Telomerase reverse transcriptase-dependent telomere equilibration mitigates tissue dysfunction in $m$Tert heterozygotes

Marie Meznikova$^1$, Natalie Erdmann$^1$, Rich Allsopp$^2$ and Lea A. Harrington$^{1,*}$

**SUMMARY**

Autosomal dominant mutations in telomere-associated factors elicit a disease known as dyskeratosis congenita (DKC), and patients suffer proliferative abnormalities associated with telomere erosion. Mice that are heterozygous for telomerase genes (Tert or Terc, hereafter referred to as $m$Tert and $m$Terc) are useful models of telomerase haploinsufficiency, but do not strictly mimic DKC. In strains with long telomeres (>60 kbp), animals that are heterozygous for $m$Tert undergo telomere erosion for nine generations and remain phenotypically normal. In an $m$Terc heterozygous strain with short telomeres (<15 kbp), early mortality arises after five to six generations, but dyskeratosis occurs only upon the further loss of $m$Pot1b. We show that prolonged $m$Tert heterozygosity (for greater than ten generations) did not elicit disease, even upon heterozygote interbreeding, and that telomeres reset to wild-type lengths. This lengthening did not occur in nullizygotes, and short telomeres inherited from $m$Tert null parents were rescued only in heterozygous progeny. In the bone marrow, nullizygotes remained competent for radioprotection for three generations. Thus, gradual telomere erosion in the presence of telomerase may enable subsequent telomere extension, similar to that described in budding yeast. We speculate whether such adaptation occurs in normal human cells (or whether it could be induced in DKC-derived cells), and whether it might mitigate the impact of telomerase inhibition upon stem cells during cancer therapy.

**INTRODUCTION**

In humans, X-linked mutation of dyskerin, an RNA pseudouridylase, leads to destabilisation of ribosomal RNAs and the telomerase RNA, and results in a disease termed dyskeratosis congenita (DKC) (Kirwan and Dokal, 2009). Other autosomal-dominant (AD) forms of aplastic anaemia, DKC and pulmonary lung fibrosis are linked to inactivating mutations in the telomerase or dyskerin complexes (the telomerase RNA, TERT, NHP2, NOP10) or components of the telomere-associated shelterin complex (TRF1, TRF2, TIN2) (Savage et al., 2006; Vulliamy et al., 2008; Walne et al., 2008; Carroll and Ly, 2009; Kirwan and Dokal, 2009). Patients carrying mutations in telomerase appear to exhibit a true haploinsufficiency (Theimer et al., 2003; Armanios et al., 2005; Yamaguchi et al., 2005; Xin et al., 2007; Errington et al., 2008), suffering telomere erosion in haematopoietic stem cell (HSC) compartments and other organs that leads to proliferative defects, including bone marrow (BM) failure and cancer (Goldman et al., 2008; Aslan et al., 2009; Calado et al., 2009; Kirwan and Dokal, 2009). Patients diagnosed with DKC often succumb to the illness before 30 years of age (Garcia et al., 2007).

Many proteins cooperate to establish and maintain telomere integrity and replication in mammals (de Lange, 2005). Telomerase replenishes telomere sequences that are lost during end processing and replication through its telomere polymerisation activity at single-stranded guanine (G)-rich overhangs, conferred by the telomerase reverse transcriptase (TERT) and the reverse transcription of an internal RNA component (TER, or TERC) (Hug and Lingner, 2006). Telomerase addition by telomerase does not occur at every telomere in each cell cycle, yet an equilibrium average length is often maintained, with longer telomeres exhibiting a decreased propensity for elongation compared with shorter telomeres (Hug and Lingner, 2006). DKC serves as but one illustrative case in yeast and mammals that this equilibrium can be perturbed by changes in the dosage of telomere-elongation activities or by factors that limit telomerase access (Smogorzewska and de Lange, 2004; Hug and Lingner, 2006).

