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14. ABSTRACT
Recent work over the past year from our laboratory has forged an intimate link between a common age-associated hematopoietic disorder, MPD, and telomere dysfunction in aging telomere dysfunctional mTerc null mice. This finding is particularly notable because one might expect telomere dysfunctional mice to exhibit complete bone marrow failure, rather than the selective expansion and loss of particular hematopoietic lineages. Over the past year we have solidified our initial observations, which include an age- and telomere dysfunction associated peripheral blood red cell anemia, neutrophilia, and thrombocythemia. These alterations were accompanied by substantial increases in plasma erythropoietin and several inflammatory cytokines, notably IL-6. These alterations in peripheral blood were accompanied by substantial changes elsewhere in the hematopoietic system. In the spleen we noted extensive extramedullary hematopoiesis and splenomegaly, and subsequent loss of lymphoid follicles and replacement by granulocytic lineages. Finally, in the bone marrow, we noted hypercellular bone marrows primarily made up of developing granulocytic lineages with a corresponding loss of developing erythroid and lymphoid lineages. In total, these phenotypes are reminiscent of myeloproliferative disorders (MPDs) that increase in incidence in elderly humans, and thus telomere dysfunctional mTerc null mice may represent a good model system to understand these age-related pathologies. We have initiated quantification and purification of hematopoietic stem cells (HSC) from these mutant mice and also initiated short term transplantation experiments.

15. SUBJECT TERMS
MPD, essential thrombocytosis, telomere and telomerase

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Introduction:

The molecular mechanisms and genetic pathways involved in the pathogenesis of myeloproliferative disease (MPDs) are not well understood. Telomere maintenance and the cellular responses to telomere dysfunction have been proposed to play crucial roles in the processes of genomic stability, aging, organ homeostasis and tumorigenesis. Our recent observation linking telomere dysfunction to an MPF like phenotype with thrombocytosis in the telomerase mutant mouse model provides an unique genetic platform to explore the molecular mechanisms by which telomere dysfunction contributes to the pathogenesis of MPD and essential thrombocytosis (ET). We hypothesize that mice engineered to have critically short telomeres will develop genomic instability which predispose these mice to the development of MPD/ET. Detailed on going *in vitro* and *in vivo* analyses of the hematopoietic compartment from these mutant mouse cohorts as a function of age and generation will further elucidate the genetic pathways that are involved in the pathogenesis of MPDs/ET.

Body:

Task 1: Identification of the cellular basis of MPD in aged telomere dysfunctional mice.

Telomere analyses of bone marrow derived from telomere dysfunction mice with MPD (Maximum 25 mice) (Months 1-12). We will utilize modified telomere fluorescent probe hybridization and FACS (FLOW-FISH) on sorted bone marrow cells from the different mutant mice and quantitative fluorescent telomere probe hybridization to metaphase chromosomes derived from sorted bone marrow culture of various mutant mice to determine the telomere lengths.

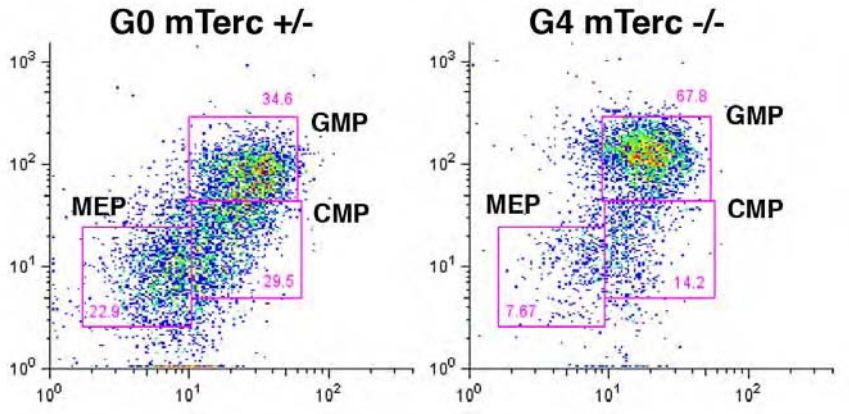
For the past year, we have been optimizing the conditions for FLOW-FISH in the hematopoietic cells and are anticipating the technique will be ready for analyses of our experimental cohort in the coming year.

