Title: Telomerase gene therapy rescues telomere length, bone marrow aplasia and survival in mice with aplastic anemia

Running title: Aplastic anemia reversal by telomerase gene therapy

Scientific category: Hematopoiesis and stem cells

Authors: Christian Bär, Juan Manuel Povedano, Rosa Serrano, Carlos Benitez-Buelga, Miriam Popkes, Ivan Formentini, Maria Bobadilla, Fatima Bosch and Maria A. Blasco

Author Affiliation:
1 Telomeres and Telomerase Group, Molecular Oncology Program, Spanish National Cancer Centre (CNIO), Melchor Fernández Almagro 3, Madrid, E-28029, Spain.
2 Human Genetics Group, Spanish National Cancer Research Centre (CNIO), Melchor Fernández Almagro 3, Madrid 28029, Spain.
3 Roche Pharma Research and Early Development (pRED), Neuroscience, Ophthalmology and Rare Disease, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd, Grenzacherstrasse 124, 4070 Basel, Switzerland.
4 Roche Partnering, EIN, F. Hoffmann-La Roche Ltd, Grenzacherstrasse 124, 4070 Basel, Switzerland.
5 Centre of Animal Biotechnology and Gene Therapy (CBATEG), and Department of Biochemistry and molecular Biology, School of Veterinary Medicine. Universitat Autònoma de Barcelona, Bellaterra, E-08193, Spain.

† Current address: Institute of Molecular and Translational Therapeutic Strategies, Hannover Medical School, Carl-Neuberg-Str. 1, 30635 Hannover, Germany

* Correspondence: Maria A. Blasco
Tel.: +34.91.732.8031
Fax: +34.91.732.8028
Email: mblasco@cnio.es

Abstract length: 208 words
Article length: 3997 words
Number of figures: 5
Number of references: 42
Number of tables: 0
Keywords: Telomerase, telomeres, aplastic anemia, gene therapy, AAV9
Key points

- Telomerase gene therapy in a mouse model of aplastic anemia targets the bone marrow and provides increased and stable telomerase expression.
- Telomerase expression leads to telomere elongation and subsequently to the reversal of aplastic anemia phenotypes.
Abstract

Aplastic anemia is a fatal bone marrow disorder characterized by peripheral pancytopenia and marrow hypoplasia. The disease can be hereditary or acquired and develops at any stage of life. A subgroup of the inherited form is caused by replicative impairment of hematopoietic stem and progenitor cells owing to very short telomeres due to mutations in telomerase and other telomere components. Abnormal telomere shortening is also described in cases of acquired aplastic anemia, most likely secondary to increased turnover of bone marrow stem and progenitor cells. Here, we test the therapeutic efficacy of telomerase activation by using AAV9 gene therapy vectors carrying the telomerase Tert gene in two independent mouse models of aplastic anemia owing to short telomeres (Trf1 and Tert-deficient mice). We find that a high dose of AAV9-Tert targets the bone marrow compartment including hematopoietic stem cells. AAV9-Tert treatment following telomere attrition in bone marrow cells rescues aplastic anemia and mouse survival compared with mice treated with the empty vector (AAV9-empty). Improved survival is associated with a significant increase in telomere length in peripheral blood and bone marrow cells as well as improved blood counts. These findings indicate that telomerase gene therapy represents a novel therapeutic strategy to treat aplastic anemia provoked or associated with short telomeres.
Introduction

Aplastic anemia is a potentially life-threatening, rare and heterogeneous disorder of the blood in which the bone marrow cannot produce sufficient new blood cells due to a marked reduction of immature hematopoietic stem and progenitor cells (HSPC)\(^1,2\). The main disease manifestations are pancytopenia and marrow hypoplasia, which can emerge at any stage of life but are more frequent in young individuals (age 10-25 years) and in the elderly (>60 years)\(^3\). Aplastic anemia can be acquired or inherited. The acquired type is often of idiopathic origin, involves autoimmune processes but can also be triggered by environmental factors such as exposure to radiation, toxins, and viral infections\(^4\). The congenital form is rarer and mutations in more than 30 genes involved in DNA repair, ribosome biogenesis and telomere maintenance pathways have been identified to date\(^5\). A frequently observed clinical feature of aplastic anemia is the presence of short telomeres in subpopulations of peripheral blood cells (in particular neutrophiles and less prominent in lymphocytes)\(^6\), even in the absence of mutations in the telomere maintenance machinery.

Telomeres, the termini of vertebrate chromosomes, are specialized nucleoprotein structures composed of tandem repeat sequences (TTAGGG in vertebrates) bound by a six protein complex (TRF1, TRF2, TIN2, RAP1, TPP1 and POT1) known as shelterin\(^7,8\). Telomeres are essential for chromosome integrity by preventing telomere fusions and telomere fragility. Telomere length is controlled by the ribonucleoprotein enzyme telomerase, which can add telomeric sequences onto telomeres \textit{de novo}. Because telomeres shorten associated with every cell division (a phenomenon known as “the end
replication problem”) and somatic cells do not express sufficient telomerase to compensate this, telomeres shorten throughout life. When telomeres reach a critically short length, their protective function is impaired, eliciting a persistent DNA damage response at chromosome ends which leads to cellular senescence or cell death\textsuperscript{9,10}. Hematopoietic stem cells, in contrast to most somatic cells, can activate telomerase, however, this is insufficient to prevent telomere attrition with aging, thus eventually leading to loss of the regeneration potential of hematopoietic stem cells\textsuperscript{11}. In line with this, recipients of bone marrow transplants have shorter telomeres than their donors, suggesting that telomerase cannot fully compensate for the increased cell proliferation that occurs during the engraftment phase of the transplanted bone marrow\textsuperscript{12}. Telomeres also show an accelerated rate of shortening in patients with aplastic anemia compared to healthy individuals, most likely owing to a higher than normal number of cell divisions in the aplastic anemia cases\textsuperscript{13}.

