Telomerase is required to slow telomere shortening and extend replicative lifespan of HSC during serial transplantation

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ABSTRACT

Telomeres shortening ultimately limits the replicative lifespan of cultured human somatic cells. Telomeres also shorten during replicative aging in vivo in hematopoietic cells, including early hematopoietic progenitors and hematopoietic stem cells (HSC), from humans and mice, despite readily detectable levels of telomerase in these cells. To assess the relevance of telomerase to the long-term replicative capacity of HSC in vivo, we serially transplanted HSC from wild-type and telomerase-deficient mice until exhaustion and monitored telomere length in HSC during this process. Telomerase-deficient HSC could only be serially transplanted for 2 rounds, whereas wild-type HSC could be serially transplanted for at least 4 rounds. Furthermore, the rate of telomere shortening was increased ~2 fold during serial transplantation of telomerase-deficient HSC. These findings suggest that, one role for telomerase in the HSC is to partially counter the rate of telomere shortening during division of HSC, thereby preventing pre-mature loss of telomere function and providing added replicative capacity.
INTRODUCTION

The inability to serially transplant whole bone marrow in mice more than 4-6 times has led to speculation that HSC, like various somatic cell types grown in vitro, have a limited replicative lifespan\(^1\,^2\). Telomeres, essential genetic elements which cap and protect the ends of chromosomes, have been shown to shorten during replicative aging of many different human somatic cell strains\(^3\). Over-expression of telomerase reverse transcriptase (TERT), the catalytic component of telomerase\(^4\), in cultured human somatic cells results in the cessation of telomere shortening and immortalization\(^5\), demonstrating a direct causal role for telomere shortening in replicative senescence in vitro. Telomeres also shorten in human hematopoietic cells, including HSC, during aging in vivo\(^6\,^7\), and during transplantation of bone marrow (BM)\(^8\), and in HSC from mice during serial transplantation\(^9\). However, unlike most other somatic cell types in humans, telomerase activity can be detected in the hematopoietic system, especially early progenitors\(^10\) and HSC\(^11\). Thus the role of telomerase in HSC is unclear. Here we assess the effect of telomerase deficiency on long-term transplantation capacity and telomere shortening during serial transplantation of HSC.
MATERIALS AND METHODS

Mice
The derivation of the mTR knock-out mice and mTERT knock-out mice has been previously described\textsuperscript{12,13}. The mTR\textsuperscript{-/-} mice and mTERT\textsuperscript{-/-} mice were back-crossed 6 and 4 times respectively to the C57Bl/Ka-Thy1.1(Ly5.1) strain at the Stanford University animal facility prior to use in this study. In all transplant experiments, the Thy1.1/Ly5.1 mice were used as HSC donors and the congenic C57Bl/Ka-Thy1.2(Ly5.2) strain was used as recipients. The initial donor mice and all the recipient mice were 2-3 months old. All mice were bred and maintained on acidified water (pH 2.5).

HSC analysis, purification and transplantation
Bone marrow cells were isolated and stained with fluorophor-conjugated antibodies as previously described\textsuperscript{9}. The antibodies used in the immunofluorescence staining for HSC detection are as previously described\textsuperscript{9}. The HSC population is defined as c-ki\textsuperscript{hi}Sca-1\textsuperscript{hi}Thy1.1\textsuperscript{lo}lineage\textsuperscript{neg}. Double-sorted HSC (n=150) were used in each round of transplantation. Every stage of serial transplantation was performed with long-term reconstituted mice as donors (≥4 months post-transplant). All recipient mice were lethally irradiated with a dose of 960r (\textsuperscript{137}Cs). All analyses and cell sorting were performed on a dual-laser Vantage (Becton-Dickinson) FACS machine.

Fluorescent in situ hybridization
Telomeres were detected by FISH using a FITC-conjugated peptide nucleic acid telomeric probe ((CCCTAA)\textsubscript{3}, Applied Biosystems), as described\textsuperscript{14}. Background noise was corrected using unstained slides for each sample processed in parallel. All quantitative analysis were performed using a Zeiss confocal microscope.

Analysis of telomerase activity
The TRAP assay and quantification of telomerase activity was performed using the TRAP assay kit from Intergen, as described\textsuperscript{15} but with following modifications. The final concentration for all dNTPs was 10mM, and 0.2µg of TS primer was used per 25µl reaction.
RESULTS AND DISCUSSION

