Mus musculus*[37]. Once significant telomere loss is reached, some cell types seem to be more adversely affected than others. The basis for the more prominent phenotype in the haematopoietic and germ cells could relate to a higher level of cell turnover and resultant greater telomere loss; however, other highly proliferative organs such as the intestine appear to be unaffected. Some cell types might be intrinsically less tolerant of genetic instability, perhaps reflecting a strategy to ensure faithful transmission of genetic information in the case of sperm cells or to guard against haematopoietic malignancies over a lifetime of intense proliferation. A detailed comparison of telomere lengths and karyotypes among these various cell types will be necessary to better resolve this issue.

Telomerase deficiency ultimately produced a reduction in proliferation and an increase in apoptosis in the mouse male germ cells. Such a response as the reduction of telomeres to 'critical length' in cultured human cells correlates with cessation of proliferation and maintenance of a viable senescent state. Human cells will remain in this state unless this checkpoint is commandeered by viral oncoproteins or genetic mutation. The difference between mice and humans may be related to the fact that the rodent cells find it easier to escape from the slow-growth 'senescent' phase than do human cells, the growth of which is tightly regulated. Thus the mouse cells continue more readily towards crisis.

These studies demonstrate that, although telomerase activity *per se* does not play a direct biological role in mammalian development, telomerase is vital in cell survival and organ homeostasis through maintenance of adequate telomere structure during cell division. The mouse model described here provides a system with which to understand the complexities of telomere dynamics and how they relate to cell physiology. These studies provide new insights into basic telomere biology and help us anticipate the systemic effects of telomerase inhibitors in cancer therapy. The maintenance of normal peripheral blood cell counts, immune competence and gastrointestinal function in the telomerase-deficient mouse indicates that diverse stem-cell compartments may sustain organ function after systemic administration of telomerase inhibitors. The high

proliferative index of most cancer cells compared with the more sporadic cycling of normal stem-cell populations suggests that telomerase inhibition will be well tolerated in clinical settings. □

## Methods

**Mice.** The genetic background of the mice (WW6/C57BL/6J) is identical to that of our previous work[20]. Age- and sex-matched animals were used in all experiments, with ages ranging from 6 to 14 weeks old. For analysis of female reproductive system, females 6–14 weeks old of the indicated genotypes were mated with CBA/B6 $F_1$ stud males. Fertilized eggs were cultured in KSOM media (Specialty).

**Haematopoietic colony assay.** Murine colony assay was performed according to the manufacturer's recommendation (StemCell Technologies). The colony counts for CFU-GM were performed on days 7–10 and for CFU-GEMM and HPP-CFC on days 12–14. HPP-CFC was defined as those colonies measuring 0.5 mm on day 12–14 (ref. 38).

**Incorporation of $^3$H-labelled thymidine and FACS analyses.** Splenocytes were resuspended in RPMI1640/10% FBS/0.55 μM β-mercaptoethanol. The mitogens used (Sigma or Pharmingen) were as follows: PMA (20 nM), ionomycin (1 μM), Con A (4 μg ml$^{-1}$), hamster anti-mouse CD3e (10 ng ml$^{-1}$) and hamster anti-mouse CD28 (10 ng ml$^{-1}$). After a pulse of $^3$H-labelled thymidine, the cells were collected at 48 h following the addition of mitogen. The incorporated c.p.m. was determined by a liquid scintillation counter. We processed samples in quadruplicate and calculated averages, standard deviations and *P* values. To analyse haematopoietic subpopulations by FACS, cells from each organ were incubated with antibodies (Pharmingen or Caltag) and analysed by FACScan (Becton Dickinson Immunocytometry Systems; LYSIS II). The antibodies were CD45-PE, IgM-PE, CD4-PE, CD3-fluorescein isothiocyanate (FITC), B220-FITC, CD8-ITC and MAC-1-FITC.

**Cell-cycle analysis and apoptosis assay.** Splenocytes were activated by PMA/Ion for 48 h. Cell-cycle and apoptosis assays were then used. For cell-cycle analysis we modified the method in ref. 39. For apoptosis assays, we used annexin-V-Fluos (Boehringer) to detect the apoptotic cell population according to the manufacturer's recommendation. For tissue sections, *in situ* apoptosis and BrdU incorporation assays were performed as described[40].

**Cytogenetic and FISH analyses.** Metaphase spreads of splenocytes were prepared and processed for G-banding by standard methods[41] following activation by Con A or PMA/Ion and colcemid treatment. For FISH analysis, hybridization of FITC-conjugated $(TTAGGG)_n$ (Oncor) was performed according to the manufacturer's recommendations.

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