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Restoration of telomerase activity rescues chromosomal instability and premature aging in *Terc*$^{-/-}$ mice with short telomeres

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Reconstitution of telomerase activity is proposed as a potential gene therapy to prevent, or rescue, age-related diseases produced by critical telomere shortening. However, it is not known whether or not short telomeres are irreversibly damaged. We addressed this by re-introducing telomerase in late generation telomerase-deficient mice, *Terc*$^{-/-}$, which have short telomeres and show severe proliferative defects. For this, we have crossed these mice with *Terc*$^{+/-}$ mice and analyzed telomere length, chromosomal instability and premature aging of the progeny. The *Terc*$^{-/-}$ progeny had one set of chromosomes with normal telomeres, whereas the other set remained with critically short telomeres; these mice presented chromosomal instability and premature aging. In contrast, *Terc*$^{+/-}$ progeny showed all chromosomes with detectable telomeres, and did not show chromosomal instability or premature aging. These results prove that critically short telomeres can be rescued by telomerase, and become fully functional, thus rescuing premature aging. This has important implications for the future design of telomerase-based gene therapy of age-related diseases.

INTRODUCTION

Telomeres are DNA–protein complexes at the ends of eukaryotic chromosomes that protect them from degradation, recombination and DNA repair activities (reviewed in Blackburn, 1991, 2000; Greider, 1996). Loss of telomeric function by loss of telomeric repeats (TTAGGG in all vertebrates), or by mutation of telomere-binding proteins (i.e. TRF2, Ku proteins, DNA-PKcs), results in increased chromosomal instability both in cultured cells and in mice (Counter *et al.*, 1992; Blasco *et al.*, 1997; van Steensel *et al.*, 1998; Hsu *et al.*, 2000; Samper *et al.*, 2000; Goytisolo *et al.*, 2001).

Telomerase, the cellular reverse transcriptase (Tert, telomerase reverse transcriptase) that elongates telomeres *de novo* using an associated RNA molecule (*Terc*, telomerase RNA component) as template (Greider and Blackburn, 1985; reviewed in Nugent and Lundblad, 1998), has been at the spotlight of biomedicine due to its potential use in designing gene therapies for both cancer and aging. On one hand, telomerase activity is upregulated in the vast majority of human tumors compared with normal somatic tissues (reviewed in Shay and Bacchetti, 1997), and its inhibition in human tumor cell lines leads to telomere shortening and loss of cell viability (Hahn *et al.*, 1999; Zhang *et al.*, 1999), suggesting that telomerase inhibition could be an effective way to abolish tumor growth (reviewed in Zumstein and Lundblad, 1999). In support of this, mice that lack telomerase and have critically short telomeres display severe proliferative defects and, in the presence of wild-type p53, they are less susceptible to developing tumors (Chin *et al.*, 1999; Greenberg *et al.*, 1999; González-Suárez *et al.*, 2000). Conversely, re-introduction of telomerase activity in cultured human primary cells results in telomere maintenance and immortal growth (Bodnar *et al.*, 1998; Jiang *et al.*, 1999; Morales *et al.*, 1999; Ramirez *et al.*, 2001).

Late generation telomerase-deficient mice, *Terc*$^{-/-}$, have short telomeres and show defects in various proliferative tissues (Blasco *et al.*, 1997; Lee *et al.*, 1998; Herrera *et al.*, 1999a,b, 2000; Rudolph *et al.*, 1999). We show here that re-introduction of one copy of the *Terc* gene in these mice via *Terc*$^{+/-}$ × *Terc*$^{-/-}$ crosses is sufficient to elongate critically short telomeres, rescue chromosomal instability and prevent severe proliferative defects

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in these mice. These data clearly establish that the pathologies described in late generation *Terc*$^{-/-}$ mice are due to telomerase deficiency and telomere shortening and not to secondary mutations. These results also support the notion that telomerase activity re-introduction in adult somatic cells or tissues could be a potential approach for gene therapy of those age-related diseases triggered by telomere exhaustion, or for premature aging syndromes characterized by a faster rate of telomere shortening such as the Werner syndrome or Dyskeratosis congenita (Mitchell *et al.*, 1999; Wyllie *et al.*, 2000).