The mouse has proven to be a valuable model with which to dissect the role of telomere attrition in cellular proliferation and disease. After several generations in the absence of telomerase genes (either Terc or Tert; the murine genes are hereafter referred to as $m$Terc and $m$Tert, respectively), the eventual loss of telomere DNA leads to end-to-end fusions and defects in many tissues and progenitor cell compartments, including the blood, skin, intestine and germline (Blasco, 2005). As with humans, mice that are heterozygous for $m$Terc or $m$Tert ($m$Terc$^{+/−}$ and $m$Tert$^{+/−}$, respectively) exhibit haploinsufficiency for the maintenance of long telomeres (Hemann et al., 2001a; Samper et al., 2001; Hatchcock et al., 2002; Chiang et al., 2004; Erdmann et al., 2004). The presence of critically short telomeres, and not of a short average telomere length, correlates with loss of tissue function (Hemann et al., 2001a; Samper et al., 2001; Erdmann et al., 2004). Similar to human telomerase haploinsufficiency, interbreeding of $m$Terc$^{+/−}$ animals in a background with short telomeres (CAST/EiJ) leads to telomere erosion and defects in the BM and other tissues, even in wild-type and $m$Terc$^{+/−}$ progeny (Hao et al., 2005). However, $m$Terc heterozygosity does not fully mimic DKC (especially in the skin) unless another component of the shelterin complex, $m$Pot1b, is also disrupted (Hockemeyer et al., 2008; He et al., 2009). Thus, further...
analysis of the consequences of telomerase haploinsufficiency in murine models is warranted.

RESULTS
Telomere re-equilibration in later mTert<sup>+/−</sup> generations
Here, we examine the long-term consequences of mTert heterozygosity on telomere length and tissue function. Previously, we showed that nine generations of mTert heterozygosity (by breeding to C57BL/6 wild-type animals) leads to telomere erosion that is comparable to that seen in mTert<sup>+/−</sup> mice; however, mTert<sup>+/−</sup> animals escape genetic instability owing to the maintenance of short telomeres (Erdmann et al., 2004). Similar to mTerc<sup>+/−</sup> animals, mice that are continually bred in the complete absence of mTert exhibit end-to-end fusions, infertility and impaired HSC renewal (Allsopp et al., 2003; Erdmann et al., 2004). To determine the consequences of prolonged mTert heterozygosity in this background (C57BL/6, telomeres >60kbp), we bred heterozygous (HG) mTert animals to wild-type (WT) C57BL/6 animals for 14 generations.

Extending and consistent with the previous analysis, the average telomere lengths in the splenocytes of mTert<sup>+/−</sup> animals up to HG10 were significantly shorter than in C57BL/6 animals (P<0.01) (Fig. 1A; supplementary material Fig. S1) (Erdmann et al., 2004). However, telomere lengths increased in mice from the later HG generations (HG12-HG14) (Fig. 1A), until the average telomere length no longer differed significantly from that seen in the BL/6 controls (supplementary material Fig. S1) (Erdmann et al., 2004). To determine the consequences of mTert heterozygosity in this background (C57BL/6, telomeres >60kbp), we bred heterozygous (HG) mTert animals to wild-type (WT) C57BL/6 animals for 14 generations.

Analysis of the progeny from mTert<sup>+/−</sup> interbreeding revealed a similar telomere equilibration (Fig. 1B). As anticipated, the HG and knockout (KO) progeny of parents with short telomeres (HG10) inherited shortened telomeres (Fig. 1B; light green). However, splenocytes from the progeny of later mTert<sup>+/−</sup> generations possessed telomere lengths that approached those of BL/6 controls (Fig. 1B; purple, blue). Specifically, telomere lengthening was observed upon comparison of the mTert<sup>+/−</sup> or mTert<sup>+/−</sup> progeny of HG10 parents with the same genotypes from later HG generations (Fig. 1B, compare the HG or KO profiles from green (HG10) to blue (HG12), P<0.001). This observation was upheld in more than 27 independent crosses without non-littermates (Fig. 1B; data not shown).

