RESEARCH REPORT

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Telomerase reverse transcriptase-dependent telomere equilibration mitigates tissue dysfunction in *mTert* 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 *mTert* and *mTerc*) are useful models of telomerase haploinsufficiency, but do not strictly mimic DKC. In strains with long telomeres (>60 kbp), animals that are heterozygous for *mTert* undergo telomere erosion for nine generations and remain phenotypically normal. In an *mTerc* heterozygous strain with short telomeres (<15 kbp), early mortality arises after five to six generations, but dyskeratosis occurs only upon the further loss of *mPot1b*. We show that prolonged *mTert* 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 *mTert* 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). Telomere 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 *mTerc* and *mTert*, 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 *mTerc* or *mTert* ($mTerc^{\Delta/+}$ and $mTert^{\Delta/+}$, respectively) exhibit haploinsufficiency for the maintenance of long telomeres (Hemann et al., 2001a; Samper et al., 2001; Hathcock 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 $mTerc^{\Delta/+}$ 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 $mTerc^{\Delta/+}$ progeny (Hao et al., 2005). However, *mTerc* heterozygosity does not fully mimic DKC (especially in the skin) unless another component of the shelterin complex, *mPot1b*, is also disrupted (Hockemeyer et al., 2008; He et al., 2009). Thus, further

[1]Ontario Cancer Institute/Campbell Family Institute for Cancer Research, Toronto, ON M5G 2C1, Canada
[2]Institute for Biogenesis Research, John A. Burns School of Medicine, Cancer Research Center of Hawaii, Honolulu, HI 96813, USA
*Author for correspondence (e-mail: l.harrington@ed.ac.uk)

analysis of the consequences of telomerase haploinsufficiency in murine models is warranted.

RESULTS

Telomere re-equilibration in later *mTert*$^{\Delta/+}$ 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*$^{\Delta/\Delta}$ mice; however, *mTert*$^{\Delta/+}$ animals escape genetic instability owing to the maintenance of short telomeres (Erdmann et al., 2004). Similar to *mTerc*$^{\Delta/\Delta}$ 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*$^{\Delta/+}$ animals up to HG_{10} 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 (HG_{12}-HG_{14}) (Fig. 1A), until the average telomere length no longer differed significantly from that seen in the BL/6 controls (supplementary material Fig. S1) (note that the longer average telomere length in HG_{13} mice is not statistically significant from BL/6 mice). Although, before HG_{10}, WT littermates had slightly shorter telomeres than BL/6 mice (Erdmann et al., 2004), the average telomere lengths of WT littermates from HG_{10}-HG_{14} did not differ significantly between generations or when compared with BL/6 controls (supplementary material Fig. S1; data not shown). This telomere equilibration was not the result of sampling variation, as splenocytes derived from several generations were measured in the same experiment and the trend was reproduced in 15 separate matings (Fig. 1). The lengthening of telomeres in the later HG generations was not the result of a contribution by long telomeres from BL/6 animals, since the same breeding strategy led to telomere attrition before HG_{10} (Erdmann et al., 2004), and the BL/6 animals used in HG_{10}-HG_{14} breeding possessed similar telomere lengths when compared with previous BL/6 controls and with BL/6 animals purchased from another commercial source (supplementary material Fig. S1; J. Dorrens and L.A.H., unpublished data).

Analysis of the progeny from *mTert*$^{\Delta/+}$ interbreeding revealed a similar telomere equilibration (Fig. 1B). As anticipated, the HG and knockout (KO) progeny of parents with short telomeres (HG_{10}) inherited shortened telomeres (Fig. 1B; light green). However, splenocytes from the progeny of later *mTert*$^{\Delta/+}$ 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*$^{\Delta/+}$ or *mTert*$^{\Delta/\Delta}$ progeny of HG_{10} parents with the same genotypes from later HG generations [Fig. 1B, compare the HG or KO profiles from green (HG_{10}) to blue (HG_{12}), $P<0.001$]. This observation was upheld in more than 27 independent crosses using 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*$^{\Delta/+}$ founder lines is intended to guard against the fixation of genetic modifiers that are unlinked to *mTert* (Erdmann