RESULTS AND DISCUSSION

Germline *Terc* re-introduction in late generation C57Bl6 *Terc*$^{-/-}$ mice via *Terc*$^{-/-}$ × *Terc*$^{+/-}$ crosses

Mice deficient for the *Terc* component of telomerase, *Terc*$^{-/-}$, lack telomerase activity and show telomere shortening and chromosomal instability with increasing mouse generations until infertility occurs at the fourth (G4) or sixth (G6) generation depending on the genetic background (G4 for mice in a pure C57Bl6 background; G6 for mice in a mixed C57Bl6/129Sv/SJL background) (Blasco *et al.*, 1997; Lee *et al.*, 1998; Herrera *et al.*, 1999b; Rudolph *et al.*, 1999). In contrast to late generation G6 *Terc*$^{-/-}$ in the mixed background (Rudolph *et al.*, 1999), late generation G4 *Terc*$^{-/-}$ mice in the C57Bl6 background show severe phenotypes at very young ages (1–5 months of age) (Herrera *et al.*, 1999b; this paper). To study whether re-introduction of the *Terc* gene in the germline of these mice is sufficient to prevent proliferative defects associated to telomere shortening, we studied the progeny of G3 *Terc*$^{-/-}$ × *Terc*$^{+/-}$ crosses. The G4 progeny resulting from this cross will have a set of chromosomes with short telomeres from the G3 *Terc*$^{-/-}$ parent and a set of chromosomes with normal telomeres from the *Terc*$^{+/-}$ parent. However, only the G4 *Terc*$^{+/-}$ progeny will inherit a copy of the *Terc* gene and have telomerase activity reconstitution. We have focused our study on the full characterization of the parents and the progeny of a male G3 *Terc*$^{-/-}$ (number 1) × female *Terc*$^{+/-}$ (number 2) cross (Figure 1). Seven different littermates were obtained, three of them were G4 *Terc*$^{-/-}$ (numbers 4, 5 and 8 in Figure 1) and four of them were G4 *Terc*$^{+/-}$ (numbers 3, 6, 7 and 9 in Figure 1). Figure 1 shows the presence or absence of the wild-type *Terc* allele in the parents and the progeny using a PCR-based method to detect the wild-type and null *Terc* alleles (see Methods).

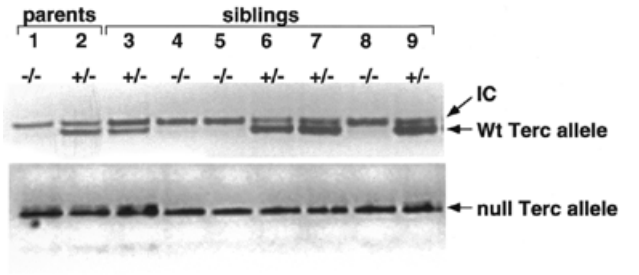

Fig. 1. Generation of G4 *Terc*$^{+/-}$ and G4 *Terc*$^{-/-}$ littermates via a G3 *Terc*$^{-/-}$ × *Terc*$^{+/-}$ cross. Wild-type and null *Terc* alleles were identified by PCR (see Methods). The positions of the PCR bands corresponding to the wild-type and null alleles, as well as that of an internal control (IC) for PCR efficiency, are indicated by arrows.

Re-introduction of the *Terc* gene is sufficient to restore short telomeres to their normal length in G4 mice

We addressed whether the G4 progeny that received the *Terc* wild-type allele rescued the critically short telomeres coming from the G3 *Terc*$^{-/-}$ father. For this, we first determined telomere length of the 'Y' chromosome in the G3 *Terc*$^{-/-}$ father and in the male G4 progeny using quantitative telomeric FISH on primary splenocytes (Q-FISH; Zijlmans *et al.*, 1997; Samper *et al.*, 2000). Table I and Figure 2A show that both p- and q-Y-telomeres were

Table I. Telomere length in parents and progeny from a *Terc*$^{+/-}$ × G3 *Terc*$^{-/-}$ cross