Accelerated telomere shortening due to defects in telomerase or other telomere maintenance genes prematurely limits the proliferation potential of cells, including stem cells, leading to decreased tissue renewal capacity\textsuperscript{9,14}. Highly proliferative tissues such as the hematopoietic system are particularly vulnerable to defects in telomere maintenance genes, leading to severe disorders such as aplastic anemia\textsuperscript{15}. As an example, the telomeropathy Dyskeratosis congenita (DKC) has been linked to mutations in 11 genes, which encode components of the telomerase complex (\textit{TERT, TERC, DKC1, NOP10, NHP2}) or of the telomere capping complex shelterin (\textit{TIN2}). Other genes altered in DKC encode for accessory proteins important for telomerase assembly and trafficking (CTC1, ACD7 (alias TPP1) and \textit{TCAB1}) or for telomere
replication (\textit{RTEL1})\textsuperscript{16}. Mutated \textit{PARN} was also recently linked to reduced mRNA levels of several key genes in telomere maintenance\textsuperscript{17}. In all these cases, DKC is characterized by very short telomeres. DKC is a multisystem syndrome comprising diverse clinical features such as nail dystrophy, oral leucoplakia, abnormal skin pigmentation and cerebellar hypoplasia\textsuperscript{16}. The most severe complication, however, is the development of aplastic anemia in 80\% of the cases, underlining that the clinical features are caused by excessive telomere shortening which eventually leads to the exhaustion of the stem cell reserve\textsuperscript{5}.

These findings suggest that telomerase activation could be a good therapeutic strategy to treat those forms of aplastic anemia associated with a limited blood forming capacity owing to the presence of very short telomeres. We have previously developed a telomerase (\textit{Tert}) gene therapy using adeno-associated virus (AAV9) vectors\textsuperscript{18}, which attenuated or reverted aging-associated telomere erosion in peripheral blood mononuclear cells (PBMCs)\textsuperscript{18}.

To test the efficacy of this strategy in the treatment of aplastic anemia, we first used a mouse model of aplastic anemia generated by us in which we depleted the TRF1 shelterin protein specifically in the bone marrow, leading to a bone marrow phenotype that recapitulates the main pathological findings of human aplastic anemia patients, including extreme telomere shortening\textsuperscript{19,20}. In particular, partial depletion of the \textit{Trf1} gene specifically in bone marrow causes severe telomere uncapping and provokes a persistent DNA damage response at telomeres, which in turn leads to a fast clearance of those HSPCs deficient for \textit{Trf1}. In this context, the remaining HSPCs that did not delete the \textit{Trf1} gene undergo additional rounds of compensatory proliferation to
regenerate the bone marrow, leading to very rapid telomere attrition. Thus, partial
depletion of the bone marrow stem cell and progenitor compartments by Trf1 deletion
recapitulates the compensatory hyperproliferation and short telomere phenotype
observed after bone marrow transplantation or in other acquired forms of aplastic
anemia, as well as in patients owing to mutations in telomere maintenance genes.
Interestingly, this mouse model allows adjustment of the rate of telomere shortening by
modulating the frequency of Trf1 deletion-mediated HSPC depletion, thus controlling the
onset of bone marrow aplasia and pancytopenia.19,20

As an additional model to mimic the presence of very short telomeres specifically
in the bone marrow, we transplanted irradiated wild-type mice with bone marrow from
late (third) generation (G3) telomerase-deficient Tert knock-out mice, which have short
telomeres owing to telomerase deficiency during three mouse generations.

Here, we tested telomerase activation by using gene therapy AAV vectors in both
mouse models of aplastic anemia produced by short telomeres. Our results show that
telomerase treatment is sufficient to attenuate telomere attrition with time as well as
HSPC depletion, thus significantly preventing death by bone marrow failure.
Material and Methods

Study approval

All experimental procedures with mice (Mus musculus) were approved by the CNIO–Instituto de Salud Carlos III (CNIO-ISCIII) Ethics Committee for Research and Animal Welfare (CEIyBA). Mice were treated in accordance to the Spanish laws and the FELASA guidelines. Approval file number: CBA PA 87_2012.

Mice and animal procedures

Mice of pure C57/BL6 background were produced and housed at the SPF animal house of the CNIO in Madrid, Spain. Trf1lox/lox Mx1-Cre and Trf1lox/lox Mx1-wt mice were generated as described21. First generation (G1) Tert−/− mice were generated by intercrossing Tert+/−. G3 Tert−/− mice were obtained by intercrossing G1 (to give G2) and subsequently intercrossing G2 mice 22. Ten weeks old Trf1lox/lox Mx1-Cre or G3 Tert−/− mice were used as bone marrow donors for transplantation into 8 weeks old lethally (12Gy) irradiated wild-type mice as described19,23. Two million cells were transplanted via tail vein injection at a donor:recipient ratio of 1:8 and mice were left for 30 days to allow bone marrow reconstitution. To induce Cre expression, mice were intraperitoneally injected with polyinosinic-polycytidylic acid (pI:pC; Sigma-Aldrich) (15 ug/g body weight) 3 times per week for a total of 5 weeks. After 1 week mice were treated with AAV9-Tert or AAV9-empty vectors. Vectors were administered via tail vein injection at a concentration of 3.5x10E12 viral genomes per mouse.

Gene therapy vector production
Viral vectors were generated and purified as described. Briefly, vectors were produced through triple transfection of HEK293T. Expression cassettes were under the control of the cytomegalovirus (CMV) promoter and contained a SV40 polyA signal for EGFP and the CMV promoter and the 3’UTR of the Tert gene as polyA signal for Tert. AAV9 particles were purified using two caesium chloride gradients, dialysed against PBS and filtered. Viral genomes particles titres were determined by a quantitative real time PCR method.

