

Genetic Engineering of Humanized Telomere Mice

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Abstract

Telomere shortening is a hallmark of human aging, and telomerase regulation plays a critical role in cellular proliferation and replicative senescence. In human cells, telomere length imposes a limit on proliferative potential, a phenomenon known as the Hayflick limit. However, species-specific differences in telomere dynamics and telomerase regulation between humans and mice present challenges to using mice as accurate models for human telomere-related research. To address this limitation, we engineered a humanized telomerase gene (*hmTert*) in mice by replacing the non-coding sequences within the mouse *Tert* locus (*mTert*) with corresponding regulatory sequences from the human *TERT* gene. Breeding of these genetically modified mice resulted in progressive telomere shortening over successive generations, ultimately reaching human-like lengths (below 10 kb). This protocol outlines the development of this humanized telomere mouse model, referred to as HuT mice, offering a robust platform for studying human telomere biology and aging-related diseases.

Key features

- This protocol describes methods to increase the success rates of knocking in large genomic fragments (~47 kb) by integrating CRISPR-Cas9 with homologous recombination.
- It enables precise engineering of a humanized telomerase gene (*hmTert*), faithfully recapitulating human TERT regulation and telomere length dynamics in mice.

Keywords: Genetic engineering, Telomere length, Humanized telomerase gene, Human-like telomere homeostasis, CRISPR-Cas9

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[illegible]

****Telomere length is measured in splenocytes by Flow-FISH.**

Telomeres, repetitive sequences at chromosome ends, are essential for maintaining genomic stability. In most human somatic cells, telomeres progressively shorten with each cell division, a process counteracted by the telomerase enzyme complex, which consists of the TERT protein and TERC RNA subunits. Most human adult tissues contain low or no telomerase, and telomere shortening eventually triggers cell cycle arrest, leading to replicative senescence or the Hayflick limit. Telomerase elongates telomeres, delaying senescence. However, telomere homeostasis and telomerase regulation differ markedly between species. For instance, while telomere lengths in human tissues typically range from 10 to 15 kb, laboratory mouse strains, such as C57BL/6, often exhibit telomeres over ~50 kb. Moreover, telomerase remains active in most adult mouse tissues, unlike in humans, where it is largely repressed [1]. As a result, mouse cells do not exhibit replicative senescence or the Hayflick limit.

Our laboratory focuses on the regulation of the *TERT* gene, which encodes the rate-limiting catalytic subunit of telomerase. We have identified key regulatory regions within the human *TERT* gene—including the 5' intergenic region, intron 2, and intron 6—that modulate *TERT* expression [4]. Replacing those genetic contexts on the mouse locus with human counterparts disrupted *Tert* repression in differentiated mouse ESCs [5]. To investigate the role of these elements in vivo, we engineered a humanized telomerase gene (*hmTert*) in mouse embryonic stem cells (mESCs) by replacing the corresponding non-coding regions in the mouse *Tert* locus with a 47 kb hybrid sequence from a bacterial artificial chromosome (BAC) containing the

human regulatory elements [6–8]. Initial attempts to introduce this large fragment via homologous recombination were hindered by low efficiency and incomplete integration. To overcome this, we employed CRISPR-Cas9 technology to enhance homologous recombination efficiency, increasing the success rate from 0.05% to 11%, in mESCs [8]. The *hmTert* gene in mESCs accurately recapitulated human *TERT* regulation during in vitro differentiation [5]. We subsequently established a mouse strain carrying germline *hmTert* alleles. Investigation of these mice confirmed human-like *Tert* regulatory patterns. With successive intercrossing of heterozygous mice, telomere lengths gradually shortened across generations, eventually reaching a human-like length of less than 10 kb. Despite their much shorter telomeres, *Tert^{h/h}* and *Tert^{h/-}* mice maintained normal body weight and tissue homeostasis. These mice, which exhibit physiologically relevant telomerase regulation, serve as a powerful model for studying human telomere biology, aging, and associated diseases. This protocol outlines the key steps for generating and validating the humanized telomere mouse model, known as HuT mice.

Materials and reagents

Biological materials

1. Mouse embryonic stem cells G4 [9]
2. Drug-resistant (DR4) feeder cells: mouse embryonic fibroblast cells derived from E13.5–14.5 embryos and treated as previously described [7]
3. pSpCas9 (BB)-2A-GFP (PX458, Addgene #48138), a gift from Feng Zhang [10]

Reagents

1. 7-AAD (Thermo Fisher, catalog number: 00-6993-50)
2. Agarose (Invitrogen, catalog number: 16500-500)
3. LB broth (Affymetrix, catalog number :75852)
4. Agar (VWR, catalog number: J637)
5. DMEM medium (HyClone, catalog number: SH30243)
6. Penicillin/Streptomycin solution 100× (Gemini, catalog number: 400-109-100)
7. NEAA 100× (HyClone, catalog number: SH30238.01)
8. Fetal bovine serum (FBS) (Atlanta, catalog number: S12450)
9. RPMI medium (HyClone, catalog number: SH30027.LS)
10. Lipofectamine 2000 (Thermo Fisher, catalog number: 11668027)
11. Ladderman DNA Labeling kit (Takara, catalog number: 6046)
12. Isopropanol (MP Biomedical, catalog number: 0219400690)
13. Ethanol absolute, pure (200 Proof) (Avantor, catalog number: 71001-754)
14. Chloroform (MP Biomedical, catalog number: 0219400225)
15. TRI reagent (Ambion, catalog number: 155960118)
16. PowerUp™ SYBR™ Green Master Mix for qPCR (Thermo Fisher, catalog number: A25778)
17. Deoxynucleotide (dNTP) solution mix (NEB, catalog number: N0447)
18. QIAEX II Gel Extraction kit (Qiagen, catalog number: 20021)
19. SuperScript™ III reverse transcriptase (Thermo Fisher, catalog number: 18080044)
20. TaKaRa LA Taq® DNA polymerase (Takara, catalog number: RR002B)
21. Reprobing charged Nylon (GVS, catalog number: 1226556)
22. Restriction digestion enzyme: all restriction enzymes were purchased from New England Biolabs (NEB)
23. NEB® 5-alpha Competent *E. coli* (NEB, catalog number: C2987H)
24. Taq DNA polymerase with ThermoPol® buffer (NEB, catalog number: M0267L)
25. T4 polynucleotide kinase (NEB, catalog number: M0201S)
26. T4 DNA ligase (NEB, catalog number: M0202L)
27. TelC-FAM probe (PNA Bio, catalog number: F1001): a PNA probe for leading strand of telomere sequences (CCCTAA)₃ conjugated with FAM
28. GenElute™ Plasmid Miniprep kit (Sigma, catalog number: PLN70)
29. Wizard® Genomic DNA Purification kit (Promega, catalog number: A1125)