Rescue from telomere erosion is mTert-dependent
The breeding of non-littermates derived from two independently generated mTert<sup>+/−</sup> founder lines is intended to guard against the fixation of genetic modifiers that are unlinked to mTert (Erdmann...
Telomere equilibration mitigates dysfunction in later HG and KO generations

Successively bred telomerase null animals eventually exhibit a decrease in fertility and testes mass (Hemmann et al., 2001b; Erdmann et al., 2004). In keeping with the retention of functional telomeres in later generations of HG animals, no change in the average number of pups per breeding pair, testes mass or intestinal crypt cell apoptosis was observed in mTert<sup>Δ/+</sup> animals (Fig. 3A–C; data not shown). This amelioration was mTert-dependent, as later generations of mTert<sup>Δ/+</sup> animals exhibited a significant reduction in litter size (Fig. 3A, *P*<0.05), a decrease in testes mass (Fig. 3B, *P*<0.01) and an increase in intestinal cell apoptosis (Fig. 3C, *P*<0.01).

A measure of haematopoietic function is the ability of the BM to protect the viability of recipients whose own BM is ablated with radiation. We isolated the BM from 114 animals that were generated during the same breeding analysis depicted in Fig. 1, and assessed their ability to radioprotect an irradiated recipient (supplementary material Table S1; Fig. 4). No defects were observed in the ability of the BM from mTert<sup>Δ/+</sup> progeny to radioprotect, even after three generations of mTert<sup>Δ/+</sup> inbreeding. Even when donor BM was derived from mTert<sup>Δ/+</sup> progeny (from HG10 or HG12 parents), radioprotection was successful in all but three of 33 transplantations (supplementary material Table S1). Three generations of successive interbreeding of mTert<sup>Δ/+</sup> animals (of HG10 or HG12 parents, *n*=26) also successfully rescued recipients, and no signal-free ends were observed in BM cells after transplantation (Fig. 4; data not shown). Furthermore, 50–100 HSCs from fourth-generation mTert<sup>Δ/+</sup> animals successfully repopulated progenitor BM compartments in irradiated recipients, even upon competitive transplantation with wild-type HSCs (Derrick Rossi and Irv Weissman, personal communication). Serial transplantation of HSCs from the primary recipients of mTert<sup>Δ/+</sup> HSCs into a second lethally irradiated recipient also proved competent for radioprotection, with shorter telomeres observed in the repopulated BM but no telomere signal-free ends (data not shown).

**Fig. 3. Germline and intestinal cell apoptosis in successive mTert generations.** (A) Average number of pups per mating pair in 6 months (labels and colours are the same as in Fig. 1). (B) Average testes mass (normalized to body mass) for littermates of an HG<sub>10</sub> × HG<sub>10</sub> cross (green), BL/6 controls (grey), and for two different third-generation KO litters of KO × KO progeny beginning at HG<sub>12</sub> (light blue). The sample number is indicated above each column. (C) Average number of apoptotic nuclei in the small intestine of the indicated genotypes. Standard deviations were not included: instead, significance was determined using analysis of variance (ANOVA) (asterisks; see Methods). In B, note the variation in testes mass between the two separate KO litters, as reported previously (Erdmann et al., 2004). A similarly variable increase in intestinal cell apoptosis and decrease in testes mass was observed in the first-generation KO progeny of HG<sub>10</sub> × HG<sub>10</sub> matings (data not shown).
Telomerase-null animals that exhibit apoptosis in the intestine or germ line also typically exhibit impaired BM or HSC function (Samper et al., 2002; Allsopp et al., 2003; Hao et al., 2005; Blasco, or germline also typically exhibit impaired BM or HSC function to radioprotect.

Unfortunately, the time elapsed between $mTert^{+/+}$ generations ten and 14 precluded an inter-generational comparison of telomere heterochromatin. Alternatively, compensatory changes in the expression of telomerase (or other telomere-associated factors) could occur during embryogenesis (Prowse and Greider, 1995; Martin-Rivera et al., 1998; Liu et al., 2007). We did not detect an upregulation of $mTert$ mRNA in later-generation $mTert^{+/+}$ animals (B. Snow, data not shown). We did not examine $mTert$ or other mRNAs (telomere-associated or otherwise) during embryogenesis. Telomere equilibration was also observed upon successive passaging of $mTert^{+/+}$ embryonic stem (ES) cells in culture (Wang et al., 2005), which may argue against the necessity of gametogenesis (or a spurious effect of breeding strategy) on telomere equilibration.