Successive generations of breeding mice in which the gene encoding the RNA component of telomerase (mTR) has been knocked out eventually results in the critical shortening of telomeres and telomere dysfunction\textsuperscript{16}, and in attenuation of hematopoietic cell function and proliferation\textsuperscript{17}. To address the biological significance of telomerase in HSC in more detail, we compared the extent of telomere shortening and replicative capacity of HSC from wild-type (mTR+/+) and generation 1 (G1) mTR-/- mice during serial transplantation. We observed an abrupt decrease in the frequency of donor-derived cells in the 5th and 3rd round of transplantation during serial transplantation of HSC, the c-ki\textsuperscript{hi}Sca-1\textsuperscript{hi}Thy1.1\textsuperscript{lo}lineage\textsuperscript{neg} BM sub-population, from mTR+/+ and mTR-/- mice respectively (Fig. 1A). During serial transplantation of HSC from mTR+/+ mice, the frequency of donor-derived cells decreased slightly after the first 2 rounds of transplantation, and then stabilized at 75-80% until the 5\textsuperscript{th} round of transplantation. However, earlier studies report a gradual decrease in frequency of donor-derived cells at each step of serial transplantation until donor-derived cells are no longer detectable\textsuperscript{2,18}. We believe this discrepancy is likely due, at least in part, to the method of serial transplantation. Notably, we find that it is necessary to avoid use of radioprotective BM and to remove any host-derived cells prior to transplantation of HSC or whole BM cells, particularly at the 3\textsuperscript{rd} and 4\textsuperscript{th} round of serial transplantation, in order to achieve a high frequency (ie. \geq40\%) of donor-derived cells in the long-term recipient mice (not shown).
To assess whether the finite reconstitutive potential of HSC and reduction of telomeric size during serial transplantation of HSC might be attributed to a concomitant decrease in telomerase expression, we assessed telomerase activity in FACS purified HSC from long-term reconstituted recipients at each stage of serial transplantation (Fig. 1B,C). As shown in figure 1, no significant change in telomerase activity in HSC was observed (P=0.26) indicating that this is not a factor.

During the serial transplantation of HSC, we measured telomere length using FISH in mTR+/+ and mTR-/- HSC from long-term (>4 months post-transplant) multi-lineage reconstituted mice at each stage of serial transplantation (Fig. 2A,B). This analysis revealed a significantly reduced telomere length in HSC from 1º recipients and 2º recipients that were reconstituted with mTR/-/- HSC as compared to 1º (P=0.008) and 2º (P=0.006) recipient mice reconstituted with mTR+/+ HSC. Also, the rate of telomere loss was ~2 fold higher during transplantation of mTR/-/- HSC as compared to mTR+/+ HSC (Fig. 2B). This increased rate of telomere shortening was associated with a decreased transplantation capacity for mTR-/- HSC (Fig. 2C). We also observed an increased rate of telomere shortening in donor-derived BM and a reduced transplantation capacity during serial transplantation of BM cells from mTR/-/- mice (not shown). FISH analysis of telomere length during serial transplantation of HSC from mTERT+/+ mice and mTERT-/- mice also revealed a more rapid loss of telomere length (~1.7 fold) and a reduced transplantation capacity for HSC deficient in telomerase (Fig. 2D,E).

Furthermore, we observed both end-to-end dicentric chromosomes and an elevated frequency of signal-free chromosome ends (<2% and 95% of metaphase spreads from mTR+/+ and mTR-/- 2º HSC respectively; see Fig. 2F) in 2º mTR/-/- HSC, supporting the notion that the attenuated transplantation capacity of telomerase-deficient HSC is caused by critical shortening of telomeres.
Our finding of an accelerated loss of telomeric DNA during serial transplantation of telomerase-deficient HSC is in contrast to the recent study by Samper et al. who found little decrease in telomere fluorescence after transplantation of mTR-/- BM\textsuperscript{18}. However, the mice used as donors by Samper et al. were G3 mTR-/- mice and therefore likely had a shorter, possibly near critical, initial telomere length, perhaps, as proposed\textsuperscript{18}, leading to the selection of HSC or more primitive stem cells in the G3 mTR-/- embryo in which alternative mechanisms\textsuperscript{19} for telomere maintenance were active.

Interestingly, in 1 out of 8 2º mTERT-/- HSC recipients that were used as donors for the third round of serial transplantation, we observed robust hematopoietic reconstitution and increase in telomere length in HSC from long-term 3º recipients (Fig 2D,E). Furthermore, we have also been able to serially transplant HSC derived from this 2º donor into 4º recipients and observe increased telomere length in 4º HSC as well (Fig 2D,E). As expected, telomerase activity could not be detected in donor-derived cells from these 3º or 4º recipients (not shown). This data indicates that a telomerase-independent alternative (ALT) mechanism\textsuperscript{19} for telomere replication has been activated these HSC, in agreement with a previous study on the effect of immunization on telomere length in B cells from late generation mTR-/- mice\textsuperscript{20}.

We find that HSC frequency remains constant during serial transplantation until donor-derived cells drop to very low levels (Fig. 2C and E), in contrast to a recent study which reports a rejuvenation of the hematopoietic stem cell compartment in recipient mice back to just ~5% of that observed in bone marrow from resting adult mice\textsuperscript{21}. However, this discrepancy is likely accounted for by differences in both analysis of HSC and transplantation methodology. These differences include: (i) measurement of the HSC population on the basis of cell surface phenotype, as opposed to limit dilution; (ii) calculation of HSC frequency within the donor-derived hematopoietic cell compartment, as opposed to whole bone marrow; (iii) the transplantation of just FACS sorted HSC or donor-derived whole bone marrow as opposed to the inclusion of competitor cells, (iv) differences in number of HSC transplanted and radiation dose of the recipient mice.