30. ^{32}P -dCTP (PerkinElmer, catalog number: BLU513H250UC)
31. Ampicillin sodium salt (Fisher Scientific, catalog number: BP1760-5)
32. Puromycin dihydrochloride (Sigma, catalog number: P8833)
33. Ganciclovir (GCV) (Sigma, catalog number: SML2346)
34. Roche blocking reagent (Roche, catalog number: 11096176001)
35. Trypsin solution (HyClone, catalog number: SH30042)
36. GlutaMax™ supplement (Gibco, catalog number: 35050061)
37. LIF recombinant mouse protein (Gibco, catalog number: A35935)
38. β -Mercaptoethanol (Sigma, catalog number: M6250)

Solutions

1. Ampicillin solution (see Recipes)
2. 50 mg/mL puromycin (see Recipes)
3. 10 mM GCV (see Recipes)
4. 10% blocking buffer (see Recipes)
5. LB medium (see Recipes)
6. 0.025% trypsin solution (see Recipes)
7. mESCs culture medium (see Recipes)
8. 0.5× TBE (see Recipes)
9. Denature buffer (see Recipes)
10. Neutralization buffer (see Recipes)
11. Church and Gilbert's hybridization buffer (Church buffer) (see Recipes)
12. 20× SSC (see Recipes)
13. DNA wash solution (see Recipes)
14. Hank's balanced salt solution (HBSS) (see Recipes)
15. PBS (see Recipes)
16. Red blood cell (RBC) lysis buffer (see Recipes)
17. Hybridization buffer (see Recipes)
18. Wash solution I (see Recipes)
19. Wash solution II (see Recipes)
20. Maleic acid buffer (see Recipes)

Recipes

1. Ampicillin solution

A stock solution (100 mg/mL) is prepared by dissolving 100 g of ampicillin sodium salt into 10 mL of Milli-Q water and filtering it through a 0.22 μm filter. The stock solution is added to the LB medium at a final concentration of 100 $\mu\text{g/mL}$.

2. 50 mg/mL puromycin

A stock solution (50 mg/mL) is prepared by dissolving 0.5 g of puromycin dihydrochloride into 10 mL of Milli-Q water and filtering it through a 0.22 μm filter. The stock solution is added to the mESCs culture medium at a final concentration of 1.5 $\mu\text{g/mL}$.

3. 10 mM GCV

This stock solution is added to the mESCs culture medium at a final concentration of 50 μM .

4. 10% blocking buffer

Dissolve Roche blocking reagent in maleic acid buffer to a final concentration of 10% (w/v) by shaking and heating on a heating block.

5. LB medium

Dissolve 20 g of LB broth in 1 L of Milli-Q water and autoclave. Prior to autoclaving, add 15 g of agar to 1 L of LB medium to prepare the bacterial culture plate.

6. 0.025% Trypsin solution

Dilute 10 mL of 0.25% Trypsin in 40 mL of autoclaved PBS.

7. mESCs culture medium

Reagent	Final concentration
FBS	15%
GlutaMax™ supplement (100×)	1×
NEAA (100×)	1×
LIF recombinant mouse protein	10 ng/mL
β-mercaptoethanol	55 μM
Penicillin/Streptomycin solution (100×)	1×
DMEM medium	85%

8. 0.5× TBE

Reagent	Final concentration
Tris base	0.045 M
H ₃ BO ₃	0.045 M
EDTA (pH 8.3)	0.01 M

9. Denature buffer

Reagent	Final concentration
NaCl	1.5 M
NaOH	0.05 M

10. Neutralization buffer

Reagent	Final concentration
NaCl	1.5 M
Tris base (pH 8.5)	9.5 M

11. Church and Gilbert's hybridization buffer (Church buffer), pH 7.2

Reagent	Final concentration
Na ₂ HPO ₄	0.34 M
NaH ₂ PO ₄	0.16 M
SDS	7% (w/v)
EDTA	0.001 M
BSA	1% (w/v)

12. 20× SSC, pH 7.0

Reagent	Final concentration
NaCl	3 M
Na ₃ C ₆ H ₅ O ₇	0.3 M

A series of SSC solutions used in the paper were diluted from 20× SSC with an appropriate volume of MilliQ water.

13. DNA wash solution

Reagent	Final concentration
Na ₃ C ₆ H ₅ O ₇	0.3 M
Ethanol	10% (v/v)

14. Hank's balanced salt solution (HBSS), pH 7.4

Reagent	Final concentration
NaCl	0.14 M
KCl	0.005 M
CaCl ₂	0.001 M

MgSO ₄	0.0004 M
MgCl ₂	0.0005 M
Na ₂ HPO ₄	0.0003 M
KH ₂ PO ₄	0.0004 M
D-Glucose	0.004 M
Na ₂ CO ₃	0.004 M

15. PBS, pH 7.2–7.4

Reagent	Final concentration
NaCl	0.137 M
KCl	0.0027 M
Na ₂ HPO ₄	0.01 M
KH ₂ PO ₄	0.0018 M

16. Red blood cell (RBC) lysis buffer, pH 7.4

Reagent	Final concentration
NH ₄ Cl	155 M
KHCO ₃	10 M
EDTA	0.1 M

17. Hybridization buffer

Reagent	Final concentration
Tris-HCl (pH 7.2)	20 mM
Deionized formamide	60% (v/v)
Blocking buffer	0.5% (v/v)

18. Wash solution I

Reagent	Final concentration
Tris-HCl (pH 7.2)	20 mM
Deionized formamide	60% (v/v)
BSA	0.1% (w/v)
Tween 20	0.1% (v/v)

19. Wash solution II

Reagent	Final concentration
BSA	0.1% (w/v)
Tween 20	0.1% (v/v)
PBS	Add to final volume

20. Maleic acid buffer (pH 7.5)

Reagent	Final concentration
Maleic acid	0.1 M
NaCl	0.15 M

Laboratory supplies

1. Cell culture multi-well plates (Greiner Bio-One, 12-well plate, 48-well plate, and 96-well plate)
2. 1.5 mL microcentrifuge tubes (Fisher Scientific, catalog number: 05-408-129)
3. 15 mL conical tubes (VWR, catalog number: 89039-658)
4. 50 mL conical tubes (VWR, catalog number: 21008-089)
5. 1 mL cryovials tubes (VWR, catalog number: I210-000001)
6. 5 mL serological pipettes (Greiner Bio-One, catalog number: 606107)
7. 10 mL serological pipettes (Greiner Bio-One, catalog number: 607107)

8. 0.1–10 μ L pipette tips (Fisher Scientific, catalog number: 02-707-454)
9. 5–300 μ L pipette tips (Fisher Scientific, catalog number: 02-707-447)
10. 100–1,250 μ L pipette tips (Fisher Scientific, catalog number: 02-707-400)
11. 0.2 mL PCR tube (Corning, catalog number: PCR-02-A)
12. qRT-PCR plates (Thermo Fisher, catalog number: 4346907)