	Parents		Progeny (siblings)						
Generation	G3		G4	G4	G4	G4	G4	G4	G4
Genotype	–/–	+/–	+/–	–/–	–/–	+/–	+/–	–/–	+/–
Mouse number/sex	1 (xy)	2 (xx)	3 (xy)	4 (xy)	5 (xy)	6 (xy)	7 (xy)	8 (xy)	9 (xx)
Q-FISH[a]	p: 6.4 ± 0.4		p: 6.4 ± 0.4	p: 3.6 ± 0.4	p: 3.0 ± 0.3	p: 9.0 ± 0.5	p: 9.6 ± 0.7	p: 4.8 ± 0.3	
Y-chromosome[b]	q: 21.0 ± 1.6	female	q: 51.0 ± 1.9	q: 19.1 ± 1.7	q: 19.6 ± 1.4	q: 51.2 ± 2.0	q: 24.2 ± 1.8	q: 21.6 ± 1.5	female
	t: 13.7 ± 0.7		t: 28.7 ± 0.8	t: 11.4 ± 0.7	t: 11.3 ± 0.6	t: 30.1 ± 0.9	t: 16.9 ± 0.8	t: 13.2 ± 0.6	
Q-FISH[a]	p: 11.1 ± 1.2	p: 15.5 ± 0.6	p: 15.5 ± 0.5	p: 12.0 ± 0.5	p: 8.1 ± 0.9	p: 12.5 ± 0.6	p: 7.8 ± 0.3	p: 13.5 ± 0.7	p: 19.1 ± 0.7
chromosome 2[c]	q: 4.1 ± 0.3	q: 14.1 ± 0.6	q: 9.5 ± 0.5	q: 6.5 ± 0.6	q: 6.1 ± 0.9	q: 11.8 ± 0.7	q: 10.3 ± 0.4	q: 6.8 ± 0.6	q: 11.9 ± 0.4
	t: 7.6 ± 0.9	t: 14.8 ± 0.5	t: 12.5 ± 0.5	t: 9.2 ± 0.6	t: 7.1 ± 0.8	t: 12.1 ± 0.5	t: 9.0 ± 0.4	t: 10.1 ± 0.8	t: 15.5 ± 0.7
Q-FISH[a]	p: 16.3 ± 0.3	p: 18.6 ± 0.2	p: 14.6 ± 0.2	p: 13.8 ± 0.2	p: 13.7 ± 0.3	p: 18.2 ± 0.2	p: 16.9 ± 0.3	p: 14.3 ± 0.3	p: 20.6 ± 0.3
(all chromosomes)[b]	q: 16.1 ± 0.3	q: 22.7 ± 0.3	q: 18.3 ± 0.3	q: 16.6 ± 0.3	q: 16.0 ± 0.3	q: 20.8 ± 0.3	q: 18.2 ± 0.3	q: 16.5 ± 0.2	q: 21.1 ± 0.3
	t: 16.2 ± 0.3	t: 20.7 ± 0.2	t: 16.4 ± 0.2	t: 15.2 ± 0.3	t: 14.9 ± 0.3	t: 19.5 ± 0.3	t: 17.6 ± 0.3	t: 15.4 ± 0.3	t: 20.9 ± 0.3

[a]Data also shown in Figure 2A.
[b]Only males are included.
[c]Both males and females are included.
p, p-arm; q, q-arm; t, average of p- and q-arms.

