Quiescent hematopoietic stem cells with short telomeres accumulate genomic damage but are blocked from hematopoietic activation by senescence and apoptosis

Jianwei Wang¹, Xin Lu², Christoph A. Klein²,³, and Karl Lenhard Rudolph¹*  

1- Leibniz Institute of Age Research, Fritz Lipmann Institute e.V., Jena, 07745, Germany, and the Research Group on Molecular Aging, Faculty of Medicine, Friedrich-Schiller-University, Jena, Germany  
2- Experimental Medicine and Therapy Research, University Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany  
3- Fraunhofer-Institut für Toxikologie und Experimentelle Medizin, Project Group Personalized Tumor Therapy, Regensburg

*Corresponding Author: Karl Lenhard Rudolph  
Email: klrudolph@fli-leibniz.de  
Tel: +49 3641 65 6350  
Fax: +49 3641 65 6351  
Mailing Address: Beutenbergstraße 11, 07745 Jena, Germany

Running Title: Dilemma of quiescent HSCs with shorten telomere
**Key Points:** DNA damage induced by shortening telomere resides in the most quiescent hematopoietic stem cells.

Senescence and apoptosis compromise the activation of hematopoietic stem cell with dysfunctional telomere.

**Abstract**

Telomere shortening limits proliferative capacity of human cells and age dependent shortening of telomeres occurs in somatic tissues including hematopoietic stem cells (HSCs). It is currently unknown whether genomic and molecular damages occur in HSC induced by telomere shortening are transmitted to the progenitor cells. Here we show that telomere shortening results in DNA damage accumulation and gene expression changes in quiescent HSCs of aged mice. Upon activation, a subset of HSCs with elevated levels of DNA damage and p16 expression is blocked from cell cycle entry and apoptosis is induced in HSCs entering cell cycle. Activation of both checkpoints associates with normalization of DNA damage and gene expression profiles at early progenitor stages. These findings indicate that quiescent HSCs have an elevated tolerance to accumulate genomic alterations in response to telomere shortening but the transmission of these aberrations to the progenitor cell level is prevented by senescence and apoptosis.

**Introduction**

Telomere shortening limits the proliferative capacity of human cells and may contribute to aging associated decline in hematopoietic stem cell (HSC) function. Studies on DNA repair deficient mice and telomerase knockout mice revealed that DNA damage accumulates in HSC compartment and limits the functionality of HSCs by induction of DNA damage checkpoints. Recent studies indicated that DNA damage is repaired when HSCs enter the cell cycle. However, telomere free chromosome ends cannot easily be repaired since telomerase recruitment requires telomere repeats. Moreover, the formation of chromosomal fusion represents an aberrant repair pathway that interferes with maintenance of chromosomal integrity in dividing cells. Whether telomere shortening induced DNA damage leads to accumulation of DNA damage and gene expression changes in HSCs and whether these alterations are transmitted to HPCs is currently unknown.

**Material and Method**
Animals: All mice used are C57BL/6 background. mTerc<sup>−/−</sup> were crossed to generate G1 mTerc<sup>−/−</sup>. mTerc<sup>−/−</sup> mice were crossed until the third generation (G3mTerc<sup>−/−</sup>). Mice were maintained and experiments were conducted according to protocols approved by the state government of Thuringia (Reg. Nr. 03-006/13).

Isolation of cells: Bone marrow cells were isolated by crushing bones from donor mice. Cells were stained and sorted by using the following surface markers combination: HSC (CD34<sup>lo</sup> Flt3<sup>−</sup> Sca1<sup>+</sup> cKit<sup>+</sup> Lineage<sup>−</sup>), MPP (CD34<sup>+</sup> Flt3<sup>+</sup> Sca1<sup>+</sup> cKit<sup>+</sup> Lineage<sup>−</sup>), myeloid cells (CD11b<sup>+</sup>). Quiescent HSCs and cycling HSCs were purified by using Pyronin Y (Sigma-Aldrich, P9172) and Hoechst33342 (Sigma-Aldrich, B2261). Bone marrow cells were stained with antibodies for HSCs first, then incubated with Hoechst33342 (DNA dye, 1mg/ml) at 37°C for 30 minutes and Pyronin Y (RNA dye, 100ug/ml) for further 15 minutes under light free condition. Samples were analyzed by ARIA (BD Biosciences).