Equipment

1. PhosphorImager system (GE Healthcare)
2. CO₂ incubator (Eppendorf, model: Galaxy 170S)
3. Incubator (VWR)
4. Incubator shaker (New Brunswick, model: I2400)
5. Water bath (Fisher Scientific, model: Isotemp215)
6. Centrifuge (Eppendorf, model: 5424R)
7. Laboratory centrifuge with rotors for 15- and 50-mL conical tubes (Eppendorf, model: 5804R)
8. Thermal cycler machine (Bio-Rad, model: C1000Touch)
9. Applied Biosystems™ StepOnePlus™ Real-Time PCR System
10. CytoFLEX (Bechman Coulter)
11. Tissue cell culture hood
12. Liquid nitrogen (N₂) tank
13. Freezer (-20 °C)
14. Refrigerator (2–8 °C)
15. Thermomixer (Eppendorf, model: 5436)
16. Hybridization incubator (Fisherbiotech)
17. UV-crossing link machine (Spectrolink, model: XL-1000)
18. pH meter (VWR, model: B10P)
19. Vortex (Fisher Scientific, catalog number: 12812)

Procedure

A. Single guide RNA (sgRNA) design

To design sgRNAs, we used the Synthego CRISPR Design Tool (<https://www.synthego.com/>) to identify guide RNA sequences that target regions near the 5' and 3' ends of the intended replacement region in the mouse genome (chr13:73769156-73787274), as illustrated in Figure 1. The input sequences for the design tool are provided in the supplemental file SEQ3 and SEQ4. sgRNA 1 (chr13:73770111-73770130) targeted the 5' intergenic region (5'IR) between *mTert* and *mCrr9* loci, while sgRNA 2 (chr13:73786833-73786852) targeted the distal end of *mTert* intron 6. Although the *TERT* locus exhibits conserved synteny between mouse and human, sharing flanking genes and orientation, the non-coding regions, including intergenic sequences, differ significantly. The selected sites were unique within the mouse genome and not conserved in the human genome, minimizing the risk of off-target effects. Upon entering the target sequences, the tool generated a ranked list of sgRNAs based on predicted on-target efficiency and off-target potential. Three potential sgRNAs with the highest scores are presented in Table S1. We selected sgRNA oligonucleotides after careful evaluation of these scores to ensure both specificity and efficiency.

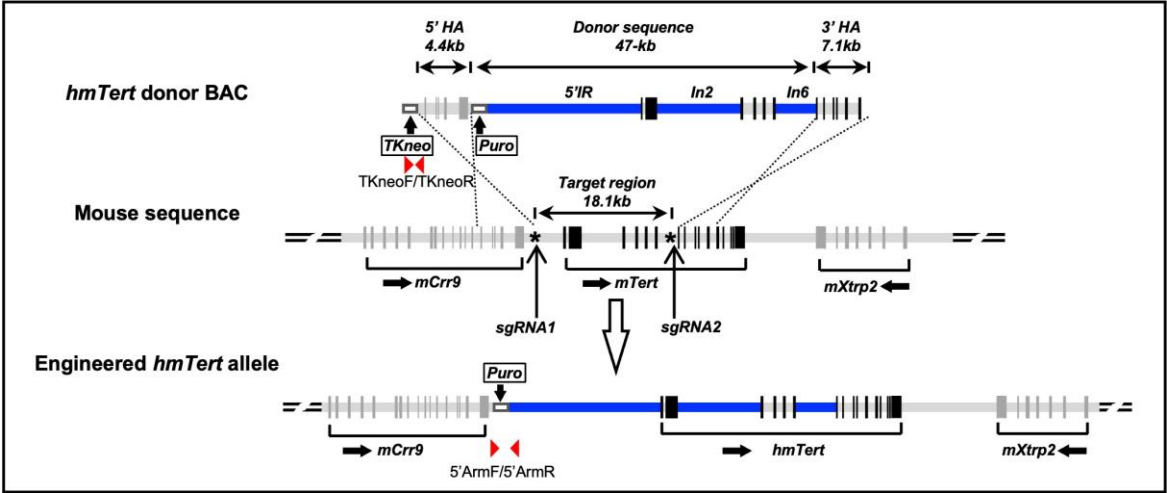


Figure 1. Schematic representation of the donor bacterial artificial chromosome (BAC) construct and engineering strategy for the *hmTert* allele. The donor BAC contained the *mTert* gene components (light grey bars). Regions within *mTert* locus, the 5' intergenic region (IR), intron 2 (In2), and intron 6 (In6), were substituted with corresponding sequences from the human *TERT* gene, shown as blue bars. The donor sequence spanned 47 kb and was flanked by 5' and 3' homologous arms (HAs). The 5' HA is a 4.4 kb sequence from the 3' end of *mCrr9* gene, whereas the 3' HA is a 7.1 kb region spanning exons 7–12 of the *mTert* gene. In addition, a TKneo and a puromycin-resistant cassette (white boxes) were inserted upstream and downstream of 5' HA, respectively. The puromycin cassette was surrounded by a pair of lox511 sites and was removed via transient transfection of a Cre expression plasmid in the mouse embryonic stem cells (mESCs) after homologous recombination. The target region was an 18.1 kb region from 5'IR to intron 6 of the *mTert* gene. Asterisk (*) represents Crispr/Cas9-sgRNAs targeting sites. Black vertical bars represent exons. PCR primers are indicated by red arrowheads.

B. sgRNA plasmid cloning

1. Digest plasmid PX458 (Figure S1) [10] as shown in Table 1 (time: 6 h):

Table 1. Restriction enzyme reaction

Components	Amount
10× NEB buffer 2.1	5.0 μL
PX458	2.0 μg
BbsI	2.5 μL
ddH ₂ O	volume to 50 μL
Total	50 μL

Perform digestion at 37 °C for 4 h. A correctly digested PX458 backbone has two fragments of 9.3 kb and 22 bp. The 9.3 kb fragment is isolated using QIAEX II Gel Extraction kit and eluted with 20 μL of water for a target concentration of 50 ng/μL.

2. Double-stranded oligos were produced by annealing forward and reverse oligos for sgRNA1 or sgRNA 2, as listed in Table 2. Oligos sequences are listed in Table S2 (time: 40 min).

Table 2. sgRNA targeting site sequences

sgRNA targeting site	Sequence (5'-3')	Oligonucleotides
sgRNA1	AAGGATGAGGTTGGGCCAAT	sgRNA1-U/ sgRNA1-B
sgRNA2	TCTGCAATGGCGTGGTCCCA	sgRNA2-U/ sgRNA2-B

Annealing reactions were set up in 0.2 mL PCR tubes as shown in Table 3.