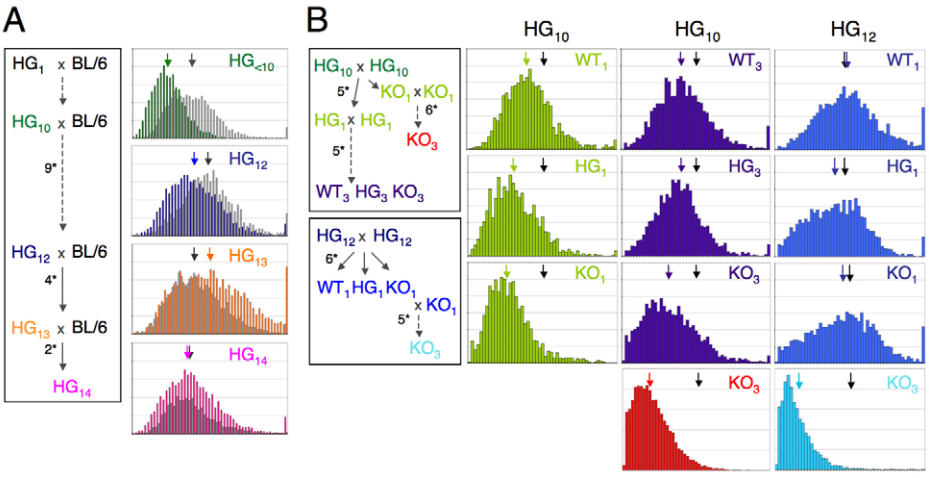

Fig. 1. Telomere length in successive *mTert*$^{\Delta/+}$ generations. (A) Left, *mTert*$^{\Delta/+}$ mice (HG) were backcrossed to C57BL/6 wild-type mice (BL/6) for 14 generations (HG_1-HG_{14}) (the asterisk indicates the number of independent mating pairs at each generation). Right, telomere quantitative-fluorescent in situ hybridisation (Q-FISH) profiles for each *mTert*$^{\Delta/+}$ generation compared with their wild-type littermates (grey). Each profile is pooled from two or three animals (i.e. 3200-4800 individual telomere measurements). The *y*-axis indicates the number of events per fluorescence category and the *x*-axis shows telomere fluorescence in arbitrary units (although values were omitted, all *x*-axes are scaled equivalently). (B) Left, heterozygote breeding strategies for HG_{10} and HG_{12}; see Methods for details. Right, telomere (Q-FISH) profiles of the first-generation (G1) progeny of an $HG_{10} \times HG_{10}$ cross (WT_1, HG_1, KO_1; green); the third-generation (G3) progeny of HG × HG crosses beginning at HG_{10} (WT_3, HG_3, KO_3; purple); the G1 progeny of HG_{12} parents (blue); and the G3 KO progeny of KO × KO crosses beginning at $HG_{10} \times HG_{10}$ (red) or $HG_{12} \times HG_{12}$ (light blue). With the exception of the HG_{10} progeny (green, $n=1$ for each genotype; however, distribution profiles were similar for a second litter), profiles were pooled from two or three animals, where progeny were derived from at least two independent breeding pairs. Axes are the same as in A. Arrows: average telomere fluorescence for *mTert* mice of the genotypes indicated (coloured) and BL/6 controls (black, $n=3$).

et al., 2004). Furthermore, the resumption of telomere erosion upon further interbreeding of KO progeny is consistent with the *mTert*-dependence of the equilibration (Fig. 1B; red, light blue). Nonetheless, we tested the *mTert*-dependence of telomere lengthening directly by mating an $mTert^{\Delta/+}$ animal with longer telomeres (HG_{14}) to an animal with shorter telomeres (third-generation KO animals from HG_{12} parents) (Fig. 2A, top panel; Fig. 2C), and analysed the splenocytes from ten littermates (Fig. 2A, middle panel). Within one generation, a statistically significant rescue of telomere signal-free ends was observed in the $mTert^{\Delta/+}$ progeny compared with their $mTert^{\Delta/\Delta}$ parents or littermates (Fig. 2A,B). The specific rescue of critically short telomeres in the $mTert^{\Delta/+}$ progeny (and the Gaussian rather than bimodal telomere distribution curve) is consistent with previous intercrosses of $mTerc^{\Delta/+}$ and $mTerc^{\Delta/\Delta}$ (Hemann et al., 2001a; Samper et al., 2001) or $mTert^{\Delta/+}$ and $mTert^{\Delta/\Delta}$ mice (Chiang et al., 2004; Erdmann et al., 2004). These data establish that limited telomerase activity in later $mTert^{\Delta/+}$ generations was necessary and sufficient to rescue signal-free ends, and that the lengthening segregated with the *mTert* allele.