DISCUSSION

Telomeres equilibrate in $mTert$ heterozygotes

Fig. 4. Bone marrow (BM) reconstitution and Q-FISH analysis of $mTert^{+/+}$ intercrosses. Q-FISH analysis of whole BM isolated from irradiated recipients of the indicated genotype (as in supplementary material Table S1), at 16-20 weeks after transplantation ($n=1$ for irradiated recipients, and $n=3$ for BL/6 and KO3 controls, at the right). The $y$-axis indicates the number of events per fluorescence category, and the $x$-axis indicates the relative telomere fluorescence in arbitrary units. All axes are scaled equivalently. Coloured arrows point to the average telomere fluorescence. Black arrows indicate the average telomere fluorescence of the BL/6 control samples ($n=3$).

It is not the length of telomeres per se that protects against loss of chromosome end function, but the ability to maintain sufficient length to form a functional telomere cap. Although equilibrium telomere lengths vary dramatically between yeast, mice and humans, they share a dosage-sensitive balance between telomere loss and replenishment. In heterozygous $mTert$ mice that have been bred for many generations, we propose that initial telomere attrition followed by eventual recovery reflects such a length-dependent equilibrium. In $S. cerevisiae$ and humans, long telomeres inhibit telomerase access owing to cis-inhibition by telomere-bound factors such as Rif1/Rif2 and TRF1/TRF2, respectively (Smogorzewska and de Lange, 2004; Hug and Lingner, 2006). Once telomeres become short, the dosage of telomere-bound factors is reduced, leading to loss of cis-inhibition and a switch to a telomerase-extendible state (Smogorzewska and de Lange, 2004; Hug and Lingner, 2006). Upon telomere shortening, murine cells lacking telomerase undergo changes in heterochromatic structure that, if manifested similarly in an $mTert$ heterozygote with short telomeres, might promote telomere extension (Blasco, 2007b). Unfortunately, the time elapsed between $mTert^{+/+}$ generations ten and 14 precluded an inter-generational comparison of telomere heterochromatin. Alternatively, compensatory changes in the expression of telomerase (or other telomere-associated factors) could occur during embryogenesis (Prowse and Greider, 1995; Martin-Rivera et al., 1998; Liu et al., 2007). We did not detect an upregulation of $mTert$ mRNA in later-generation $mTert^{+/+}$ animals (B. Snow, data not shown). We did not examine $mTert$ or other mRNAs (telomere-associated or otherwise) during embryogenesis. Telomere equilibration was also observed upon successive passaging of $mTert^{+/+}$ embryonic stem (ES) cells in culture (Wang et al., 2005), which may argue against the necessity of gametogenesis (or a spurious effect of breeding strategy) on telomere equilibration.

Long and heterogeneous telomeres, a hallmark that is typical of humans, are selectively expanded during transplantation. It may be possible to distinguish these hypotheses by extending the serial transplantations (e.g. to tertiary or quaternary recipients) and testing the ability of fewer than 50 HSCs to radioprotect.

Clearly, humans carrying TERT mutations cannot avert the consequences of telomere attrition (Kirwan and Dokal, 2009). However, these patients inherit shorter telomeres from affected parents [a phenomenon termed genetic anticipation (Vulliamy and Dokal, 2008)]; by analogy to CAST/EiJ murine strains, telomere reserves may be insufficient to permit adaptation. Enforced expression of exogenous hTERT in DKC-derived fibroblasts (or of the telomerase RNA in DKC-derived keratinocytes) is sufficient to maintain telomere lengths and extend the proliferative life span (Gourronc et al., 2009; Kirwan et al., 2009). To our knowledge, telomere lengths have not been examined in DKC patient-derived
cancer cells, to determine whether telomere length stabilisation occurs during tumourigenesis. An induction of telomerase expression [e.g. through chemical activation of hTERT transcription (Fauce et al., 2008)], perhaps combined with a partial and transient depletion of essential cis-inhibitory telomere factors such as TRF1 or TRF2 (van Steensel and de Lange, 1997; Smogorzewska et al., 2000; Karleseder et al., 2003), might shift the balance toward telomere maintenance or extension in DKC-affected cells, similar to a strategy suggested by Garcia and colleagues (Garcia et al., 2007). In normal human cells, we speculate that telomere reserves could be sufficiently long to permit telomere equilibration without intervention, thus inuring stem cells to the deleterious effects of partial telomerase inhibition during tumour therapy.