Table 3. sgRNA annealing reaction mix

Components	Amount
Upper oligonucleotide (100 µM)	1.0 µL
Bottom oligonucleotide (100 µM)	1.0 µL
10× T4 DNA ligation buffer	1.0 µL
T4 polynucleotide kinase	0.5 µL
ddH ₂ O	6.5 µL
Total	8 µL

Annealing was done using the following program (Table 4) in a thermocycler:

Table 4. sgRNA annealing reaction

Temperature	Time
37 °C	30 min
95 °C	5 min
	ramping down to 25 °C at 5 °C/min
4 °C	Hold

3. Assemble PX458-sgRNA: set up assembling reaction in a 0.2 mL PCR tube as shown in Table 5 (time: 16 h):

Table 5. Fragment assembling reaction

Components	Amount
Digested PX458 (50 ng/µL)	0.5 µL
Annealed oligonucleotide (1:250 dilution)	0.5 µL
10× T4 DNA ligation buffer	1.0 µL
T4 DNA ligase	0.25 µL
ddH ₂ O	7.75 µL
Total	10 µL

Run the reaction at 16 °C overnight in the thermocycler.

4. Transform 5 µL of the ligated product from step B3 using NEB Chemical Competent DH5α *E. coli* according to the manufacturer's protocol. Upon transformation, spread all competent cells onto LB plates containing 100 µg/mL ampicillin (time: 1 h).

5. Bacterial colony identification: Culture three colonies in 3 mL of LB medium with 100 µg/mL of ampicillin. Shake overnight at 37 °C. Isolate plasmid DNAs from the cultures using the GenElute™ Plasmid Miniprep kit according to the manufacturer's instructions. Plasmids were digested with BbsI and AgeI to identify and confirm the correct constructs (Figure S1) (time: 3 h). Set up the digestion reaction as shown in Table 6.

Table 6. Restriction enzyme digestion reaction

Components	Amount
10× NEB buffer 1.1	1 µL
BbsI	0.4 µL
AgeI	0.2 µL
PX485-sgRNA plasmid DNA	1.0 µL
ddH ₂ O	7.4 µL
Total	10 µL

Incubate at 37 °C for 2 h in a water bath. PX458-sgRNA plasmids were digested to a 9.3 kb band, while the parental PX458 plasmid was digested into 8.3 kb and 971 bp bands (Figure 2).

6. PX458-sgRNA plasmids were validated by Sanger sequencing using the U6-Fwd primer: 5'-GACTATCATATGCTTACCGT-3'.

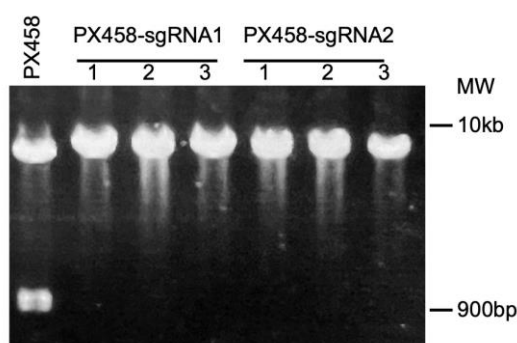


Figure 2. Identification of sgRNA plasmids. Correct PX458-sgRNA plasmids were identified and digested with BbsI and AgeI. MW: molecular weight markers.

C. Homologous recombination in mouse ESCs (total time: 24 d)

hmTert donor BAC was co-transfected with two sgRNA-expressing plasmids into mESCs (G4) [9]. The construction of the *hmTert* donor BAC has been previously described [5]. Briefly, in addition to incorporating the human *TERT* regulatory sequences specified above, we introduced the following modifications: (1) insertion of a puromycin resistance (Puro) cassette at the 3' end of *mCrr9*; (2) replacement of *mCrr9* exons 1–12 with a thymidine kinase-neomycin resistance (TK-neo) selection cassette; and (3) deletion of sequences downstream of *mTert* exon 12. These modifications collectively generated extended homologous recombination arms, as indicated in Figure 1, with their sequences provided in the supplemental file SEQ1 and SEQ2.

1. Day 1: Seed 3.5×10^6 mESCs into each well of a 12-well-plate with DR4 feeder cells. Transfect cells with 0.25 μ g of PX458-sgRNA1 and PX458-sgRNA2 plasmids, together with 1.0 μ g of donor BAC construct using lipofectamine 2000 according to the manufacturer's instructions. Eight hours later, passage transfected mESCs into 6-well plates. A brief protocol of cell passaging is as follows:

- For a well in a 12-well plate, aspirate the medium and wash the cells twice with 2 mL of PBS.
- Then, add 0.25 mL of prewarmed 0.025% Trypsin solution and incubate for approximately 2 min or until the cells are uniformly dispersed into small clumps.
- Next, add 2.5 mL of mESCs culture medium to neutralize the trypsin, and transfer the cells to a new 6-well plate pre-coated with fresh DR4 feeder cells.

Note: DR4 feeder cells are mouse embryonic fibroblasts (MEFs) derived from 13.5-day-old mouse embryos and inactivated by X-ray irradiation. These cells are resistant to multiple antibiotics, including puromycin, making them suitable for selection-based experiments. In our setup, DR4 feeder cells were seeded at a density of 5×10^4 cells per well in a 12-well plate. The optimal feeder cell density corresponds to approximately 50% confluence of the plate surface area, which provides sufficient support for mESC growth without overcrowding. Feeder cells are maintained in standard MEF culture medium and should be used within a few days post-irradiation to ensure viability and support function.

- Day 2: Select mESCs with 1.5 μ g/mL puromycin for two days.
- Day 4: Wash mESCs once with PBS and culture with fresh mESCs culture medium.
- Day 6: Select mESCs with 1.5 μ g/mL puromycin for two days again.
- Day 8: Wash mESCs once with PBS and culture with fresh mESCs culture medium.
- Day 10: Select 800 puromycin resistant colonies and seed them individually into 96-well plates with fresh DR4-feeder cells.
- Day 11: Culture mESCs in culture medium containing 1.5 μ g/mL puromycin.
- Day 13: Wash cells once with PBS and culture them in fresh mESCs culture medium.
- Day 14: Culture puromycin-resistant colonies in mESCs culture medium containing 50 μ M GCV for two days.

Note: Following GCV selection, many colonies were eliminated. GCV served as a negative selection agent by killing cells that retained the TK-neo cassette, indicating random integration of the donor BAC at non-targeted chromosomal sites.

10. Day 16: Wash surviving mESC colonies (approximately 5,000–10,000 mESCs of each colony) once with PBS and passage them into 96-well plates coated with fresh DR4-feeder cells.

11. Expand surviving colonies for further analysis. The duration of colony expansion depends on the initial size of the colony when picked from the primary screening dish, typically requiring 4–6 days to reach sufficient confluence.

Note: Cells were split into 48-well plates (for frozen) and 24-well plates (for genomic DNA).

D. Identification of recombinants

1. PCR identification: extract genomic DNAs from puromycin- and GCV-selected mESC colonies using Wizard® Genomic DNA Purification kit (time: 9 h).

a. 5' end recombination was identified by PCR using primers (5'ArmF/5'ArmR) designed out of 5' homology arm (Figures 1 and 3A). Colonies with correct 5' end recombination had a 4.9 kb PCR band. Primer sequences are listed in Table S2. Set up PCR reactions as shown in Table 7 and run reactions following the program in Table 8.