Comet Assays: Comet Assays were conducted by using OxiSelect™ Comet Assay Kit according manufacturer’s protocol. HSCs were sorted into ice-cold PBS with the concentration (1×10<sup>5</sup> cells/ml) and fixed onto OxiSelect™ Comet together with Comet Agarose. Then, electrophoresis was applied and Vista Green DNA Dye was used to develop tails.

Result and Discussion

Telomere dysfunction induces DNA damage accumulation and gene expression changes in quiescent hematopoietic stem cells but not at progenitor cell level

To analyze gene expression changes in response to telomere shortening, HSCs (CD34<sup>−</sup> Flt3<sup>−</sup> LSK), multipotent progenitor cells (MPPs: CD34<sup>+</sup> Flt3<sup>+</sup> LSK) and myeloid cells (CD11b<sup>+</sup>) were freshly isolated from 12-month-old G3mTerc<sup>−/−</sup> mice and age-matched mTerc<sup>+/+</sup> mice (Suppl. Table 1). Gene expression profiling (original profile were uploaded to GEO: GSE60164) revealed that 63 genes (fold change>2, p<0.05) were differently regulated in telomere dysfunctional HSCs compared to mTerc<sup>+/+</sup> HSCs (Fig. 1A). In contrast, the comparison of gene expression from mTerc<sup>+/+</sup> vs. G3mTerc<sup>−/−</sup> revealed no differentially expressed genes at the level of MPPs, and only 11 genes at the level of myeloid cells (Suppl. Table 2, Fig. 1A). Among the 63 genes differently expressed in HSCs from G3mTerc<sup>−/−</sup> compared to mTerc<sup>+/+</sup>, several DNA damage or apoptosis related genes were present (highlighted in Fig. 1A).

γH2AX staining (a marker of DNA breaks) revealed a significant increase of DNA damage in freshly isolated HSCs (Fig. 1B), but not in MPPs and myeloid cells between G3mTerc<sup>−/−</sup> and mTerc<sup>+/+</sup> (Fig. 1C, D). Similar results were obtained for 53BP1 staining (data not shown).
Furthermore, analysis of DNA breakage by the Comet-Assay revealed that 47% of the HSCs from G3mTerc<sup>−/−</sup> carried more than 30% DNA in comet tails compared to only 18% of HSCs from mTerc<sup>+/+</sup> (Fig. 1E, p<0.001), but no significant difference in MPPs and myeloid cells from G3mTerc<sup>−/−</sup> compared mTerc<sup>+/+</sup> (Fig. 1F, 1G).

**Apoptosis and senescence limit survival and cell cycle entry of quiescent telomere dysfunctional HSCs**

The above results indicated that HSCs accumulate DNA damage and gene expression changes in response to telomere dysfunction but these alterations are not transmitted to the progenitor cell level. To determine at what stage of HSCs amasses the alterations, 12 genes, that are differently expressed in HSCs between G3mTerc<sup>−/−</sup> and mTerc<sup>+/+</sup>, were selected (based on a reported high expression level in HSC compartment<sup>12</sup>) to be investigated in quiescent and cycling HSCs from G3mTerc<sup>−/−</sup> compared to mTerc<sup>+/+</sup> (n=11-13 mice per group pooled into 2 pools per group). The results revealed that most of the gene expression changes were present in quiescent HSC but not in cycling HSCs (Fig. 2A, n= 3 technical repeats, n= 2 pools per group). The higher magnitude of gene expression changes in quiescent compared to cycling HSCs correlated with a significant elevated rate of DNA damage foci in quiescent vs. cycling HSCs from G3mTerc<sup>−/−</sup> compared to mTerc<sup>+/+</sup> (Fig. 2B, C). Together, these results indicated that only the non-damaged HSCs contributed to the pool of cycling HSCs in aged telomere dysfunctional mice.