Histology

Bone marrow samples (sternum or tibia bone) were fixed in 4% PFA and paraffin embedded after decalcification. 5µm tissue sections were stained with Hematoxylin-Eosin. Immunohistochemistry was performed on deparaffinized sections. After antigen retrieval, samples stained with anti-EGFP (rabbit anti-EGFP, 1:200; Abcam, ab290). EGFP positive cells were counted in a semi-automated way using ImageJ software.

FACS sorting

For sorting of HSPCs, whole bone marrow cells were extracted from the long bones (femur & tibia) as described. Erythrocytes were lysed for 10min in 10ml erythrocyte lysis buffer (Roche), washed once with 10ml PBS, and resuspended in FACS buffer (PBS, 2mM EDTA, 0,3% BSA) containing Fc-block (1:400) at a concentration of 5-10x10^6 cell/100µl. Cells were incubated for 10min and washed once in FACS buffer. Cells were then resuspended in FACS buffer at 20-25x10^6 cell/ml and the antibody cocktail was added as follows: Anti–sca-1–PerCP-Cy5.5 (1:200), lin
cocktail-eFluor450 (1:50) (all eBioscience), and anti–c-kit–APC-H7 (1:100) (BD Pharmingen). Cells were incubated for 30 min. After washing cells twice with PBS, 2μL of DAPI (200g/mL) was added and cells were sorted in a FACS ARIA IIu (Becton Dickinson, San Jose, CA) into HSPCs (lin negative, sca1 and c-kit positive) and lineage positive fractions.

**Colony forming assay**

Short-term colony-forming assay (CFA) was performed by plating 1x10⁴ and 2x10⁴ freshly isolated mononucleated bone marrow cells in 35-mm dishes containing MethoCult media (both StemCell Technologies) following the manufacturer’s protocol. All experiments were performed in duplicates and the number of colonies counted after 12 days incubation at 37°C.

**Blood counts**

Peripheral blood was drawn from the facial vein (~50μl) and collected into anticoagulation tubes (EDTA). Blood counts were determined using an Abacus Junior Vet veterinary hematology analyzer.

**Quantitative real-time PCR and Western blots**

Total RNA from whole bone marrow extracts or FACS sorted bone marrow cells was isolated using Qiagen’s RNeasy mini kit according to the manufacturer. Quantitative real-time PCR was performed using an ABI PRISM 7700 or QuantStudio 6 Flex (both Applied Biosystems). Primers sequences for Tert and reference genes Act1
and TBP are as follows: Tert-Forward 5'GGATTGCCACTGGCTCCG; Tert-Reverse 5'TGCCTGACCTCCTCTTGTGAC; Actin-Forward 5'GGCACCACACCTTCTACAATG; Actin-Reverse 5'GTGGTGGTGAAGCTGTAG; TBP-Forward 5'CTTCCTGCCACAATGTCACAG; TBP-Reverse 5'CCTTTCTCATGCTTGCTTCTTG.

QFISH telomere analysis

QFISH on paraffin-embedded tissue sections was performed as described. Confocal images were acquired as stacks every 0.5 μm for a total of 1.5 μm using a Leica SP5-MP confocal microscope and maximum projections were done with the LAS-AF software. Telomere signal intensity was quantified in at least 6 images per mouse using Definiens software with a specific script allowing for individual spot background correction.

HT-QFISH on peripheral blood leukocytes was done using 120-150 μl of blood as described. Confocal images were captured using the OPERA (Perkin Elmer) High-Content Screening system. TL values were analysed using individual telomere spots (>10,000 telomere spots per sample). The average fluorescence intensities of each sample were converted into kilobase using L5178-R and L5178-S cells as calibration standards, which have stable TLs of 79.7 and 10.2 kb, respectively. Samples were analysed in duplicate.

Real time PCR-based measurement of relative telomere length was done on genomic DNA isolated from whole bone marrow samples following a protocol previously described.
Results

AAV9-Tert targets bone marrow and hematopoietic stem cells

First, we tested the ability of AAV9 vectors to transduce bone marrow cells upon intravenous mouse injection. In particular, to determine the location and percentage of transduced cells, we first treated wild-type mice with an AAV9-EGFP reporter virus (3.5E12 viral genomes per mouse) via tail vein injections. We found that 2% of the BM cells were positive for EGFP upon immunohistochemistry with anti-EGFP antibodies in middle bone sections and this increased to 10% in bone regions adjacent to the joints, which showed the highest AAV9-transduction (Fig. 1A,B). We then injected wild-type mice with the same amount of AAV9-Tert particles and determined Tert mRNA expression by qRT-PCR in whole bone marrow isolates at two weeks and 8 months after virus injection. Two weeks post-treatment with the AAV9 vectors, Tert mRNA expression was significantly increased in the AAV9-Tert treated mice compared to those treated with the AAV9-empty vector and this increased expression was maintained up to 8 months after the initial treatment (Fig. 1C-E). In agreement with the known tropism of the AAV9 serotype, we found a stronger induction of Tert in organs such as heart and liver, which are preferential AAV9 targets (Supplementary Fig. 1A-C). We then studied Tert mRNA expression specifically in the blood-forming compartments of the bone marrow. To this end, we performed FACS sorting of c-kit and Sca-1 positive HSPCs and lin+ lineage committed cells. We found a significant increase in Tert mRNA in both HSPCs (10-fold) and lineage committed bone marrow cells (3.5-fold) in AAV9-Tert treated mice compared to mice treated with the empty vector (Fig. 1F,G),
demonstrating that bone marrow cells including HSPCs are targeted by *Tert* gene therapy. Of note, the higher expression in whole bone marrow compared with isolated hematopoietic (HPSC and lin⁺) cells could suggests that additional bone marrow cells corresponding to the stroma, i.e. adipocytes, maybe also infected. In this regard, we previously demonstrated that adipocytes are efficiently targeted by AAV9\(^{33}\). Moreover, the relative lower fold changes in *Tert* in HSPCs compared to total bone marrow may also be due to higher level of endogenous *Tert* in HPSC compared to whole bone marrow. As control, AAV9-*Tert* treatment of wild-type mice did neither affect the relative numbers of lineage positive or negative cells, nor the proportion of HSPCs (Supplementary Fig. 2). Given the increased *Tert* expression in HSPCs, we next addressed whether this affected their proliferation/colony forming potential. To this end, we performed a colony forming cell assay (MethoCult). We observed a significantly increased number of colonies in the bone marrow from AAV9-*Tert* treated mice compared to those treated with the empty vector (Fig. 1H).