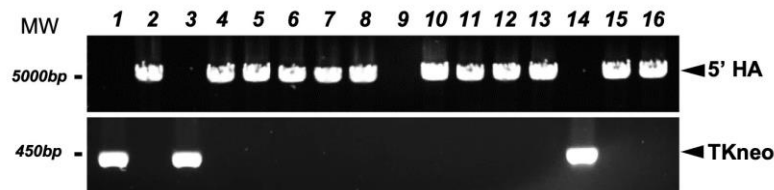


Figure 3. Characterization of mouse embryonic stem cell (mESC) clones with *hmTert* locus. PCR-based identification of candidate mESC clones. Primers designed outside of 5' HA detected a 4,888 bp fragment, indicating successful homologous recombination at the 5' end. Primers targeting the TKneo cassette amplified a 473 bp band, indicating random integration of the donor BAC.

Table 7. PCR reaction

Components	Amount
10× LA PCR buffer (Mg ²⁺ PLUS)	1 µL
LA Taq polymerase	0.1 µL
dNTP (2.5 mM)	1.6 µL
Primers (10 µM)	0.2 µL
mESCs genomic DNA (10ng/µL)	1.0 µL
ddH ₂ O	6.1 µL
Total	10 µL

Table 8. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial denaturation	94 °C	1 min	1
Denaturation	98 °C	10 s	31 cycles
Annealing	68 °C	5 min	
Final extension	72 °C	10 min	
Hold	4 °C		1

b. Detect TK-neo sequence by PCR using primers TKneoF and TKneoR. Amplification of a 473 bp PCR product suggests that the mESC clone contained randomly integrated donor BAC constructs (Figure 3A). Primer sequences were listed in Table S2. Set up PCR reactions as shown in Table 9 and run reactions following the program in Table 10.

Table 9. PCR reaction

Components	Amount
10× ThermoPol® buffer	1 µL
Taq polymerase	0.1 µL
dNTP (10 mM)	0.2 µL
Primers (10 µM)	0.2 µL
mESCs genomic DNA (10 ng/µL)	1.0 µL
ddH ₂ O	7.5 µL
Total	10 µL

Table 10. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial denaturation	94 °C	5 min	1
Denaturation	95 °C	30 s	35 cycles
Annealing	60 °C	30 s	
Extension	72 °C	45 s	
Final extension	72 °C	5 min	1
Hold	4 °C		

Among 110 puromycin- and GCV-resistant clones, 84 yielded a 4.9 kb PCR fragment, indicating that these clones had undergone homologous recombination at the 5' end.

2. For further verification, verify correct recombination with Southern blot analysis of the entire chimeric *hmTert* region (time: 4 d).

a. Day 1: Digest 2.0 µg mESCs genomic DNAs with BamHI, HindIII, or EcoRV, followed by hybridization with different probes. Correctly recombined *hmTert* sequences and wildtype *mTert* alleles were identified by comparing the size of restriction fragments (Figure 4A). Set up restriction enzyme digestion reactions as shown in Table 11.

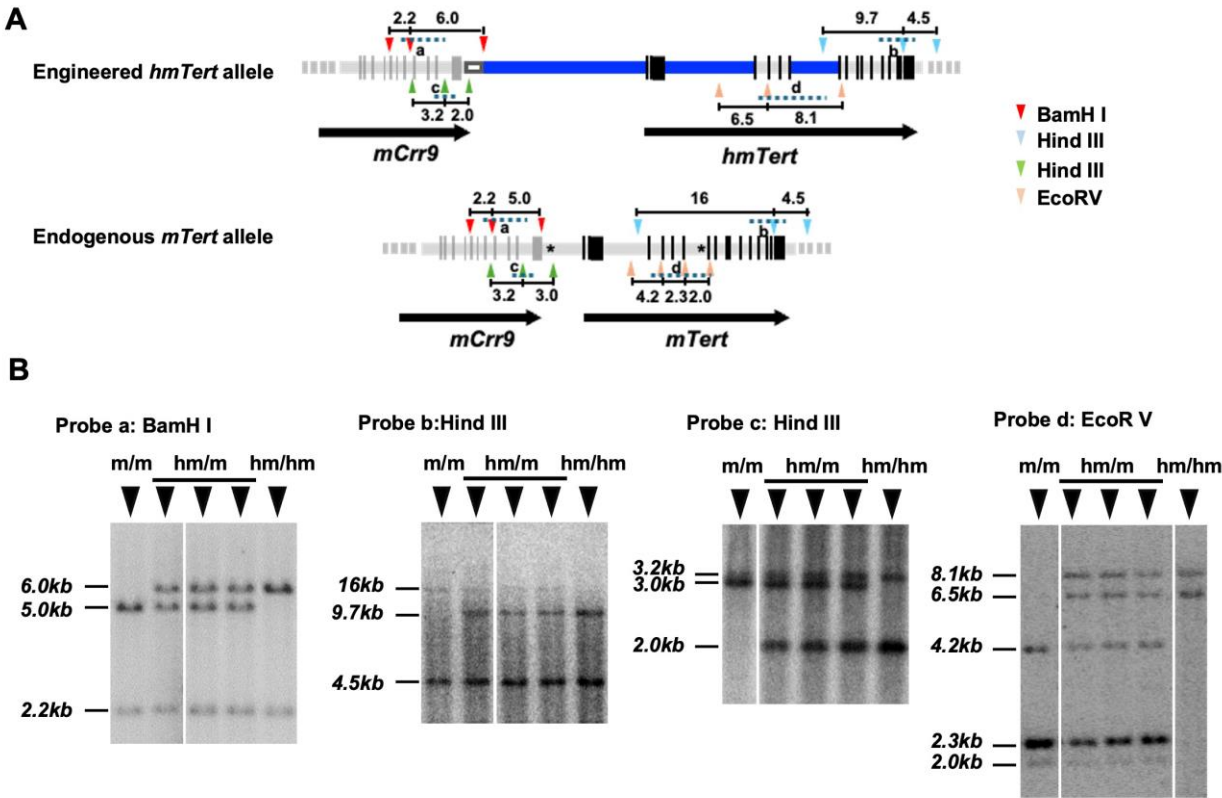


Figure 4. Characterization of the *hmTert* alleles by Southern blot analysis. (A) Schematic representation of the *hmTert* and *mTert* alleles. Restriction enzyme sites (BamHI, HindIII, and EcoRV) used in the analysis are indicated by arrowheads on corresponding colors. Distance between restriction sites are labeled in kilobases (kb). Probes used for hybridization are shown as dashed lines. Black vertical bars represent exons, and asterisks (*) mark the sgRNA cleavage sites. (B) Representative Southern blot images using four different DNA probes. Genomic DNA (2 µg) from individual mouse embryonic stem cell (mESC) clones were digested with the indicated enzymes and hybridized with ³²P-labeled DNA probes. Genotypes are indicated as follows: *m/m*, homozygous *mTert* alleles (*Tert*^{+/+}); *hm/m*, heterozygous *hmTert/mTert* alleles (*Tert*^{h/+}); *hm/hm*, homozygous *hmTert* alleles (*Tert*^{h/h}).