Hematopoietic stem cell (HSC) analysis of aged telomere dysfunctional mice. (Maximum 25 mice) (Months1-12). We will ascertain the combined impact of age and telomere erosion on the population of HSC, and other committed progenitors including CMP (common myeloid progenitor), GMP (granulocyte monocyte progenitor), and MEP (megakaryocyte erythroid progenitor) populations by determining the percentage of these cells in the bone marrow of the different cohorts of telomerase mutant mice using high speed five color flow cytometric analysis.

We have begun to determine the cellular and molecular basis for this dramatic alteration of hematopoiesis in aging telomere dysfunctional mice. First, we have used 5 color, 4-way sorting to quantify and purify hematopoietic stem cells (HSCs), as well as various progenitor populations such as granulocyte-macrophage (GMP), common myeloid (CMP), and megakaryocyte-erythroid progenitor (MEP) populations. Consistent with the increase in maturing granulocyte lineages in whole bone marrow, the percentage

of GMPs is also increased relative to the CMP and MEP populations in telomere dysfunctional mice compared to wildtype mice (figure 1). We are currently assessing the RNA expression profile of these purified progenitor populations to ascertain whether master regulatory hematopoietic transcription networks are perturbed by telomere dysfunction.

Fig. 1. **Ratio of GMPs is increased in telomere dysfunctional G4 null mice.**



Growth factor independent clonogenic growth of bone marrow cells from aged telomere dysfunctional mice (Maximum 15 mice) (Months 1-12). We will test the ability of progenitor cells from aged telomere dysfunctional mice to grow in the absence of exogenously added growth factors using methycellulose-containing media without exogenous cytokines and scoring for colony forming ability.

Preliminary experiments performed suggested that hematopoietic progenitors isolated from the aged telomere dysfunctional mice have impaired ability to from colonies in the absence of exogenously added growth factors. We are in the process of optimizing the conditions and formally quantify our results.

In vitro characterization of HSC progenitor populations from aged telomere dysfunctional mice (Maximum 25 mice) (Months 1-12). We will determine the effect of telomere dysfunction in the bone marrow cell populations of aged telomerase deficient mice using cell culture-based colony forming assays, long-term colony initiating cell assays, and serial replating assays.

We will begin these experiments in the very near future as we can now purify and isolate these hematopoietic progenitors (as shown above).

Cytokine production by telomere dysfunctional bone marrow derived cells (Maximum 25 mice) (Months1-12). We will test whether the cytokine production is intrinsic to the MPD cells in aged telomere dysfunctional mice using the Luminex xMAP technology (bead-bound sandwich ELISA; Luminex, Austin, TX), allowing simultaneous detection of up to 20 cytokines from each sample

Over the past year we have solidified our initial observations, which include an age- and telomere dysfunction associated peripheral blood red cell anemia, neutrophilia, and thrombocythemia (Fig. 5). These alterations were accompanied by substantial increases in plasma erythropoietin and several inflammatory cytokines, notably IL-6.