In summary, intravenous injection of AAV9 vectors administered at a high dose can target *Tert* to hematopoietic cells, including HSPCs.

**AAV9-*Tert* treatment rescues survival in a mouse model of aplastic anemia**

We next tested whether treatment with AAV9-*Tert* was effective in increasing survival upon induction of lethal aplastic anemia owing to critically short telomeres. First, we used the conditional *Trf1* mouse model recently developed by us, *Trf1*\(^{lox/lox}\) *Mx1-Cre* mice, in which we induce partial *Trf1* deletion specifically in the bone marrow\(^{19}\). To this end, we transplanted lethally irradiated wild-type mice with bone marrow isolated from
Trf1\textsuperscript{lox/lox} Mx1-Cre mice followed by administration of pl:pC to induce the expression of Cre recombinase and Trf1 deletion\textsuperscript{19,20}. Genotyping confirmed that the new bone marrow solely consists of donor cells with excisable Trf1 (Supplementary Fig. 3). Thus, Trf1\textsuperscript{lox/lox} Mx1-Cre mice allow to study the effects of Trf1 depletion exclusively in the bone marrow. We previously showed that partial Trf1 deletion in the bone marrow results in rapid death and removal of the Trf1 deleted cells, while cells that fail to delete Trf1 undergo compensatory rounds of cell division, leading to rapid telomere shortening and replicative senescence, eventually resulting in bone marrow failure\textsuperscript{19,20}.

Here, we induced Trf1 deletion with pl:pC injections at a frequency of 3 times per week for 5 weeks, at which point these mice started to show signs of aplastic anemia\textsuperscript{19,20}. One week after the last pl:pC injection, mice were treated with either AAV9-\textit{Tert} or AAV9-empty vectors (Fig. 2A). Mouse survival was monitored for 100 days following treatment with the AAV9 vectors (Note: beyond 100 days after virus treatment none of the mouse cohorts developed signs of aplastic anemia. At 120 days post virus administration all mice were sacrificed for further analyses). Strikingly, AAV9-\textit{Tert} treatment significantly increased survival, as we found 87% of mice still alive at 100 days after virus administration in the AAV9-\textit{Tert} treated group compared to only 55% of mice alive in the empty vector-treated group (Fig. 2B). In particular, while only 4 out of 31 mice treated with AAV9-\textit{Tert} developed aplastic anemia (which represents 13% of the mice), 16 out of 36 mice (44%) died with clear signs of aplastic anemia in the group treated with the empty vector (Fig. 2C). In both groups, aplastic anemia was determined as the cause of death in those mice that showed a drastic drop in platelet counts and in haemoglobin levels at the time of death (Fig. 2D,E), and that presented with severe
bone marrow hypo- and aplasia after post-mortem histopathologic analysis of bone marrow sections (Fig. 2F). Interestingly, among those mice that died from aplastic anemia, we observed a tendency to show a milder bone marrow aplasia phenotype in the AAV9-\textit{Tert} treated group compared with the AAV9-empty treated group as indicated by higher bone marrow cellularity in the AAV9-\textit{Tert} treated group (Fig. 2F). Quantification of the bone marrow cellularity confirmed a drastic decrease of cellularity in aplastic anemia mice compared to the wild-type control mice. Importantly, the decrease in cellularity was significantly attenuated in the AAV9-\textit{Tert} treated cohort compared with the AAV9-empty treated group (Fig. 2G,H). Our results suggest that AAV9-\textit{Tert} treatment of mice with induced severe telomere shortening significantly reduces the mortality of these mice by aplastic anemia.

\textbf{Telomerase treatment reverses telomere shortening in peripheral blood and bone marrow cells in a mouse model of aplastic anemia}