Table 11. Restriction enzyme reaction

Components	Amount (μL)
10× NEB buffer	2.5 μL
Restriction enzyme	0.4 μL
mESCs genomic DNA	2.0 μg
ddH ₂ O	up to 25 μL
Total	25

Incubate at 37 °C overnight in a water bath.

b. Day 2: Analyze digested genomic DNAs on a 0.7% agarose gel with 0.5× TBE for 4 h. Wash agarose gels with denature buffer twice for 20 min each, followed by washing with neutralization buffer twice for 20 min each at room temperature. Rinse the agarose gels with 10× SSC buffer and transfer DNAs from gel to nylon membranes through the capillary transfer method at room temperature overnight.

c. Day 3: Wash the nylon membranes with 2× SSC once and UV-crosslink. Rinse the membranes with 2× SSC and pre-hybridize with Church buffer at 60 °C in rotating tubes for 4 h. Meanwhile, label the probes with ³²P-dCTP using the Ladderman DNA Labeling kit according to the manufacturer's instructions. Hybridize the membranes with 5 mL of fresh Church buffer containing 2 μL of ³²P-labeled DNA probes in rotating tubes overnight at 60 °C.

d. Day 4: Wash membranes three times with 1× SSC (+0.1% SDS) for 10 min each at room temperature and in 0.2× SSC (+0.1% SDS) twice for 15 min each at 60 °C. Detect signals using the PhosphorImager system (GE Healthcare) (Figure 4B).

Note: Our data showed that approximately 80% (130 out of 156) of puromycin- and GCV-resistant clones underwent successful homologous recombination following CRISPR/Cas9-mediated double-stranded breaks. Among these, 29% (45 out of 156) harbored the correctly targeted hmTert locus.

E. Removal of the puromycin cassette

Four mESC clones with the correctly targeted *hmTert* locus were selected for removal of the puromycin resistance cassette.

1. Transient transfection with pCBM plasmid (Cre recombinase expression vector) (time: 6 d).

a. Day 1: Seed 1.8×10^6 *hmTert* knocked-in mESCs [9] into each well of a 24-well plate containing DR4 feeder cells. Cells were transfected with 0.2 μg of the pCBM plasmids using Lipofectamine 2000. Eight hours post-transfection, passage cells into a 6-well plate.

b. Day 2: Culture cells with fresh mESCs culture medium.

c. Day 4–6: Seed individual mESC colonies per well in a 96-well plate for expansion and further analysis.

2. Identification of puromycin-negative colonies (time: 1 d).

Colonies that had successfully excised the puromycin cassette were identified by PCR amplification of a 333-bp fragment using primer pair RePuroF1/RePuroR1, which flanks the puromycin cassette (Figure 5). Primer sequences are listed in Table S2. Set up PCR reactions as shown in Table 12 and run the reactions following the program in Table 13.

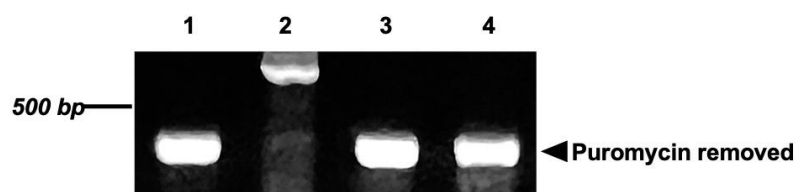


Figure 5. Identification of puromycin cassette removal in *hmTert* knock-in mouse embryonic stem cell (mESC) clones. Genomic DNA was amplified using the primer pair RePuroF1/RePuroR1. Clones 1, 3, and 4 produced a 333 bp PCR fragment, confirming successful excision of the puromycin resistance cassette.

Table 12. PCR reaction

Components	Amount
10× ThermoPol® buffer	1 µL
Taq polymerase	0.1 µL
dNTP (10 mM)	0.2 µL
Primers (10 µM)	0.2 µL
Genomic DNA (10 ng/µL)	1.0 µL
ddH ₂ O	7.5 µL
Total	10 µL

Table 13. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94 °C	5 min	1
Denaturation	95 °C	20 s	36 cycles
Annealing	60 °C	30 s	
Extension	72 °C	45 s	
Final Extension	72 °C	5 min	1
Hold	4 °C		

F. Generation of chimeric mice

1. Inject G4 mESCs (*Tert*^{h/+}), carrying one *hmTert* and one wildtype *mTert* allele, into C57BL/6J embryos (Transgenic Core, Cornell University, US). Six chimeric mice were obtained as indicated by coat coloration (time: 18 d).
2. Detect *hmTert* or *mTert* mRNA expression (time: 6 h):
 - a. Isolate total RNAs from various tissues of chimeric mice using TRI reagent according to the manufacturer's instructions.
 - b. Synthesize first-strand cDNAs using SuperScript™ III reverse transcriptase.
 - c. *hmTert* or *mTert* mRNA expression levels were determined by quantitative RT-PCR using PowerUp™ SYBR™ Green Master Mix (Figure 6A). Primer sets are listed in Table 14. *Tert* mRNAs from *hmTert* or *mTert* allele were distinguished by nine silent nucleotide substitutions near the 3' end of exon 2 of the *hmTert* locus, which overlapped with the forward primers [5]. Primer sequences are listed in Table S2.

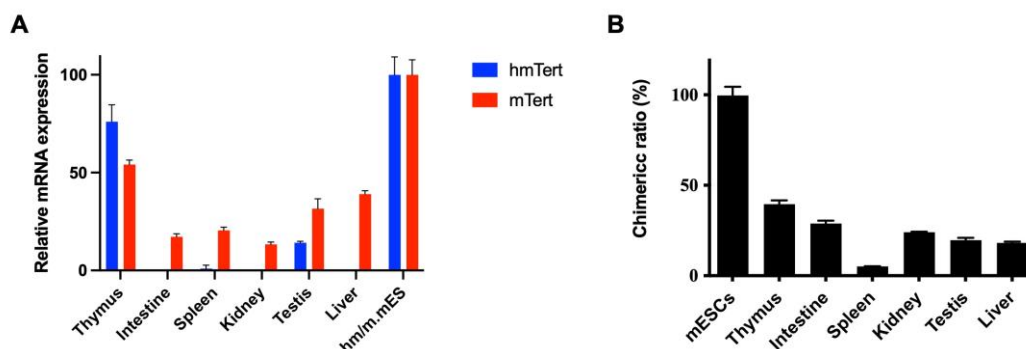


Figure 6. Analysis of chimera mice. (A) Detection of *hmTert* mRNA in tissues of a chimera mouse. Total RNAs were isolated from tissues, and *hmTert* and *mTert* mRNA levels were quantified by qRT-PCR. Expression levels were normalized to the chimeric ratio in the corresponding tissues (see panel B). *Tert*^{h/+} mouse embryonic stem cells (mESCs) were used as the control, and mRNA levels were normalized to 18S rRNA. (B) Chimeric ratio in mouse tissues. The chimeric contribution of *hmTert*/*mTert* in various tissues was determined by quantitative genomic PCR targeting *hmTert* and *mTert* sequences. The ratio of mESCs (*Tert*^{h/+}) was defined as 100%. Data are presented as mean values with SD.