Telomere equilibration mitigates dysfunction in later HG and KO generations

Successively bred telomerase null animals eventually exhibit a decrease in fertility and testes mass (Hemann 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 $mTert^{\Delta/+}$ animals (Fig. 3A-C; data not shown). This amelioration was *mTert*-dependent, as later generations of $mTert^{\Delta/\Delta}$ 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 $mTert^{\Delta/+}$ progeny to radioprotect, even after three generations of $mTert^{\Delta/+}$ inbreeding. Even when donor BM was derived from $mTert^{\Delta/\Delta}$ progeny (from HG_{10} or HG_{12} parents), radioprotection was successful in all but three of 33 transplantations (supplementary material Table S1). Three generations of successive interbreeding of $mTert^{\Delta/\Delta}$ animals (of HG_{10} or HG_{12} 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 $mTert^{\Delta/\Delta}$ 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 $mTert^{\Delta/\Delta}$ 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).

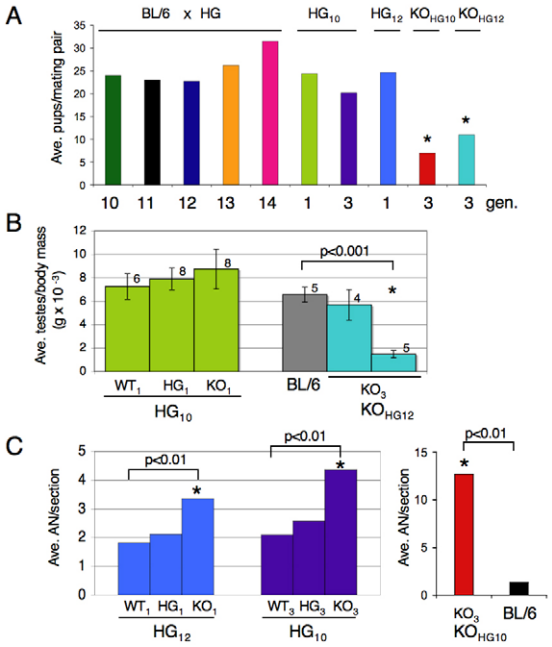

Fig. 3. Germline and intestinal cell apoptosis in successive *mTert* generations. (A) Average number of pups per mating pair in 6 months (labels and colours are the same as in Fig. 1). (B) Average testes mass (normalized to body mass) for littermates of an $HG_{10} \times HG_{10}$ cross (green), BL/6 controls (grey), and for two different third-generation KO litters of KO × KO progeny beginning at HG_{12} (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 $HG_{10} \times HG_{10}$ matings (data not shown).

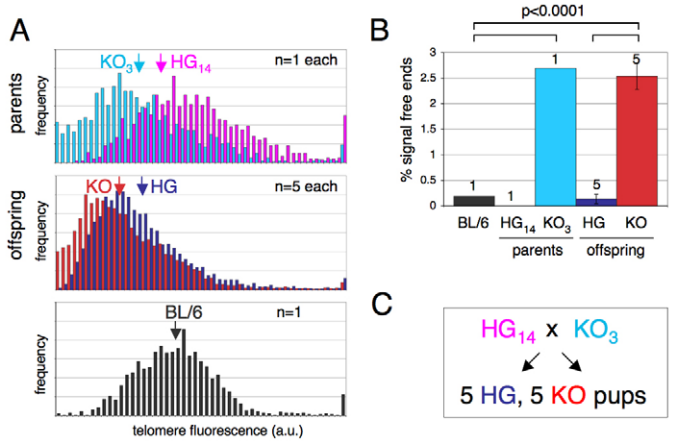

Fig. 2. Telomere elongation and rescue is *mTert*-dependent. (A) Telomere Q-FISH profiles of splenocytes from $mTert^{\Delta/+}$ (HG_{14}) and $mTert^{\Delta/\Delta}$ parents (third-generation KO mice were derived from KO × KO crosses beginning at HG_{12}) (top panel); pooled telomere Q-FISH profiles from ten offspring ($mTert^{\Delta/+}$, $n=5$; $mTert^{\Delta/\Delta}$, $n=5$) (middle panel); and a BL/6 wild-type control analysed in the same experiment (bottom panel). (B) Percentage of signal-free ends for the genotypes indicated in A. *P* values were calculated using Fisher's exact test.