A possible explanation for the obtained results suggested that DNA damage checkpoints were less active in quiescent HSCs compared to activated HSCs, thus damaged HSCs were eliminated or arrested at the transition from quiescent to activated stage. To test this interpretation, quiescent and activated HSCs were purified by Pyronin Y and Hoechst33342<sup>13</sup> from 12-month-old G3mTerc<sup>−/−</sup> that were treated with Interferon-α to activate quiescent HSC to enter cell cycle<sup>14</sup>. Cell cycle analysis showed significant cell cycle entry of HSCs in response to Interferon-α treatment (Suppl. Fig. 1A, B). To monitor the activation of DNA damage checkpoints, the expression levels of p21<sup>7</sup>, p16<sup>15</sup>, BATF<sup>16</sup>, and PUMA<sup>17</sup> were analyzed by qPCR in freshly isolated quiescent and activated HSCs. The experiments revealed that Puma was significantly increased in cycling compared to quiescent HSCs of G3mTerc<sup>−/−</sup> (Fig. 2D), but there was no difference for p21 and Batf expression (Suppl. Fig. 1G, H). Several other apoptosis regulating genes were also up-regulated in cycling HSCs compared to quiescent HSCs from G3mTerc<sup>−/−</sup> (Suppl. Fig. 1C), but not in cycling HSCs from mTerc<sup>+/+</sup> stimulated with Interferon-α (Suppl. Fig. 1D), indicating that apoptosis was induced in damaged HSCs of aged G3mTerc<sup>−/−</sup> upon activation. Furthermore, annexin-5 staining revealed a significant increase in apoptosis in activated HSCs compared to quiescent HSCs of G3mTerc<sup>−/−</sup> (Fig. 2E, Suppl. Fig. 1E), but not in mTerc<sup>+/+</sup> (Suppl. Fig. 1F).

The expression of p16 (a cell cycle inhibitor associated with senescence) was significantly elevated in quiescent HSCs from G3mTerc<sup>−/−</sup> compared to mTerc<sup>+/+</sup> and this increase was even
higher in quiescent HSCs from Interferon-α stimulated G3mTerc<sup>-/-</sup> (Fig. 2F). These data suggested that a subset of quiescent HSCs of telomere dysfunctional mice was arrested in senescence and could not exit quiescence to enter the cell cycle upon stimulation. In agreement with this interpretation, γH2AX staining revealed a significantly elevated amount of DNA damage in HSCs from G3mTerc<sup>-/-</sup> mice that remained in G0 gate after Interferon-α stimulation compared to G0 HSCs from control group (Fig. 2G). To further prove this hypothesis, a number of HSCs (CD34<sup>lo</sup> KSL) from 12-month-old G3mTerc<sup>-/-</sup> (332) and age-matched mTerc<sup>+/+</sup> (310) were purified and cultured individually. At 6 and 12 days after seeding, cell number of each colony was accounted (Fig. 2H, 2I). On day 6, 7.6% of HSCs from G3mTerc<sup>-/-</sup> died compared to 0.32% of the HSCs from mTerc<sup>+/+</sup> (Fig. 2H, p= 0.0083). Moreover, the percentage of HSCs that stayed alive as single cells and could not enter cell cycle to form colonies was higher in HSCs purified from G3mTerc<sup>-/-</sup> compared to mTerc<sup>+/+</sup> by day 6 (Fig. 2H, 9.2% vs 2.9%, p= 0.043).

Together with the in vivo data these experiments indicate that both senescence cell cycle arrest and apoptosis represent checkpoint responses that block damaged HSCs from G3mTerc<sup>-/-</sup> to enter cell cycle upon stimulation and to generate committed progenitor cells.