Table 14. Primer sets used for quantitative RT-PCR

Allele	Primer sets
<i>hmTert</i>	mTert.Mut F/ mTert.Endo R
<i>mTert</i>	mTert.Endo F/ mTert.Endo R
18s	18s RNA control F/18s RNA control R

- d. The chimeric ratios of various tissues were determined by quantitative PCR of 5' end recombination site. Isolate genomic DNAs from the phenol phase and interphase of the Trizol/chloroform step of total RNA isolation. Add 1/3 volume of 100% ethanol per TRI reagent and mix solution by inversion. Incubate the samples at room temperature for 5 min and then centrifuge at 2,000× g for 5 min at 4 °C to precipitate DNA. Wash the DNA pellets twice with DNA wash solution for 30 min at room temperature, and enrich the DNAs again by centrifuging samples at 2,000× g for 5 min at 4 °C. Wash the DNA twice with 75% ethanol. Dry DNA pellets at room temperature, and dissolve pellets in 8 mM NaOH. Adjust the pH of the solution with 0.1 M HEPES to pH 8.0. Add EDTA to the final concentration of 1 mM to stabilize DNA.
- e. The ratios of *hmTert* and *mTert* alleles were assessed by quantitative PCR using PowerUp™ SYBR™ Green Master Mix and the primer sets listed in Table 15 (Figure 6B). Primer sequences are listed in Table S2.

Table 15. Primer sets used for quantitative PCR

Allele	Primer sets	Chromosome position
<i>hmTert</i>	hmTert up5k F/ hmTert up5k R	chr5: 1300555-1300574/chr5: 1300435-1300454
<i>mTert</i>	mTert up5k F/ mTert up5k R	chr13: 73770040-73770059/chr13: 73621998-93622018

G. Reducing telomere length in mice with the *hmTert* allele

1. Cross male chimeric mice with C57BL/6J female mice, resulting in progeny with a germline *hmTert* allele (F0). Cross again the mice with C57BL/6J mice to generate F1 *Tert^{hm/+}* mice with 88% C57BL/6J genetic background. Mouse genotypes were determined by PCR using primers specific for *hmTert* or *mTert* alleles. Isolate genomic DNAs from the toes of 7-day-old mice using the Wizard® Genomic DNA Purification kit and the primer sets listed in Table 16. Primer sequences are listed in Table S2 (time: 6 months).

Table 16. Primer sets used for mouse genotyping

Allele	Primer sets	PCR amplicon length (bp)
<i>hmTert</i>	hmTert F/ hmTert R	363
<i>mTert</i>	mTert F/ mTert R	467

Set up PCR reactions as shown in Table 17 and run the reaction following the PCR program in Table 18.

Table 17. PCR reaction

Components	Amount
10× ThermoPol® buffer	1 µL
Taq polymerase	0.1 µL
dNTP (10 mM)	0.2 µL
Primers (10 µM)	0.2 µL
Mouse genomic DNA (10 ng/µL)	1.0 µL
ddH ₂ O	7.5 µL
Total	10 µL

Table 18. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial denaturation	94 °C	5 min	1
Denaturation	94 °C	30 s	35
Annealing	60 °C	30 s	
Extension	72 °C	30 s	
Final extension	72 °C	5 min	1
Hold	4 °C		

2. *Tert^{h/-}* mice were generated by crossing F1 *Tert^{h/+}* mice with *Tert^{+/-}* mice (C57BL/6J background, a gift of Dr. Lea Herrington). The resulting (*Tert^{h/-}*) mice were subsequently bred with *Tert*-knockout (*Tert^{-/-}*, C57BL/6J) mice for successive generations. The *Tert*-KO alleles were detected by PCR using primers (mTert-KO F/ mTert-KO R), which were designed to span the *mTert* knockout region as previously reported [11], producing a 467 bp amplicon. Primer sequences are listed in Table S2. Set up PCR reactions following Table 19 and run the reactions following Table 20.

Table 19. PCR reaction

Components	Amount
10× ThermoPol® buffer	1 μL
Taq polymerase	0.1 μL
dNTP (10 mM)	0.2 μL
Primers (10 μM)	0.2 μL
Mouse genomic DNA (10 ng/μL)	1.0 μL
ddH ₂ O	7.5 μL
Total	10 μL

Table 20. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial denaturation	94 °C	5 min	1
Denaturation	94 °C	30 s	32
Annealing	60 °C	30 s	
Extension	72 °C	30 s	
Final extension	72 °C	5 min	1
Hold	4 °C		

3. The *Tert*^{h/-} mice from the fourth-generation *Tert*-KO breeding were intercrossed for 20 generations, from G4.1 to G4.20 (Figure 7A) (time: 5 years).