METHODS

Breeding

These studies were initiated in 1998, when two independently generated mTert<sup>+/-</sup> founder lines were bred with wild-type C57BL/6 mice, for up to nine generations (Erdmann et al., 2004). In this study, we continued to breed heterozygous progeny with C57BL/6 wild-type mice for up to 14 generations (Fig. 1A). Genotyping and breeding was carried out as described previously (Erdmann et al., 2004). To promote consistency in nomenclature with other labs (Hao et al., 2005), we named these backcrossed generations HG<sub>1</sub>-HG<sub>14</sub> (they were previously termed 'BC' for 'backcross') (Erdmann et al., 2004). To further accelerate telomere attrition, in a strategy similar to that employed for mTert<sup><r/></sup> mice (Hao et al., 2005), we also interbred HG<sub>10</sub> animals and HG<sub>12</sub> animals (Fig. 1B), and examined WT, HG and KO littersmates for up to three additional HG × HG generations. The number of generations of subsequent inbreeding is indicated as a subscript. For example, in an HG<sub>10</sub> × HG<sub>10</sub> cross, WT<sub>1</sub> represents the first-generation WT progeny. Two HG<sub>1</sub> progeny (from distinct litters and with different parents) were interbred to generate HG<sub>2</sub> mice, and HG<sub>2</sub> cousans from this cross were interbred to generate WT<sub>3</sub>, HG<sub>3</sub> and KO<sub>3</sub> progeny (Fig. 1B). As a comparison to multiple generations of nullizygosity, we also bred the null progeny of an HG<sub>6</sub> × HG<sub>6</sub> intercross (KO<sub>HG6</sub>-KO<sub>1</sub>), or an HG<sub>12</sub> × HG<sub>12</sub> intercross (KO<sub>HG12</sub>-KO<sub>1</sub>), together for three generations (Fig. 1B). Non-littermates were used for all crosses, and multiple crosses were performed at each generation, with a total of 42 independent crosses performed for the data shown in Fig. 1.

Fertility and genotype distribution

No decrease in fertility and no disproportionate in genotype distribution were observed among 14 generations of mTert<sup><r/></sup> backcrossing to C57BL/6 mice; however, a slight but not statistically significant increase in the average number of pups per mating pair (over 6 months of mating) occurred in HG<sub>14</sub> mice (31.5, n=6 mating pairs), compared with HG<sub>10</sub> (24, n=9), HG<sub>11</sub> (23, n=6), HG<sub>12</sub> (22.8, n=4) and HG<sub>13</sub> (26.2, n=5) mice (Fig. 3A). First-generation HG<sub>10</sub> × HG<sub>10</sub> and HG<sub>12</sub> × HG<sub>12</sub> crosses exhibited normal fertility, with an average number of pups, per mating pair in 6 months, of 24.1 (n=7) and 24.6 (n=7), respectively (Fig. 3A). The fertility of second-generation HG<sub>10</sub> × HG<sub>10</sub> parents was somewhat lower (20.2, n=6), and a significantly decreased fertility was observed in KO<sub>HG10</sub>-KO<sub>1</sub> (7, n=3) and KO<sub>HG12</sub>-KO<sub>1</sub> animals (11, n=5) (P<0.05) (Fig. 3A). In one HG<sub>10</sub> × HG<sub>10</sub> cross, the genotype distribution was altered as follows (169 pups in total): the proportion of WT animals was higher than expected (WT:HG:KO=1:2:1), with 62 WT animals observed, compared with the expected 42.25 (P<0.01), and the proportion of KO mice was lower than expected, with 28 animals observed, compared with the expected 42.25 (P<0.01). The HG progeny from this cross did not differ from the expected ratio, with 79 HG progeny observed, compared with the expected 84.5 (P>0.05).