Because aplastic anemia in our mouse model is caused by extreme telomere shortening\textsuperscript{19,20}, we next compared the dynamics of telomere length in mice treated with AAV9-\textit{Tert} versus mice treated with the empty vector. To this end, we performed a longitudinal study to follow telomere length in peripheral blood (PBMCs) over time using the high-throughput (HT) telomere QFISH technology\textsuperscript{34}. To do so, we extracted blood at 4 different time points: 30 days after bone marrow engraftment (1), 5 weeks after pl:pC treatment (2), as well as 2 months (3) and 4 months (4) after treatment with the AAV9 vectors (Note: longitudinal telomere measurements were done on PBMCs from mice which did not develop aplastic anemia). In agreement with previous findings, we found a
dramatic drop in telomere length of around 10 kb in all mice after induction of Trf1 deletion with pl:pC and prior to treatment with the gene therapy vectors (Fig. 3A; compare timepoints 1 and 2)\(^{19,20}\). We observed a further drop in telomere length in those mice treated with AAV9-empty vector, when comparing timepoint 4 with timepoint 2 in this mouse cohort (Fig. 3A,B). Importantly, during the same period of time, mice treated with AAV9-Tert showed a net increase in average telomere of 10kb when comparing timepoint 4 with timepoint 2 (Fig. 3A,B). Indeed, throughout the course of the experiment, AAV9-empty treated mice showed a total decrease in average telomere length of 12kb, while mice treated with AAV9-Tert showed re-elongation of telomeres to the similar telomere length as before the induction of Trf1 deletion by pl:pC treatment (Fig. 3C). These findings indicate that AAV9-Tert treatment is sufficient to stop and even revert initial telomere shortening. To further confirm whether telomeres were elongated as the consequence of AAV9-Tert gene therapy specifically in the bone marrow, we performed QFISH analysis on bone marrow cross-sections at the end point of the experiment. In agreement with longer telomeres in peripheral blood cells in the AAV9-Tert treated mice, we found that AAV9-Tert treated mice also had significantly longer telomeres in the bone marrow compared with mice treated with the empty vector (Fig. 3D-F). We confirmed the AAV9-Tert mediated telomere elongation on independent samples using a real time PCR assay for relative telomere length determination\(^{30,31}\) (Supplementary Fig. 4A). Furthermore, in line with our hypothesis that aplastic anemia is the consequence of drastically shortened telomeres, mice treated with AAV9-empty that developed aplastic anemia had significantly shorter telomeres than mice that were
treated in the same manner but did not develop aplastic anemia (Supplementary Fig. 4B).

Of note, telomere length analysis on bone marrow sections or bone marrow DNA does not allow to distinguish between the various different cell populations. However, the observed telomere elongation in PBMCs, suggests a direct effect of AAV9-Tert on HSPCs.

**Telomerase gene therapy of aplastic anemia produced by short telomeres owing to Tert deletion improves blood counts and increases telomere length**

To validate the therapeutic use of telomerase gene therapy in aplastic anemia provoked by short telomeres, we used an additional mouse model for modelling short telomere length in the hematopoietic system, in this case owing to telomerase deficiency during several mouse generations, the Tert-deficient mouse model. To this end, we irradiated wild-type mice and transplanted them with bone marrow from third generation (G3) Tert knock-out mice, which have short telomeres in all mouse tissues including the bone marrow (Fig. 4A). First, we confirmed shorter telomeres in the G1 and G3 Tert knock-out bone marrow donors compared to the wild-type bone marrow donors by performing HT-QFISH analysis on peripheral blood mononuclear cells. In particular, Tert deficiency leads to progressive telomere shortening with mice from the third generation (G3) having an average telomere length of ~25kb compared to ~40kb in the wild-type controls (Fig. 4B). One month after transplantation of irradiated wild-type mice with G3 Tert knock-out bone marrow to allow for bone marrow engraftment, mice were divided in two groups and then treated with either AAV9-Tert or AAV9-empty gene
therapy vectors (3.5x10E12 viral genomes/mouse) (Fig. 4A). After treatment, we monitored mice during a follow up period of five months and observed robust expression of Tert in the bone marrow in the AAV9-Tert treated group (Supplementary Fig. 5). Importantly, in response to AAV9-Tert treatment we observed an increase in survival compared to the AAV9-empty treated group, which almost reached statistical significance ($P=0.058$) (Fig. 4C). Upon mouse sacrifice, Tert treated mice had significantly increased haemoglobin levels and higher erythrocyte and platelets counts compared with mice treated with the empty vector (Fig. 4D-F). The same trend was observed for leukocyte counts, which again were higher in AAV9-Tert treated mice compared with the AAV9-empty group although the trend did not reach statistical significance ($P=0.09$) (Fig. 4G). Finally, to analyse the mechanism by which Tert gene therapy improved survival and blood counts in these mice, we followed longitudinally telomere length in peripheral blood mononuclear cells in both mouse cohorts. To this end, we extracted blood before and 3 and 5 months after mice were injected with the viruses and performed HT-QFISH analysis. In line with the results obtained with the Trf1lox/lox mouse model (see above), we found that AAV9-Tert treatment led to net increase in average telomere length with time of 5.18Kb, while during the same period mice treated with the AAV9-empty vector suffered a slight telomere shortening of -1.76Kb (Fig. 5A,B). These findings were also confirmed by telomere QFISH analysis on bone marrow sections at 5 months after virus administration. In particular, we found significantly longer telomeres in the bone marrow of Tert treated mice compared to mice treated with the empty vector (Fig. 5C,D).
In summary, these results indicate a single treatment with the AAV9-\textit{Tert} vector in mice with previously shortened telomeres in the bone marrow owing to telomerase deficiency, is sufficient to increase telomere length in the bone marrow and in blood. Telomerase gene therapy also improved blood counts and mouse survival.
Discussion

Here, we set out to test the hypothesis of whether telomerase increased expression through systemic virus-based Tert delivery may delay or prevent the emergence of aplastic anemia provoked by short telomeres in two independent mouse models with very short telomeres specifically in the bone marrow owing to either Trf1 or Tert deficiencies\textsuperscript{20,22}.

The rational for this study was based on our previous finding showing that systemic AAV9-Tert gene therapy in wild-type mice was sufficient to delay different age-related diseases and to significantly increase mouse life span by delaying telomere shortening with age in different tissues\textsuperscript{18}. A 5-month longitudinal follow up of these mice, also revealed increased telomere length in peripheral blood mononuclear cells from mice treated with telomerase gene therapy, suggesting that the vectors were also targeting the bone marrow\textsuperscript{18}. This is in line with recent reports showing that AAV9 viral genome copies are readily detectable in bone marrow isolates even 20 weeks post injection\textsuperscript{35}, and with the fact that FACS analysis of bone marrow from neonatal mice administered with AAV9-GFP show increased amounts of GFP positive cells\textsuperscript{36}. Thus, Tert delivery via AAV9 may hold potential to treat aplastic anemia triggered or associated with short telomeres in the bone marrow, a common consequence of telomerase mutations in the so-called telomeropathies or telomere syndromes, as well as in some acquired cases of aplastic anemia\textsuperscript{37-39}.