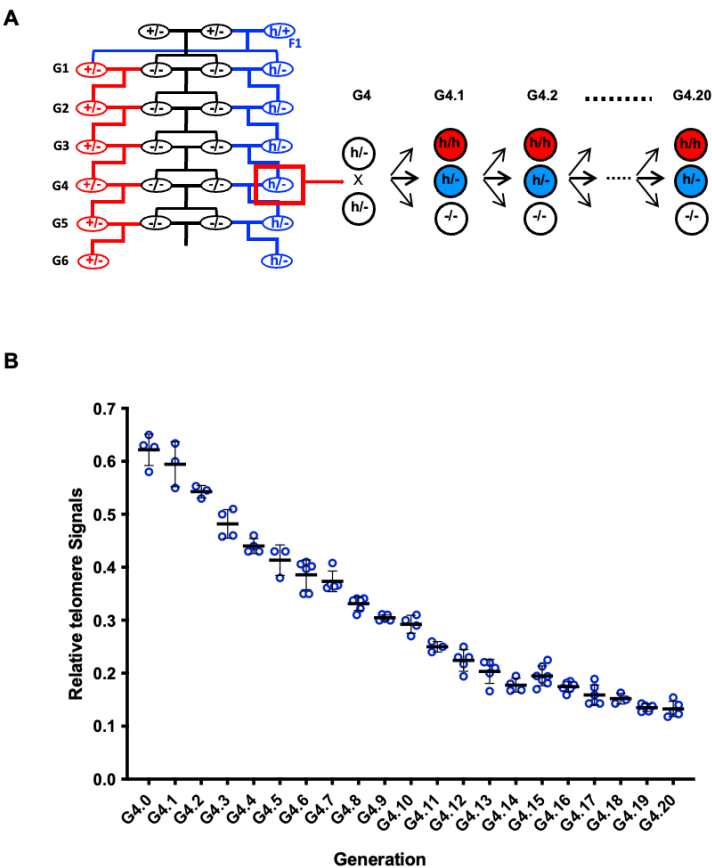


Figure 7. Telomere lengths in mice carrying the *hmTert* gene. (A) Breeding strategy. *Tert*-knockout mice (*Tert*^{-/-}) were initially generated by intercrossing *mTert* heterozygous (*Tert*^{+/-}) parents. In each subsequent generation, *Tert*^{-/-} mice were bred with the corresponding generation of either *Tert*^{+/-} or *Tert*^{h/-} mice. This strategy established and maintained two parallel heterozygous lines, *Tert*^{+/-} and *Tert*^{h/-}, through six generations (G1–G6). Beginning at generation 4 (G4), *Tert*^{h/-} mice were intercrossed over successive generations, resulting in progressive telomere shortening and the eventual establishment of

human-like telomere lengths. (B) Average telomere lengths across generations of *Tert*^{h/-} intercrosses. Telomere lengths in splenocyte were measured by Flow-FISH. Hybridization signals were normalized with those of wildtype C57BL/6J mice (*Tert*^{+/+}), which have an average telomere length of approximately 50 kb. Data are presented as mean values with SD.

4. Average telomere lengths in *Tert*^{h/-} mice were determined by Flow-FISH (time: 2 d).

- a. Collect spleens from adult mice in 1.5 mL tubes with 0.5 mL of HBSS or RPMI medium (+2% FBS).
- b. Smash spleens on a 70 μ m strainer on top of a 50 mL tube using a stick. Resuspend splenocytes in 1.5 mL of HBSS or RPMI medium (+2% FBS) and centrifuge tubes at 800 \times g for 10 min.
- c. Resuspend cell pellets in 5 mL of RBC lysis buffer for 5 min at room temperature.
- d. Halt the lysis process by adding 5 mL of complete RPMI medium (+10% FBS). Centrifuge samples at 800 \times g for 5 min. Resuspend the pellets in 10 mL of PBS (+2% FBS) and centrifuge again at 800 \times g for 5 min to enrich the cells.
- e. Filter cell suspensions through a 70 μ m strainer. Count cell numbers and move 4 million cells into 1.5 mL tubes. Add PBS (+0.1% BSA) to a final volume of 1 mL. Centrifuge tubes at 800 \times g for 5 min.

Note: The cell count typically ranges from 1 to 4 million. However, the loss and clogging of splenocytes during the experimental procedure can reduce the final yield of single cells, which are used for probe signal quantification. To ensure reliable data, it is recommended to start with a higher number of cells for hybridization.

- f. Resuspend cell pellets by vortexing for 10 s and then mix with 500 μ L of hybridization buffer containing the FISH probe (TelC-FAM, 0.14 μ g/mL). Three samples without the probe are included as negative controls. Incubate tubes at 80 $^{\circ}$ C for 10 min with continuous shaking, then allow to cool to room temperature and leave overnight in the dark.
- g. Centrifuge the samples at 800 \times g for 10 min at 16 $^{\circ}$ C. Vortex the pellets for 20 s and resuspend cells with 1 mL of wash solution I.

Note: Approximately 20 μ L of residual solution is left in the tube to aid in resuspending the cell pellets by vortexing, helping to reduce cell clogging.

- h. Repeat step G4g.
- i. Centrifuge the samples at 800 \times g for 10 min at 16 $^{\circ}$ C. Vortex the pellets for 20 s and resuspend cells with 1 mL of wash solution II.
- j. Centrifuge the samples at 800 \times g for 10 min at 16 $^{\circ}$ C. Vortex the pellets and incubate cells with 400 μ L of PBS with 5 μ L of 7-AAD.
- k. Filter the cells through a 40 μ m strainer and analyze samples using the flow cytometry machine Cytotflex. Capture the telomere and DNA signals using the appropriate channels: B525 for FAM and Y675 for 7-AAD. Analyze the data using the CytExpert software (Figure S2). Telomere signals of *Tert*^{h/-} mice were normalized with those of wildtype C57BL/6J mice (Figure 7B, Figure S2).

Validation of protocol

A. Validation of *hmTert* allele engineering in mESC cells

The protocol has been validated and successfully applied to engineer the *hmTert* allele in an additional mESC cell line, TC1, as described in the following publications:

Zhang et al. [8]. Crispr/Cas9-mediated cleavages facilitate homologous recombination during genetic engineering of a large chromosomal region. *Biotechnology Bioengineer* (Table 2)

Cheng et al. [5]. Engineering a humanized telomerase reverse transcriptase gene in mouse embryonic stem cells. *Scientific Report* (Figure 3, Figure S1, and Table S1)

B. Validation of telomere length shortening in mice with the *hmTert* allele

This protocol has also been validated and applied in the following publication to establish a parallel breeding line, successfully generating *Tert*^{h/-} heterozygous mice with human-like telomere lengths:

Zhang et al. [12]. Modification of the telomerase gene with human regulatory sequences resets mouse telomeres to human length. *Nature Communications* (Figure 8)

General notes and troubleshooting

General notes

1. Since the larger DNA fragment (about 47 kb) was used in this protocol, Southern blotting and PCR analyses were conducted to ensure the accuracy of engineered sequences.
2. Cells are more prone to clogging after hybridization with a FISH probe, primarily due to the high temperature involved in the process. To minimize background noise and prevent cell aggregation, it is crucial to thoroughly wash and resuspend the cells. This includes vortexing the cell pellets before adding the washing buffer and mixing the suspension thoroughly by pipetting up and down.

Supplementary information

The following supporting information can be downloaded [here](#):

1. Table S1. Scores of potential sgRNAs
2. Table S2. Sequences of oligo primers
3. Figure S1. Illustration map of pSpCa9 (BB)-2A-GFP
4. Figure S2. Mice telomere length was quantified using flow cytometry analysis of TelC-FAM fluorescence intensity
5. SEQ 1. Sequence of 5' end homologous recombination arm (4,361 bp, chr.13: 73764794-73769155)
6. SEQ 2. Sequence of 3' end homologous recombination arm (7,152 bp, chr13: 73787275-73794428)
7. SEQ 3. Input sequence for sgRNA1 design (1,000 bp, chr13: 73769582-73770581, 5' intergenic region between *mTert* and *mCrr9*)
8. SEQ 4. Input sequence for sgRNA2 design (1,000 bp, chr13: 73786261-73787260, intron 6 of *mTert*)

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Competing interests

The authors declare no competing interests.

Ethical considerations

All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Animals and were approved under protocols ASAF#6659 and ASAF#6693 by the Institutional Animal Care and Use Committee of Washington State University.

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