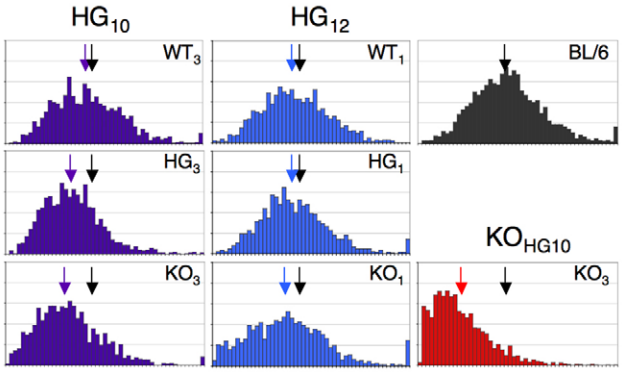

Fig. 4. Bone marrow (BM) reconstitution and Q-FISH analysis of *mTert*$^{\Delta/+}$ intercrosses. Q-FISH analysis of whole BM isolated from irradiated recipients of the indicated genotype (as in supplementary material Table S1), at 16-20 weeks after transplantation (n=1 for irradiated recipients, and n=3 for BL/6 and KO_3 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).

Telomerase-null animals that exhibit apoptosis in the intestine or germline also typically exhibit impaired BM or HSC function (Samper et al., 2002; Allsopp et al., 2003; Hao et al., 2005; Blasco, 2007a; Choudhury et al., 2007; Rossi et al., 2007). In this study, it may appear surprising that the BM of later-generation *mTert*$^{\Delta/\Delta}$ animals retained the capacity to radioprotect, especially when the telomeres of donor animals were already short (e.g. Fig. 1B). Nonetheless, the radioprotection is consistent with the lack of signal-free ends in the *mTert*$^{\Delta/\Delta}$ BM after rescue (e.g. Fig. 4, KO_{HG10}-KO_3; data not shown). This finding could reflect two non-exclusive possibilities: that telomeres began longer in *mTert*$^{\Delta/\Delta}$ animals derived from *mTert*$^{\Delta/+}$ parents with near wild-type telomeres (compared with *mTert*$^{\Delta/+}$ parents with shorter telomeres), or that a small subset of *mTert*$^{\Delta/\Delta}$ BM cells with longer telomeres (inherited from HG parents) 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.

DISCUSSION

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*$^{\Delta/+}$ 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*$^{\Delta/+}$ 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*$^{\Delta/+}$ 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 human ALT (alternative lengthening of telomeres), were not observed in later-generation *mTert*$^{\Delta/\Delta}$ animals (Fig. 1). More subtle types of telomere recombination have been reported recently in vitro, including increased p/q arm exchange in *mTerc*$^{\Delta/+}$ and *mTerc*$^{\Delta/\Delta}$ cells (Morrish and Greider, 2009). In support of the co-existence of telomerase-dependent and -independent mechanisms of telomere homeostasis in vivo, increased telomere sister-chromatid exchange (T-SCE) has been observed concomitant with telomerase-dependent telomere elongation during early development (Benetti et al., 2007; Liu et al., 2007). However, even when telomeres were short, T-SCE was not observed in *mTert*$^{\Delta/+}$ animals and ES cell lines (Wang et al., 2005). Nonetheless, the *mTert*-dependent lengthening described in this study does not rule out other contemporaneous methods of telomere maintenance.