To demonstrate this, we first confirmed that a high dose ($3.5\times10^{12}$ vg/mouse) of AAV9-EGFP reporter vector injected intravenously, was able to transduce the bone
marrow as indicated by the presence of EGFP-positive cells. Furthermore, administration of the same amount of AAV9-\textit{Tert} particles, led to robust \textit{Tert} expression in whole bone marrow isolates 2 weeks after treatment, and this increased expression was maintained at 8 months post-treatment. To rule out the possibility that AAV9 may be only targeting bone marrow stroma cells, we demonstrated significantly increased \textit{Tert} mRNA expression both in isolated hematopoietic stem cells (Lin$^-\text{, Sca1}^+\text{, c-Kit}^+$) and in lineage committed bone marrow cells (Lin$^+$) from mice treated with AAV9-\textit{Tert} compared with mice treated with the empty AVV9 vector. Importantly, bone marrow cells from AAV9-\textit{Tert} treated mice showed enhanced colony forming abilities, suggesting that telomerase expression may increase the stem cell reserve.

Indeed, AAV9-\textit{Tert} treatment of mice with aplastic anemia triggered by short telomeres owing to marrow-specific \textit{Trf1} deletion\textsuperscript{19} significantly rescued mortality by aplastic anemia, concomitant with telomere re-elongation in blood and bone marrow cells from these mice after telomerase treatment. We confirmed these findings by generating a second mouse model of aplastic anemia produced by short telomeres, in this case owing to telomerase deficiency. In particular, we generated mice with \textit{Tert} deficiency specifically in the bone marrow. In this case, treatment with \textit{Tert} gene therapy of mice with a \textit{Tert}-deficient bone marrow and short telomeres (irradiated wild-type mice transplanted with G3 \textit{Tert} knockout bone marrow) showed a moderate improvement of survival, which was not as dramatic as in the case of the \textit{Trf1}-deficient bone marrow model. This is likely due to the fact that, in contrast to the \textit{Trf1} deletion model, which shows a very severe and rapid induction of aplastic anemia\textsuperscript{19,20}, \textit{Tert}-deficiency leads to a variable penetrance of aplastic anemia with increasing mouse
generations\textsuperscript{40,41}. Similarly to the \textit{Trf1}-deficient mouse model, \textit{Tert} gene therapy of the \textit{Tert}-deficient bone marrow mouse model also resulted in increased telomere length with time in peripheral blood cells and significantly improved blood counts. In both mouse models, improvement of blood counts can be interpreted as the consequence of improved stem cell reserve. This is in line with recently published data showing that genetic \textit{Tert} re-activation in G5 \textit{Tert}\textsuperscript{+/−} mice using a Cre-inducible system restored HSPC proliferation concomitant with improved erythrocyte counts and haemoglobin levels\textsuperscript{42}.

In summary, here we provide proof of concept for a therapeutic effect of telomerase treatment using AAV9 gene therapy vectors in the treatment of aplastic anemia provoked by short telomeres. A strategy based on AAV9-\textit{Tert} treatment may be beneficial not only in the correction of monogenic bone marrow disease such as in carriers of \textit{Tert} mutations (we demonstrate improved blood counts in the \textit{Tert} knockout mice), but also in other forms of aplastic anemia associated with short telomeres and hematopoietic stem cell depletion (e.g. Fanconi anemia\textsuperscript{43}). Generally, due to an excellent safety profile owing to their low immunogenicity and the fact that they are non-integrative, AAV vectors have become an attractive gene therapy tool and many clinical trials using those vectors are already underway (see www.clinicaltrials.gov). However, in spite of the fact that AAV9 vectors carrying the \textit{Tert} gene are non-integrative, and therefore, unlikely to aid in the division of cancer cells, the association of many cancers with telomerase expression imposes specific safety concerns. In this regard, it is important to point out that in a previous study more than 1-year follow up of wild-type mice treated with AAV9-\textit{Tert} did not show increased cancer, in fact cancer onset was delayed in the same manner than other age-related diseases\textsuperscript{18}. Nevertheless,
subsequent studies should address the safety of this strategy in long-lived mammals such as primates. If those studies confirm our proof of principle findings, this gene therapy approach may also be adapted to treat hereditary forms of aplastic anemia caused by mutation other than *Tert* through swapping the cargo.

**Author contributions**

M.A.B. conceived the original idea. M.A.B. and C.B. designed the experiments. C.B. performed the majority of the experiments. R.S. performed bone marrow transplantations and monitored mice during all animal procedures. J.M.P., M.P. and C.B.B. performed experiments during the revision process. I.F. and M.B. contributed to scientific discussions and experimental design. F.B. provided viral vectors. M.A.B. and C.B. wrote the paper.

**Conflict of interest**

The authors have declared that no conflict of interest exists.