Telomere length analysis

Q-FISH was performed on fixed splenocytes, as described previously (Erdmann et al., 2004). For BM transplantation, BM single cell suspensions were plated in MarrowMax medium (Invitrogen) and cultured for 24-48 hours. After colcemid arrest (0.1 µg/ml) (Roche Diagnostics) for 60 minutes, cells were harvested and fixed in 3:1 methanol-acetic acid. To control for hybridisation differences between experiments, each experiment contains the same sample derived from a wild-type C57BL/6 mouse. Except where indicated, Q-FISH profiles are compiled from at least ten metaphase spreads (1600 telomeres) for n=2-3 animals derived from different parents, to demonstrate reproducibility between litters. Where P values are indicated, Student’s t-tests were performed on the log<sub>10</sub> of the mean telomere fluorescence of two samples (data not shown). Wilcoxon rank-sum tests yielded similar results (data not shown).

Assessment of apoptosis in the testes and small intestine

To analyse apoptosis in germ cells and intestinal stem cells, an in situ end-labelling technique (ISEL) was performed (Wijssman et al., 1993), which measures the incorporation of biotinylated nucleotides at DNA breaks. Tissue samples of small intestine and testes were collected and fixed in 10% v/v neutral buffered formalin immediately after dissection. Tissues were processed in a Ventana tissue processor through ascending graded alcohol, to xylene and paraffin wax. Intestine samples were embedded and cross-sectioned to ensure round vertical sections, at a thickness of approximately 100 µm per slice. Paraffin sections were dewaxed and treated with 1% w/v pepsin in 0.01 N HCl (pH 2.0). Endogenous peroxidase was blocked using 3% v/v aqueous hydrogen peroxide, and endogenous biotin was masked using an avidin/biotin blocking kit (Lab Vision). Sections were treated with buffer A [50 mM Tris-HCl (pH 7.0), 50 mM MgCl<sub>2</sub>,6H<sub>2</sub>O, 100 mM β-mercaptoethanol, 0.005% w/v bovine serum albumin (BSA)] for 5-10 minutes, incubated with biotin-nucleotide cocktail in a water bath at 37°C for 60-90 minutes, and then washed in phosphate-buffered saline (PBS). After labelling with Ultra Streptavidin-horseradish peroxidase labelling reagent (ID Labs Inc) for 30 minutes at room temperature, and washing in PBS, staining was developed with freshly prepared Nova Red solution (Vector Laboratories, Inc.). Slides were counterstained with Mayer’s haematoxylin. As a result, apoptotic and necrotic cells appear red, and normal nuclei appear blue.

Apoptotic nuclei in testes were counted in at least 200 seminiferous tubules (cross sections). In the intestine, round vertical sections of the small intestine were used to define the incidence of apoptosis in intestinal crypts. Twelve to 18 sections were analysed per mouse, and three mice of each genotype from each generation were used to calculate the average number of signals per section for each generation. No increase in intestinal
Disease Models & Mechanisms

**Clinical issue**

Dyskeratosis congenita (DKC) is a collection of autosomal dominant diseases that result in abnormalities in the skin, nails, bone marrow and lungs. DKC arises from hereditary mutations in factors that replenish or cap telomeres, which are at the ends of the chromosomes. When the level of telomere-replenishing factors dips, telomeres erode and chromosomes rearrange. The affected cells either die or become unable to divide. Patients with DKC often die before the age of 30, usually from infection because of bone marrow dysfunction, or as a result of their increased predisposition to cancer.

**Results**

The authors use a mouse model that resembles DKC in humans to determine the potential for adaptive extension of telomeres. They use mTert mice, which express one functional allele and one disrupted allele of the telomere-replenishing factor, telomerase reverse transcriptase (Tert). The initial generations of mTert heterozygotes exhibit the same telomere erosion as seen in DKC patients. The authors show that, after several generations, telomeres in mTert mice are restored to nearly the same lengths as those found in normal animals. This restoration depends on the inheritance of Tert, which actively supports lengthening of the telomeres. The telomere length restoration that is observed prevents cell death, and the mice do not exhibit phenotypes that are normally associated with loss of telomere function in the intestine, testes and the bone marrow. This result is similar to findings in yeast but shows that telomerase can correct telomere length over time in a mammal.