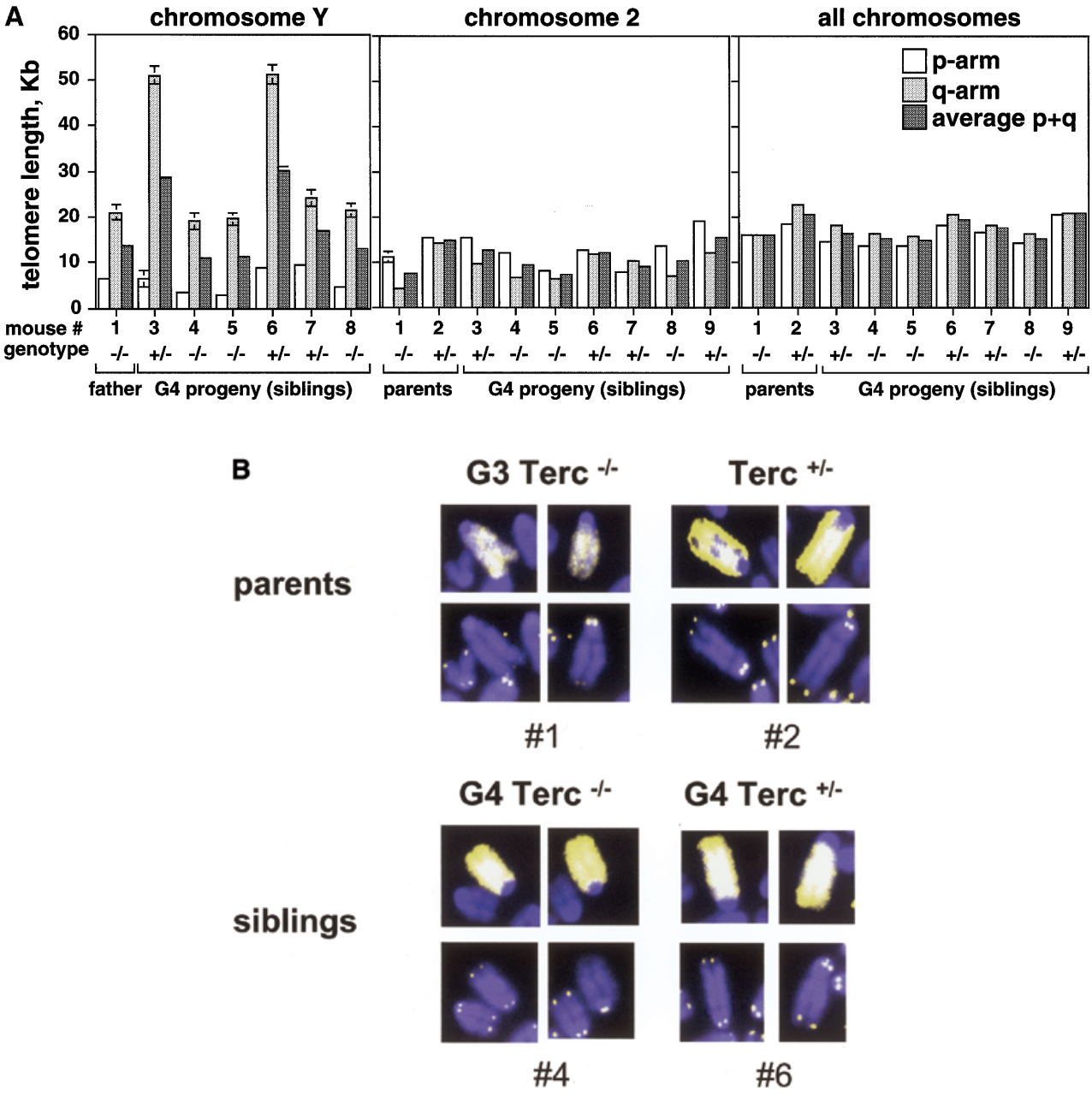

Fig. 2. (**A**) Telomere length of both p- and q-arms, as well as the average of both arms (average p+q) from chromosome Y, chromosome 2, as well as all chromosomes, are represented. Standard errors are also included; however, some of the errors are so small (see Table I) that the error bars are not visible. (**B**) Illustrative images of combined Q-FISH and chromosome 2 painting. For each case represented, both chromosome 2 are shown (mice numbers 1, 2, 4 and 6). Notice undetectable chr2-telomeres in the G3 Terc$^{-/-}$ father (number 1), as well as in the G4 *Terc*$^{-/-}$ sibling (number 4). The G4 *Terc*$^{+/-}$ sibling (number 6) and the *Terc*$^{+/-}$ mother (number 2) show detectable chr2-telomeres.