The data presented here indicate that one function for telomerase in HSC is to provide an extended replicative capacity of HSC via a reduction of the rate of telomere shortening during cell division, thereby preventing a pre-mature critical shortening of
telomeres and abrogation of telomere function. The extended replicative capacity conferred by telomerase in HSC is likely to be important throughout the organismal lifespan during times of high hematopoietic stress, such as infections and excessive blood loss, as evidenced by the delayed time for regeneration of peripheral blood cell count in old mTR-/- mice following exposure to 5-FU. Moreover, the autosomal dominant form of dyskeratosis congenita, a progressive bone-marrow failure syndrome, has recently been shown to be specifically associated with mutations in the human hTR gene, as well as accelerated telomere shortening. The ability of small numbers telomerase-deficient murine HSC to provide efficient hematopoietic recovery after 2 rounds of transplantation as compared to the progressive BM failure observed within the lifetime of those afflicted with dyskeratosis congenita may be explained by the 2-3 times longer telomere length in murine cells, as well as possible additional unknown deleterious consequences of expression of mutant hTR in the hematopoietic system including HSC. The apparent reduction of replicative capacity of HSC following extensive telomere shortening will be important to take into consideration during therapeutic transplantation of bone marrow or HSC-enriched populations in humans and any potential cancer therapies involving telomerase inhibition.

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FIGURE LEGEND

Figure 1. Analysis of donor-derived cell frequency and telomerase activity during serial transplantation of HSC. A. FACS purified HSC (n=150) from donor mTR-/- mice (n=4) and mTR+/+ mice (n=4) were serially transplanted until exhaustion of transplantation capacity. The fraction of donor-type (Ly5.1+) cells in the bone marrow of long-term (>4 month) reconstituted mice (n≥5) was determined by FACS analysis at each stage of serial transplantation. The decrease in frequency of donor-derived BM cells during the first and second round of serial transplantation of mTR+/+ HSC and mTR-/- HSC was statistically significant (P<0.05; Student’s t test). Error bars (standard deviation) are shown. B. HSC (n=250) from adult mice and recipient mice at each stage of serial transplantation were sorted directly into lysis buffer. Telomerase activity was measured for duplicate sample extracts at each stage of serial transplantation by the TRAP assay. A telomerase extract from NIH3T3 cells (n=2500) was included as a positive control. The internal control for PCR efficiency is indicated by the arrow on the right. C. Telomerase activity was measured for HSC samples from 3 or more mice at each stage of serial transplantation and averaged for all samples. The mean level of activity and error bars (standard deviation) are shown. We did not observe a significant change in telomerase activity level in HSC during serial transplantation (P=0.26).

Figure 2. Analysis of telomere length and replicative capacity during serial transplantation of HSC from telomerase-deficient mice. A. At each stage of transplantation, ~2000 HSC were purified via FACS from a total of 1-2 mice, and cytopspun onto glass slides. All HSC samples were collected at 15 months (mTR+/+ donor) or 7 months (mTR-/- donor) after the initial transplant. Detection of telomeres by FISH was performed using a FITC-tagged peptide nucleic acid telomeric probe as previously described. Individual interphase nuclei are indicated by arrow-heads and the size scale (5 µm; bottom right) is shown in the top left panel. B. The fluorescent signal intensity was measured for 25 nuclei at each stage of transplantation using a Zeiss confocal microscope. During serial transplantation of HSC from both mTR-/- and mTR+/+ mice, the change in fluorescent signal intensity was significant (P<0.05) for all stages except 3° → 4° for mTR +/- HSC (P=0.21; Student’s t test). C. The average
frequency of donor-derived HSC from long-term (> 4 month) reconstituted mice (n=5) was measured at each stage of serial transplantation. The HSC frequency is shown relative to the fraction of donor-derived cells. Error bars (standard deviation) are shown. All mTR+/+ and mTR-/- mice used in this analysis and FISH analysis of telomere length were also used in the analysis of frequency of donor-derived BM cells (Fig. 1). D. and E. Fish analysis of telomere length and analysis of donor-derived HSC frequency was performed during serial transplantation of HSC from mTERT+/+ mice and mTERT-/- mice as described above. At least 4 mice were used in the assessment of donor HSC at each stage of transplantation. Signal intensity was significantly diminished in HSC from 1º recipients and 2º recipients that were reconstituted with mTERT-/- HSC as compared to 1º (P=0.006) and 2º (P=0.009) recipient mice reconstituted with mTERT+/+ HSC. The analysis of telomere signal intensity and HSC frequency for the 2º mTERT-/- HSC donor and successive 3º and 4º recipients in which telomere length and replicative lifespan were observed to increase are indicated by the open circle and dashed line. F. HSC from 2º recipients of either mTR+/+ or mTR-/- HSC were FACS sorted into media containing 10% FBS, IL-3, IL-6, IL-11 and Steel factor (all 10ng/ml) and cultured for 5 days. Metaphase spreads were then prepared using standard procedures, and telomeres were detected using FISH. End-to-end chromosome fusions are indicated by the arrow-heads.
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