**Implications and future directions**

The ability to maintain a functional telomere cap appears to be more important than the actual length of the telomere itself. In yeast cells, long telomeres are bound by factors that inhibit telomere extension by telomerase. Short telomeres are not bound by as many inhibitory factors and telomerase can gain access and extend the telomere. The authors speculate that such an equilibration process might protect normal human stem cells. Furthermore, activation of the process in DKC-affected cells might reduce symptoms of the disease that result from lost telomere function. The capacity to lengthen telomeres in normal tissues with low levels of telomerase activity might also protect against the inhibition of telomerase during cancer therapy, and improve its efficacy.

doi:10.1242/dmm.004481

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**Radioprotection assays**

Bone marrow was harvested from the femurs and tibias of donor mice (age-matched, 3-4 months old) using appropriate guidelines. Cells were washed once in PBS and resuspended into red blood cell lysis buffer (ACK) (Biosource). After incubation for 5 minutes at room temperature, ACK was substituted with PBS. Cells from three donor mice of each respective genotype were counted (Beckman Coulter Vi-Cell XR 2.03 analyser) and mixed equally to a final concentration of 2 × 10⁶ cells/ml. Each lethally irradiated (9 Gy) wild-type C57BL/6 (11-week-old) female recipient was injected with 1 × 10⁶ cells. Transplanted animals were monitored for at least 16 weeks after transplantation, at which point they were sacrificed or dissected, and the BM isolated and analysed through F-QISH.

**RT-PCR analysis of mTert mRNA**

Trizol reagent (Invitrogen) was used to purify total RNA from C57BL/6 mouse spleen and testes. First-strand cDNA was prepared from total RNA using oligo-dT and random hexamer primers, and the Superscript III first-strand cDNA synthesis kit (Invitrogen), according to the manufacturer’s instructions. For reverse transcription PCR, a touch-down/touch-up PCR protocol was performed, as described (Snow et al., 1997), using the following primers: mTERT-KO_RTPCR_U2 (5'-CCA TTG CAC CCA GCC TCT CAA G-3') and mTERT_KO_RTPCR_L2 (5'-ACA GTA CGT GCT CTC CGA CGA CTG G-3').

**ACKNOWLEDGEMENTS**

We thank Derrick Rossi and Irv Weissman for HSC analysis; J. Dorrens for Q-FISH analysis; B. Snow for RT-PCR analysis of mTert mRNA; D. Bouchard for FACS analysis; and C. Clarke, W. Earnshaw, C. Greider, Y. Liu, H. Pickersgill, I. Stancheva, M. Tyers and lab members for comments and discussion. L.A.H. acknowledges support from the National Institute on Aging (RO1 AG02398), the HHMI International Scholar Award Program (55005945), and the Wellcome Trust (84637). Deposited in PMC for release after 6 months.

**COMPETING INTERESTS**

The authors declare no competing financial interests.

**AUTHOR CONTRIBUTIONS**

M.M., N.E. and L.A.H conceived and designed the experiments; M.M., N.E. and R.A. executed the experiments; M.M., N.E. and L.A.H. analysed the data; and L.A.H. wrote the paper with input from M.M., N.E. and R.A.

**SUPPLEMENTARY MATERIAL**

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.004069/-/DC1

Received 7 July 2009; Accepted 17 August 2009.

**REFERENCES**


Table S1. Number of deaths recorded within 16 weeks after bone marrow transplantation of the indicated genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HG_{10} 1^{st} gen</th>
<th>HG_{12} 3^{rd} gen</th>
<th>KO_{HG10} 1^{st} gen</th>
<th>KO_{HG12} 3^{rd} gen</th>
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<td>0 (7)</td>
<td>0 (13)</td>
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<td>2 (12)</td>
<td>1 (9)</td>
<td>0 (12)</td>
<td>0 (15)</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent the total number of recipients. Blank boxes: not determined.