This study reveals that DNA damage and gene expression changes accumulate specifically in quiescent HSCs in the context of telomere shortening. Activation of senescence locks a subset of damaged HSCs in G0 stage, which is consistent with recent report showing similar phenotype in muscle stem cell from geriatric mice<sup>18</sup>. Some damaged HSCs from G3mTerc<sup>-/-</sup> entered cell cycle upon stimulation lead to apoptosis induction. Taken together, both senescence and apoptosis checkpoints may cooperate to prevent damaged HSCs from generating hematopoietic progenitor cells. A similar mechanism has been disclosed in intestinal stem cell system<sup>17</sup>. In addition to the activation of checkpoints it is also possible that DNA repair contributes to the decline of DNA damage and gene expression changes at the transition from quiescent HSCs to progenitor cells. Along these lines, a recent study showed that DNA repair signaling is compromised in quiescent HSCs, but it is activated when HSCs enter cycle<sup>6</sup>. Of note, many of the differentially expressed genes play a role in DNA damage repair and apoptosis, which may be a response and/or contributing factor to the accumulation of DNA damage. It is conceivable that some gene expression changes are induced by chromosomal, genetic and epigenetic alterations that occur in response to telomere dysfunction<sup>17,19,20</sup>. Together, these findings improve our understanding of the evolution and dynamics of gene expression changes and DNA damage accumulation in aging hematopoietic stem and progenitor cells in the context of telomere dysfunction. The findings may influence the selection of aberrant HSC clones that are characteristic of human aging and leukemia development<sup>21,22</sup>.

**Author Contribution:** J.W. performed experiments, analyzed the data and wrote the paper; X.L., C.K. performed the gene array experiment and analyzed the data; K.L.R. designed research, analyzed the data and wrote the paper.
Conflict of Interest: The authors declare no competing financial interests.

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Reference


Figure legends

Figure 1: DNA damage and gene expression changes in response to telomere shortening accumulate in quiescent HSCs but not in hematopoietic progenitors and myeloid cells.

A) The histogram shows the number of genes that are differentially expressed in hematopoietic stem cells (HSCs), multiple potential progenitor cells (MPPs) and myeloid cells isolated from 12-month-old G3mTerc−/− compared to age-matched mTerc+/+ mice. 50-200 freshly isolated cells were freshly isolated for the analysis of gene expression profiles (n= 6-7 mice per group). Several DNA damage or apoptosis related genes were differently expressed in HSCs between G3mTerc−/− compared to age-matched mTerc+/+ mice, including Polq (polymerase, theta), Fancc (Fanconi anemia, complementation group C), Rb1 (Retinoblastoma 1), Ccdc14 (coiled-coil domain containing 14), Bmf (Bcl2 modifying factor), Aatf (apoptosis antagonizing transcription factor), Hspa12b (heat shock protein A12B), Anxa9 (Annexin 9), Ddx10 (Dead box 10), Dact2 (Dapper homolog 2), Rbm10 (RNA binding motif protein 10), Map3k12 (mitogen-activated protein kinase kinase kinase 12) and Soc56 (suppressor of cytokine signaling 6). B-G) To analyze DNA damage, HSCs, MPPs and myeloid cells from 12-month-old G3mTerc−/− mice and mTerc+/+ mice were freshly isolated for DNA damage quantification assay including γH2AX staining and alkaline comet assay. (B-D) The histograms show the percentage of cell nuclei staining positive for the indicated numbers of γH2AX foci in (B) HSCs, (C) MPPs, and (D) myeloid cells (100 nuclei were counted per cell type and group). (E-G) DNA fragmentation was analyzed using the alkaline comet assay. Fragmented DNA is visualized as a tail moving out of the gel-embedded nuclei. More than 500 HSCs, MPPs and myeloid cells from G3mTerc−/− and mTerc+/+ were scored by alkaline comet assay. The histogram shows the distribution of tail DNA percentage of HSCs (E), MPPs (F) and myeloid cells (G) from 12-month-old G3mTerc−/− and age-matched mTerc+/+ mice (n>500 nuclei per group). (H) Representative images of HSCs from 12-month-old G3mTerc−/− and age-matched mTerc+/+ mice analyzed by the alkaline comet assay.