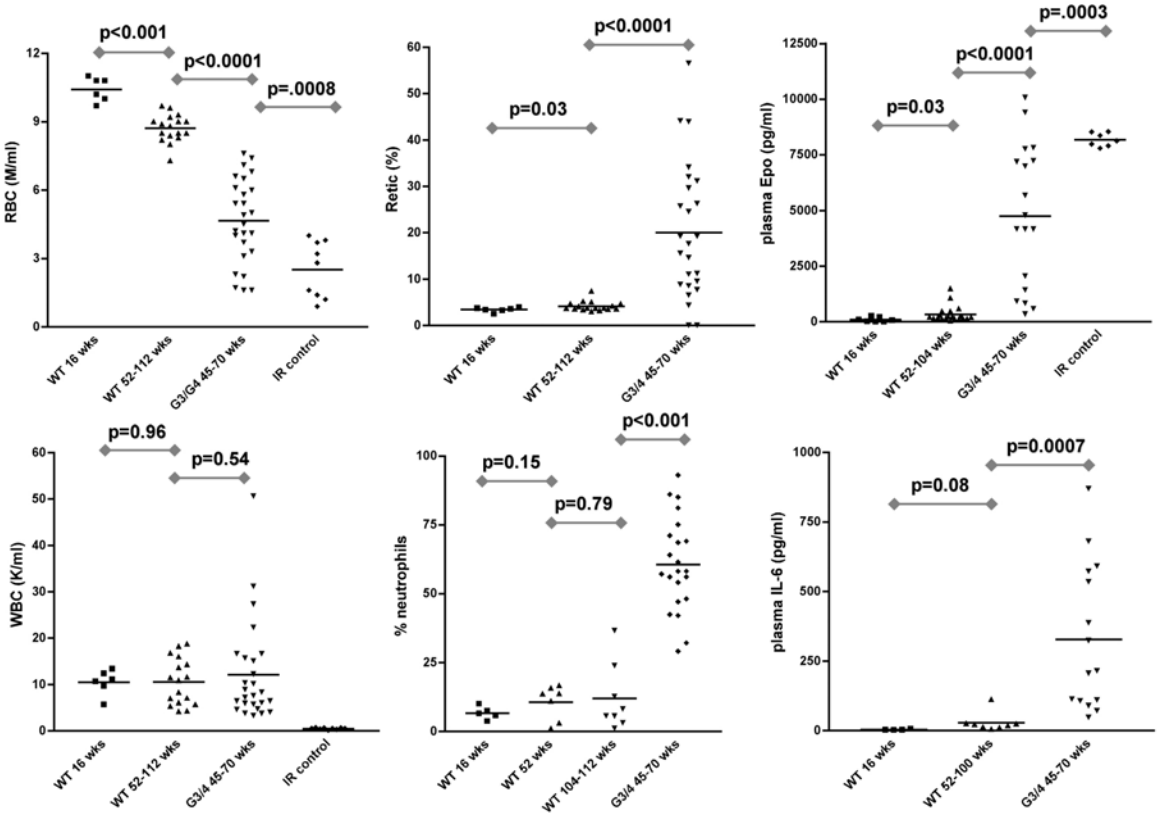


Fig. 2. **Altered CBC and cytokine values in aging telomere dysfunctional (G3/G4) mice.**

These alterations in peripheral blood were accompanied by substantial changes elsewhere in the hematopoietic system. In the spleen we noted extensive extramedullary hematopoiesis and splenomegaly, and subsequent loss of lymphoid follicles and replacement by granulocytic lineages. Finally, in the bone marrow, we noted hypercellular bone marrows primarily made up of developing granulocytic lineages with a corresponding loss of developing erythroid and lymphoid lineages. In total, these phenotypes are reminiscent of myeloproliferative disorders (MPDs) that increase in incidence in elderly humans, and thus telomere dysfunctional *mTerc* null mice may represent a good model system to understand these age-related pathologies.