**Acknowledgements**

The Blasco lab is funded by the Spanish Ministry of Economy and Competitiveness, the Fundación Botín and the Roche Extended Innovation Network (EIN).
References


Figure legends

**Figure 1 | High dose of AAV9 particles targets bone marrow including HSPCs. (A)** Representative anti-EGFP immunohistochemistry images of bone marrow corresponding to the tibia. Mice were injected with AAV9-EGFP or AAV9-empty vectors at a concentration of $3.5 \times 10^{12}$ vg/mouse. EGFP positive cells were mainly located towards the end of the bones. Scale bar: 500 μm left hand images and 50 μm for magnifications. **(B)** Percentage of EGFP positive cells relative to the total number of cells. Cells were separately counted in joint adjacent areas and in the middle of the bone. **(C)** Tert mRNA expression level in total bone marrow isolated 2 weeks and **(D)** 8 months after virus injection (VI) with $3.5 \times 10^{12}$ vg/mouse AAV9-Tert relative to the expression of mice injected with the same amount of AAV9-empty vector. **(E)** Delta Ct values (Tert minus Act1) of the qRT-PCR shown in D. **(F)** qRT-PCR determined relative Tert expression in FACS sorted HSPCs and **(G)** Lineage committed cells. **(H)** Colony forming assay in MethoCult with whole bone marrow cells isolated from mice injected with AAV9-Tert or AAV9-empty. For all experiments n indicates number of mice. Data are mean ± SEM. Statistical analysis: two-sided Student’s t-test, P-values are shown.

**Figure 2 | AAV9-Tert treatment rescues the aplastic anemia phenotype in Trf1lox/lox mice. (A)** Experimental design. Mice were lethally irradiated and the following day transplanted with Trf1lox/lox Mx1-Cre bone marrow. After engraftment, Cre expression and Trf1 excision was induced by pl:pC injections for 5 weeks. One week later, mice were injected with AAV9-Tert or AAV9-empty particles. **(B)** Kaplan Meier survival curves
showing that AAV9-Tert treatment significantly rescues mouse survival. (C) Kaplan-Meier survival curves considering only those animals that died from aplastic anemia within 100 days after virus treatment, show significant protection of AAV9-Tert treatment from deaths by aplastic anemia. (D) Platelet counts and (E) hemoglobin levels in mice of both the AAV-Tert and AAV9-empty treated groups showing clear signs of anemia compared with healthy mice from the same AAV-Tert and AAV9-empty treated groups. (F) Representative bone marrow images of healthy controls (no Cre-mediated induction of Trf1 deletion) and of mice with bone marrow aplasia. Genotypes and AAV9 treatments are indicated. Scale bar: 500μm for images on the left and 20μm for magnifications. (G) Quantification of bone marrow cellularity expressed as number of nucleated cells per field. 4-5 fields per mouse were counted. (H) Quantification of bone marrow cellularity expressed as the percentage of nuclear area (purple stain) to total areas per field. 4-5 fields per mouse were counted. In all graphs, n indicates number of mice. Data are mean ± SEM. Statistical analysis: Log-rank test in (B) and (C) and two-sided Student’s t-test in (D, E, G and H), P-values are shown. n.s. = not significant.

Figure 3 | AAV9-Tert treatment causes telomere elongation in blood and bone marrow. (A) Longitudinal HT-QFISH analysis of telomere length in peripheral blood monocytes (Trf1lox/lox Mx1-Cre transplanted mice, see also Fig. 2A). Blood was extracted at 4 different time points: (1) before pI:pC treatment, (2) after 5 weeks of pI:pC treatment (before AAV9 injection), (3) 2 months and (4) 4 months after AAV9 injection. (B) Relative variation (Δ) of telomere length in AAV-Tert and AAV9-empty treated animals between timepoints 2 and 4 and (C) between timepoints 1 and 4. (D) Relative
telomere length in bone marrow sections from AAV-Tert and AAV9-empty treated mice shown as arbitrary units of fluorescence. Each square and triangle represents the mean telomere length per nucleus of an individual mouse. (E) Frequency distribution blot of telomere length showing a higher abundance of short telomeres in the AAV9-empty treated group compared with AAV9-Tert treated mice (pooled data from D) (F) Representative images of bone marrow sections from AAV-Tert and AAV9-empty treated mice used for QFISH analysis. Cell nuclei are stained blue (DAPI) and telomeres are stained red (Cy3). White arrowheads in the bottom panels indicate nonspecific extra-nuclear signal while yellow arrowheads indicate specific telomere signals within dapi stained nuclei. Scale bars: 20 μm top row and 10 μm bottom row. In all graphs, n indicates number of mice. Data are mean ± SEM. Statistical analysis: Two-way anova (A) and two-sided Student’s t-test in (B-C), P-values are shown.

Figure 4 | AAV9-Tert treatment improves blood counts in mice with short telomeres owing to specific Tert deletion in the bone marrow. (A) Experimental design. G3 tert−/− mice with short telomeres were generated by consecutive crosses of Tert deficient mice. Bone marrow from these G3 mice was isolated and transplanted into irradiated wild-type mice. After engraftment mice were injected with AAV9-Tert or AAV9-empty virus particles. (B) HT-QFISH analysis of telomere length in peripheral blood mononuclear cells from wild-type, G1 Tert−/− and G3 Tert−/− mice reveals progressive telomere shortening with consecutive mouse generations. (C) Kaplan Meier survival curves showing that AAV9-Tert treatment improves survival of mice with very short telomeres in the bone marrow owing to Tert deficiency specifically in the bone
marrow (irradiated wild-type mice transplanted with G3 Tert\textsuperscript{−/−} bone marrow). AAV9-Tert compared to AAV9-empty treatment improves (D) erythrocyte counts, (E) hemoglobin levels, (F) platelet and (G) leukocyte counts. In all graphs, n indicates number of mice. Data are mean ± SEM. Statistical analysis: Log-rank (A) and two-sided Student’s t-test in (B, E-H), P-values are shown.