Figure 2: DNA damage and gene expression changes are reverted in stimulated, telomere dysfunctional HSCs that enter the cell cycle.

A-C) To analyze gene expression and DNA damages in quiescent and cycling HSCs under homeostatic conditions, HSCs were freshly isolated from 12-month-old, non-stimulated G3mTerc−/− mice and mTerc+/+ mice: (A) qPCR analysis of differentially expressed genes in G0-HSCs (grey bars) and G1/S/G2/M HSCs (black bars). Note that gene expression differences between HSCs of G3mTerc−/− mice and mTerc+/+ mice were more pronounced in quiescent HSCs. Three genes (Dusp2, Fyb and Clasp1), which were not differently regulated in gene array analysis of HSCs from G3mTerc−/− compared to mTerc+/+, were chosen as negative control (right bars in Fig. 2A). Values are shown as mean ± SEM. * represents p<0.05. (B, C) The histograms show the percentage of cell nuclei staining positive for the indicated numbers of γH2AX foci in (B) quiescent HSCs, (C) cycling HSCs (100 nuclei were counted per cell type and group) of G3mTerc−/− mice and mTerc+/+ mice. D-E) 12-
9

month-old G3mTerc<sup>−/+</sup> mice were treated with Interferon-α to stimulate cell cycle activity (Suppl. Fig. 1A, B). Interferon-α (10,000 Units per mouse) or PBS were injected into 12-month-old G3mTerc<sup>−/+</sup> intraperitoneally. Freshly isolated bone marrow cells were analyzed 16 hours after stimulation. (D) mRNA expression of Puma in cycling HSCs (black bars) compared to quiescent HSCs (grey bars). Data are shown as mean ± SEM (n=3 for each group). E) Rate of apoptosis (Annexin V-positive cells) in quiescent HSCs and activated HSCs of G3mTerc<sup>−/+</sup> mice treated with either PBS or Interferon-α. Data are shown as mean ± SEM (n= 3 mice per group). F) This histogram shows the relative expression of p16 in quiescent HSCs from 12-month-old G3mTerc<sup>−/+</sup> and age-matched mTerc<sup>+/+</sup> mice treated with PBS or Interferon-α. Data are shown as mean ± SEM (n= 3 mice per group). G) The histogram shows the percentage of cell nuclei staining of γH2AX foci for quiescent HSCs from G3mTerc<sup>−/+</sup> mice treated with either PBS (grey bar) or Interferon-α (black bar) (100 nuclei were counted per group). H, I) The pie charts depict the composite of clones generated from freshly isolated single HSCs from 12-month-old G3mTerc<sup>−/+</sup> (332) and age-matched mTerc<sup>+/+</sup> mice (310). Cell numbers were counted on (H) day 6 and (I) day 12 after plating. HSCs were cultured individually in stem cell medium (Stem Cell Technology) with mSCF (30ng/ml) and TPO (20ng/ml).
Figure 1:

A) Number of genes
- Repressed in G3mTerc−/−
- Increased in G3mTerc−/−

B) Percentage of cells scored
- Number of gH2AX foci per cell
- p < 0.001

C) Percentage of cells scored
- Number of gH2AX foci per cell
- n.s.

D) Percentage of cells scored
- Number of gH2AX foci per cell
- n.s.

E) Percentage of cells scored
- Number of gH2AX foci per cell
- p < 0.001

F) Percentage of cells scored
- Number of gH2AX foci per cell
- n.s.

G) Percentage of cells scored
- Number of gH2AX foci per cell
- n.s.

H) Immunofluorescence images
- HSC
- G3mTerc−/−
- mTerc+/+

For B, C, D, E, F, G:
- mTerc+/+
- G3mTerc−/−
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