C57BL/6 mice possess a longer equilibrium telomere length than other inbred, wild-derived laboratory strains of mice such as CAST/EiJ (Hemann and Greider, 2000). In mice and humans with shorter telomere reserves, chromosome ends may irrevocably cross a threshold of telomere instability before a telomerase-extendible state can be achieved. The impact of the initial telomere length on subsequent telomere dynamics is not without precedent. In *S. cerevisiae* strains that possess longer telomeres, rare cells can survive the loss of telomerase and *RAD52*, this survival does not occur in populations with shorter telomeres (Grandin and Charbonneau, 2009; LeBel et al., 2009). Our data suggest that the response to prolonged telomerase heterozygosity could similarly be influenced by the initial telomere length.

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; Karlseder 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 $mTert^{\Delta/+}$ 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_1-HG_{14} (they were previously termed 'BC' for 'backcross') (Erdmann et al., 2004). To further accelerate telomere attrition, in a strategy similar to that employed for $mTerc^{\Delta/+}$ mice (Hao et al., 2005), we also interbred HG_{10} animals and HG_{12} animals (Fig. 1B), and examined WT, HG and KO littermates for up to three additional HG × HG generations. The number of generations of subsequent inbreeding is indicated as a subscript. For example, in an $HG_{10} \times HG_{10}$ cross, WT_1 represents the first-generation WT progeny. Two HG_1 progeny (from distinct litters and with different parents) were interbred to generate HG_2 mice, and HG_2 cousins from this cross were interbred to generate WT_3, HG_3 and KO_3 progeny (Fig. 1B). As a comparison to multiple generations of nullizygosity, we also bred the null progeny of an $HG_{10} \times HG_{10}$ intercross (KO_{HG10}-KO_1), or an $HG_{12} \times HG_{12}$ intercross (KO_{HG12}-KO_1), 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 disproportion in genotype distribution were observed during 14 generations of $mTert^{\Delta/+}$ 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_{14} mice (31.5, n=6 mating pairs), compared with HG_{10} (24, n=9), HG_{11} (23, n=6), HG_{12} (22.8, n=4) and HG_{13} (26.2, n=5) mice (Fig. 3A). First-generation $HG_{10} \times HG_{10}$ and $HG_{12} \times HG_{12}$ 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 $HG_{10} \times HG_{10}$ parents was somewhat lower (20.2, n=6), and a significantly decreased fertility was observed in KO_{HG10}-KO_3 (7, n=3) and KO_{HG12}-KO_3 animals (11, n=5) (P<0.05) (Fig. 3A). In one $HG_{10} \times HG_{10}$ 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 animal. 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 $\log_{10}$ 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 (Wijsman 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 $MgCl_2.6H_2O$, 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

TRANSLATIONAL IMPACT

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 detemine 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.

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cell apoptosis was observed in the WT and HG progeny of HG_{10} and HG_{12} intercrosses (Fig. 3C). A significant increase in intestinal cell apoptosis was observed in HG_{12}-KO_1 mice (an average of 3.36 signals per section) and HG_{10}-KO_3 mice (an average of 4.36 signals per section) compared with wild-type (averages of 1.81 and 2.09 signals per section, respectively) or heterozygous (averages of 2.11 and 2.57 signals per section, respectively) animals of the same generations ($P \leq 0.01$) (Fig. 3C). First-generation $HG_{10} \times HG_{10}$ progeny exhibited variability in apoptotic nuclei in the small intestine: in one $HG_{10} \times HG_{10}$ cross, there was no significant difference between WT, HG and KO progeny; however, in other experiments a significant increase in apoptotic nuclei was observed in KO progeny (data not shown). In KO × KO intercrosses, a significant increase in apoptosis was observed in third-generation KO animals from HG_{10} parents (an average of 12.70 signals per section), compared with C57BL/6 animals (an average of 1.33 signals per section) ($P<0.01$) (Fig. 3C). Owing to the variation between animals, standard deviations and *t*-tests were not calculated; instead, ANOVA was performed to assess statistical significance between different comparison groups, assuming unequal variance between comparison data sets (InStat3, GraphPad).

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 2×10^6 cells/ml. Each lethally irradiated (9 Gy) wild-type C57BL/6 (11-week-old) female recipient was injected with 1×10^6 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 Q-FISH.

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 GCA TTT CAC CCA GCG TCT CAA G-3′) and mTERT_KO_RTPCR_L2 (5′-ACA GTA CGT GCT CTC CGA GTG CCT G-3′).

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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

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