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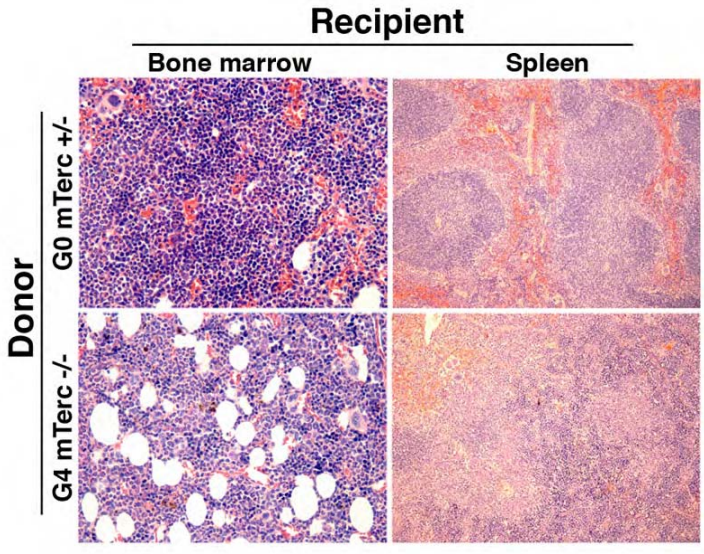
In vivo characteristics of Bone Marrow Compartment of aged telomere dysfunctional mice with MPD

Transplantability of the MPD phenotype from bone marrow cells of telomerase null mice with telomere dysfunction. (Maximum 40mice) (Months 6-18). To test whether the MPD phenotype of the telomerase null mice with telomere dysfunction can be transplanted into recipient mice, we will perform adaptive transfer experiments.

To help determine the cellular autonomous nature of the MPD phenotype in aging telomere dysfunctional mice, we have also performed short-term transplantation experiments of donor-derived MPD bone marrow from telomerase dysfunctional mice into irradiated wildtype recipient mice. Consistent with the expansion of GMP populations in these donor bone marrows, recipient mice that received telomere dysfunctional whole bone marrow cells showed bone marrow and spleen that was predominated by donor-derived developing granulocytes, whereas normal proportions of hematopoietic lineages were observed in recipients that received wild-type bone marrow. Recipient mice exhibited anemia and thrombocytopenia, most likely due to the lack of those progenitor cells in the transferred bone marrow.

***Fig. 3*. Histology of bone marrow and spleen from lethally-irradiated wildtype recipients of either G0 *mTerc* +/- or G4 *mTerc* -/- whole bone marrow transplant.**



Rescue of the MPD phenotype in telomerase null mice with telomere dysfunction with HSCs from wild type mice. (Maximum 40 mice) (Months 6-18). Either dysfunctional HSCs or/and altered bone marrow stromal environment may cause the MPD phenotype seen in the telomerase null mice with telomere dysfunction. To determine the contribution of the bone marrow stromal environment on the MPD phenotype, we will determine whether HSC from wild type mice can rescue the MPD in the telomerase null mice.

Now that we have optimized out protocol to isolate and purify HSC from our mutant mouse, we will begin these experiments shortly.

Task 2: Identification of the molecular forces driving the MPD phenotype in aged telomere dysfunctional mice.

·Conventional cytogenetics and BCR-ABL analysis. (Monthss 6-18). To differentiate the MPD phenotype in aged telomere dysfunctional mice, and to correlate the phenotype to human diseases associated with MPD, we will determine whether the MPD with neutrophilia is a form of chronic myeloid leukemia related to BCR-ABL rearrangement. Thus, we will perform fluorescent in situ hybridization (FISH) and Northern analyses to determine if telomere dysfunctional mice harbor BCR-ABL rearrangements in bone marrow precursor and maturing granulocyte lineages.

FISH and Northern analyses on the hematopoietic bone marrow precursor cells derived from the aged telomere dysfunctional mice showed that these cells do not harbor the BCR-ABL rearrangements. This demonstrated that the MPD phenotype seen in our mutant mice is not caused by the BCR-ABL rearrangement and thus the mechanism for the emergence of this phenotype might be novel.