elongated from 6.4 ± 0.4 kb (p-arm) and 21.0 ± 1.6 kb (q-arm) in the father to an average length of 8.8 ± 0.5 kb (p-arm) and 42.1 ± 1.9 kb (q-arm) in the G4 *Terc*$^{+/-}$ siblings (average of mice 3, 6, 7 and 9 in Figure 2A and Table I). This corresponds to an average elongation of 2.0 and 21.1 kb for p- and q-telomeres, respectively. It is relevant to note that q-Y-telomeres were subjected to larger changes in length than p-Y-telomeres in the presence of the *Terc* gene, suggesting that they may be more 'accessible' to telomerase. Both p- and q-Y-telomeres shortened from 6.4 ± 0.4 kb (p-arm) and 21.0 ± 1.6 kb (q-arm) in the father to an average of 3.8 ± 0.3 kb (p-arm) and 20.1 ± 1.5 kb (q-arm) in the G4 *Terc*$^{-/-}$ siblings (average of mice 4, 5 and 8 in Figure 2A and Table I). This corresponds to an average shortening of 2.6 and 0.9 kb for p- and q-Y-telomeres, respectively, in agreement

with the absence of telomerase activity in these mice. Altogether, these results indicate that telomerase activity is able to elongate short telomeres (i.e. those of the parental G3 *Terc*$^{-/-}$ Y-chromosome) extending them to different lengths in the G4 *Terc*$^{+/-}$ progeny.

We also determined the length of chromosome 2 telomeres (chr2-telomeres) in the parents and the progeny using Q-FISH and a chr2-specific painting probe on primary splenocytes (Methods) (see Figure 2B for examples). Chromosome 2 was chosen for the study because, in the mouse, this chromosome has shorter telomeres than average (Hande *et al.*, 1999). Chr2-telomeres (average of p- and q-arms) in the G3 *Terc*$^{-/-}$ father were shorter than in the *Terc*$^{+/-}$ mother, 7.6 ± 0.9 and 14.8 ± 0.5 kb, respectively (mice 1 and 2 in Table I and Figure 2A). Figure 2B shows that the G3 father has undetectable telomeres in one chromosome 2, whereas the *Terc*$^{+/-}$ mother has both copies of chromosome 2 with normal telomeres. This is also illustrated by telomere length histograms, which show that the G3 *Terc*$^{-/-}$ father has a population of very short chr2-telomeres, which are not present in the *Terc*$^{+/-}$ mother (compare numbers 1 and 2 in Figure 3A; see also Figure 2B for representative examples). In the case of the G4 progeny, chr2-telomeres were longer in the siblings that received the *Terc* allele than in those that received the null allele, 12.3 ± 0.5 and 8.8 ± 0.7 kb, respectively (average of telomere length values shown for individual mice in Table I and Figure 2A; see Figure 2B for representative examples). Importantly, histograms depicting chr2-telomere length frequencies revealed that only those siblings that received the wild-type *Terc* allele rescued the population of very short chr2-telomeres inherited from the father (G4 *Terc*$^{+/-}$; numbers 3, 6, 7 and 9 in Figure 3A; see mouse number 6 in Figure 2B for a representative example), whereas those siblings that received the null allele showed a two-peak distribution of telomeres: a peak of very short telomeres (inherited from the father) and a peak of normal telomeres (inherited from the mother) (G4 *Terc*$^{-/-}$; numbers 4, 5 and 8 in Figure 3A, see mouse number 4 in Figure 2B for a representative example). These observations indicate that telomerase is able to rescue short telomeres in a mouse, as previously shown for Y-telomeres.

Curiously, we noticed that chr2-q-telomeres were shorter than chr2-p-telomeres both in the parents and the progeny, suggesting that the general assumption that p-telomeres are shorter than q-telomeres is not always true (Zijlmans *et al.*, 1997).