Figure 5 | Telomerase gene therapy leads to telomere elongation in peripheral blood and bone marrow cells from mice with specific deletion of Tert in the bone marrow. (A) Longitudinal HT-QFISH analysis of telomere length in peripheral blood mononuclear cells of irradiated wild-type mice transplanted with bone marrow from G3 Tert\textsuperscript{−/−} mice (see also Fig. 4A). Blood was extracted at 3 different time points: after G3 Tert\textsuperscript{−/−} bone marrow engraftment and before AAV9 injection (1), 3 months (2) and 5 months (3) after AAV9 injection. (B) Relative variation (Δ) of telomere length in AAV-Tert and AAV9-empty treated animals between timepoints 1 and 3. (C) Telomere QFISH analysis on bone marrow sections from animals transplanted with G3 Tert\textsuperscript{−/−} bone marrow and treated with AAV9-empty or AAV9-Tert for five months prior to sacrifice. Each square or triangle represents the mean telomere length per nucleus (expressed as arbitrary units of fluorescence) of an individual mouse. (D) Representative images of bone marrow sections from AAV-Tert and AAV9-empty treated mice used for QFISH analysis. Cell nuclei are stained blue (DAPI) and telomeres are stained red (Cy3). White arrowheads indicate nonspecific extra-nuclear signal while yellow arrowheads indicate specific telomere signals within dapi stained nuclei. Scale bars 10 μm. For all experiments n = number of mice. Bar represents mean
± SEM. Two-way anova (A) and two-sided Student’s t-test in (B-C) was used for statistical analysis, P-values are shown.
Figure 1

A IHC anti-EGFP

B

- black: joint adjacent (EGFP)
- gray: bone middle (EGFP)
- white: no EGFP

n=4

C 2 weeks post VI

D 8 months post VI

P=0.034

E 8 months post VI

P=0.0008

F HSCs

P=0.05

G Lin+

P=0.0029

H colony forming assay (Methocult)

P=0.04
Trf1lox/lox Mx1-Cre
bone marrow transplantation
irradiation (12Gy)
AAV9-Tert treatment

A

5 weeks pi:pC treatment
3 injections / week

B

Percent survival

AAV9-Tert
AAV9-empty

P=0.0025

n=31
n=36

87%
55%

C

D

E

Figure 2

P=0.0006

n=4
n=16

P<0.0001

n=16
n=18

P<0.0001

n=8
n=18

n.s.

F

Trf1lox/lox Mx-wt

AAV9-empty

Trf1lox/lox Mx-Cre

AAV9-empty

AAV9-Tert

P<0.0001

n=3
n=8

P<0.0001

n=4

P<0.0001

n<0.0001

P=0.006

P=0.024

G

n=3
n=4

P=0.003

H

% nuclear area / total area

n=3
n=4

P=0.02
**Figure 3**

**A**

**Blood HT-qFISH**

- Chart showing telomere length (kb) over time for AAV9-empty and AAV9-Tert groups.
- Timepoints: 1 week, 2 weeks, 3 weeks, 4 weeks.
- P<0.05 for AAV9-Tert compared to AAV9-empty.

**B**

**Δ time point 2 vs. 4**

- AAV9-Tert vs. AAV9-empty with mean spot intensity (a.u.)
- P=0.042, n=8

**C**

**Δ time point 1 vs. 4**

- AAV9-Tert vs. AAV9-empty with mean spot intensity (a.u.)
- P=0.03, n=8

**D**

**Bone marrow**

- Chart showing mean spot intensity (a.u.) for AAV9-Tert and AAV9-empty.
- P=0.024

**E**

- Chart showing telomere frequency against telomere intensity (a.u.f.)

**F**

- Images comparing AAV9-empty and AAV9-Tert in bone marrow.

- AAV9-empty
- AAV9-Tert

*Note: For personal use only.*
**Figure 4**

A. Genetic inheritance model showing progressive telomere shortening in different genotypes:

- **G1**: Tert<sup>+/−</sup> X Tert<sup>+/−</sup>
- **G2**: Tert<sup>−/−</sup> X Tert<sup>−/−</sup>
- **G3**: Tert<sup>−/−</sup> X Tert<sup>−/−</sup>

Gene therapy: AAV9-Tert vs. AAV9-empty

- Wild-type mouse with short telomere haematopoiesis
- Aplastic anemia survival, blood counts, telomere length?

B. Blood HT-qFISH

- Telomere length (Kb)
  - Tert<sup>+/+</sup>
  - Tert<sup>−/−</sup>
  - Tert<sup>−/−</sup>

- P-values: 0.0062, 0.0025, 0.058

C. Percent survival

- N = 25, 27

D. Humane endpoint blood counts

- Erythrocytes (10<sup>12</sup> L<sup>−1</sup>)
  - AAV9-empty (n=11), AAV9-Tert (n=14)

- Haemoglobin (g dL<sup>−1</sup>)
  - AAV9-empty (n=11), AAV9-Tert (n=14)

- Platelets (10<sup>9</sup> L<sup>−1</sup>)
  - AAV9-empty (n=10), AAV9-Tert (n=14)

- Leukocytes (10<sup>9</sup> L<sup>−1</sup>)
  - AAV9-empty (n=11), AAV9-Tert (n=14)

- P-values: 0.006, 0.003, 0.035, 0.09, 0.09, 0.09, 0.09
Figure 5

A. Blood HT-qFISH

- Graph showing telomere length (kb) over time (1, 2, 3 months) for AAV9-Tert (n=15) and AAV9-empty (n=13).
  - **P=0.0065**

B. Bar chart showing Δ telomere length (kb) for AAV9-Tert and AAV9-empty.
  - **P=0.0007**
  - AAV9-Tert: n=15, AAV9-empty: n=13

C. Bone marrow

- Graph showing mean spot intensity (a.u.) for AAV9-Tert and AAV9-empty.
  - **P=0.028**

D. Imaging results for AAV9-empty and AAV9-Tert in bone marrow.
Telomerase gene therapy rescues telomere length, bone marrow aplasia and survival in mice with aplastic anemia

Christian Bär, Juan Manuel Povedano, Rosa Serrano, Carlos Benitez-Buelga, Miriam Popkes, Ivan Formentini, Maria Bobadilla, Fatima Bosch and Maria A. Blasco