·Spectral karyotype (SKY) analysis of MPD metaphases. (Months 12-24). Given the relationship between genomic instability and progression to CML blast phase, as well as the known genetic rearrangements associated with hematological malignancies, we will perform SKY analyses to comprehensive assess the cytogenetic profile of the bone marrow cells from aged telomere dysfunctional mice

SKY analyses of the MPD metaphases demonstrated that these cells do not harbor any gross clonal chromosomal rearrangements. However, this does not preclude the existence of focal rearrangements that cannot be detected by SKY.

Array-based whole genome analyses. (Months 18-24). Because genomic instability and bridge-fusion-breakage cycles are often associated with regional amplifications and deletions of genomic loci, we will employ oligonucleotide array comparative genomic hybridization (CGH) to determine MPD-specific genetic loci in telomere dysfunctional mice.

To be conducted in year 2

Kinase cascade analysis. (Months 18-24). A large body of evidence demonstrates the importance of tyrosine kinase mutations to the development of myeloproliferative disorders, particularly CML. Clues regarding the relative importance of various kinase pathways can be gleaned from examining the activation state of various molecular players in the kinase cascades. Thus, as a corollary analysis to the DNA- and RNA-based analyses described above, we will undertake an examination of phosphorylation events that occur in mice with MPD using the Luminex xMAP bead-bound sandwich immunoassay (Bio-Rad Laboratories, Hercules, CA).

To be conducted in year 2.

Task 3: The impact of ionizing radiation, chronic replicative stress, and other genetic alterations on the progression of the MPD phenotype in aged telomere dysfunctional mice.

The experiments described below will be conducted in year 2.

·In vivo chronic stress on the hematopoietic system of the telomerase null mice with telomere dysfunction with 5-FU. (Maximum 40 mice) (Months 12-24) We hypothesize that chronic stress on the hematopoietic compartment to expand in the aged telomere dysfunctional mice will further fuel the genomic instability generated from progressive telomere dysfunction in the stem/progenitor cells and cause either its depletion or transformation. We will treat different cohorts of telomerase mutant mice with 5FU at (50mg/kg body weight) once every 3 weeks for a total of 5 cycles and subsequently analyze their bone marrows.

·In vivo genotoxic stress on the hematopoietic compartment of the telomerase null mice with telomere dysfunction with sublethal dose of ionizing radiation. (Maximum 40 mice) (Months 12-24). Our previous studies have shown that telomerase null mice are hypersensitive to ionizing radiation and that telomerase null cells with telomere dysfunction are impaired in their ability to repair double strand DNA breaks. Exposure to ionizing radiation will generate greater genomic instability leading to greater mutational rate and stem/progenitor cell depletion. A transformed clone might emerge from the natural selection process. To test this hypothesis, we will expose various cohorts of telomerase mutant mice to 3Gy of sub-lethal ionizing radiation and then monitor them for development of worsening phenotypes.

Cooperativity of mutations in transcriptional factors and activated tyrosine kinases known to be relevant in AML on the progression of disease in the telomerase null mice. (Maximum 100 mice) (Months 12-24). To investigate whether there is cooperativity between telomere dysfunction and known mutant proteins involved in the process of MPD or leukemia, we will retrovirally introduce these mutant proteins into the bone marrow cells of the aged telomere dysfunctional mice and transplant them into recipient mice. These recipient mice will then be closely monitored for worsening of the MPD phenotype.

Key Research Accomplishments:

Over the past year we have solidified our initial observations, which include an age- and telomere dysfunction associated peripheral blood red cell anemia, neutrophilia, and thrombocythemia. These alterations were accompanied by substantial increases in plasma erythropoietin and several inflammatory cytokines, notably IL-6. We have also shown that many of the phenotypes seen are transplantable. Lastly, we showed that the phenotypes seen in our mutant mice are not due to the presence of BCR-ABL.

Reportable Outcomes: none

Conclusions: As the research and experiments are ongoing, we can not definitively draw any major conclusions presently. We anticipate the results from the second year of experiments will yield unique insights on the pathogenesis of MPD/ET.

References: none