Q-FISH analysis of all-chromosome telomeres was also performed in the parents and the progeny. The average length of all telomeres was shorter in the G3 *Terc*$^{-/-}$ father than in the *Terc*$^{+/-}$ mother, 16.2 ± 0.3 and 20.7 ± 0.2 kb, respectively (Table I; Figure 2A). Similarly, those G4 siblings that received the wild-type *Terc* allele showed longer telomeres than the ones that received the null allele; average telomere lengths were 18.6 ± 0.2 and 15.6 ± 0.3 kb, respectively (Table I; Figure 2A). Histograms depicting telomere length frequencies also indicated that the G4 *Terc*$^{+/-}$ but not the G4 *Terc*$^{-/-}$ progeny rescued the population of critically short telomeres (notice telomeres ≤1 kb in the G4 *Terc*$^{-/-}$ but not the G4 *Terc*$^{+/-}$ progeny; Figure 3B). In agreement with this, all G4 *Terc*$^{-/-}$ siblings had a very high percentage of chromosome-ends with undetectable TTAGGG repeats by Q-FISH (~6% of all ends lacked detectable TTAGGG signals) compared with the G4 *Terc*$^{+/-}$ siblings (only ~0.2% of all ends lacked detectable TTAGGG signal) (Table II shows data for each individual mouse). Again, these results suggest that telomerase is sufficient to rescue short telomeres in a mouse, as shown above for Y- and chr2-telomeres.

Table II. Chromosomal aberrations in the parents and progeny of a G3 *Terc*$^{-/-}$ × *Terc*$^{+/-}$ cross

Mouse number and genotype	Chromosomal aberrations	Undetectable telomeres (%)
#1 G3 *Terc*$^{-/-}$	3.8 RT[a]	204/1761[b]
	3.8 B/F	(11.5%)
#2 *Terc*$^{+/-}$	0 RT	6/1580
	3.7 B/F	(0.3%)
#3 G4 *Terc*$^{+/-}$	0 RT	6/1568
	0 B/F	(0.4%)
#4 G4 *Terc*$^{-/-}$	11.1 RT	83/1573
	3.7 B/F	(5.3%)
#5 G4 *Terc*$^{-/-}$	38.7 RT	105/1272
	3.2 DIC	(8.25%)
	0 B/F	
#6 G4 *Terc*$^{+/-}$	0 RT	0/1600
	0 B/F	(0.0%)
#7 G4 *Terc*$^{+/-}$	0 RT	5/1600
	0 B/F	(0.3%)
#8 G4 *Terc*$^{-/-}$	8 RT	82/1596
	12 B/F	(5.1%)
#9 G4 *Terc*$^{+/-}$	3.4 RT	0/1612
	0 B/F	(0.0%)

[a]Aberrations per 100 metaphases.
[b]Ends lacking TTAGGG signal out of total ends analyzed. The percentage of undetectable telomeres is also shown in parentheses.
RT, Robertsonian-like chromosome; B/F, breaks/fragments; DIC, dicentric chromosome.

Curiously, Q-FISH analysis showed that Y-telomeres (especially Y-q-telomeres) were longer than the average of all chromosome telomeres, whereas those of chromosome 2 were shorter than average (Figure 1A).

As a different approach to estimate telomere length that is not based on fluorescence, we carried out terminal restriction fragment (TRF) analysis using Southern blotting (Methods). Figure 4 shows TRF analysis of both parents, as well as that of the progeny. First, it is noticeable that the G3 *Terc*$^{-/-}$ father shows low molecular weight TRFs (below the 9.4 kb marker), which are not present in the *Terc*$^{+/-}$ mother (Figure 4). All siblings that received the wild-type *Terc* allele from the *Terc*$^{+/-}$ mother, G4 *Terc*$^{+/-}$ (numbers 3, 6, 7 and 9), showed low molecular weight TRFs of ≥9.4 kb, whereas siblings that received the null *Terc* allele from the mother, G4 *Terc*$^{-/-}$ (numbers 4, 5 and 8), showed low molecular weight TRFs that were below the 6.5 kb marker (also indicated by white circles in Figure 4). These observations suggest that the appearance of low molecular weight TRF bands correlates with telomere shortening in the G4 progeny, and that G4 *Terc*$^{-/-}$ mice have shorter telomeres than the G4 *Terc*$^{+/-}$ littermates. The TRF analysis is also in agreement with the notion that short telomeres are elongated by telomerase when the *Terc* wild-type allele is present, as previously shown by Q-FISH.

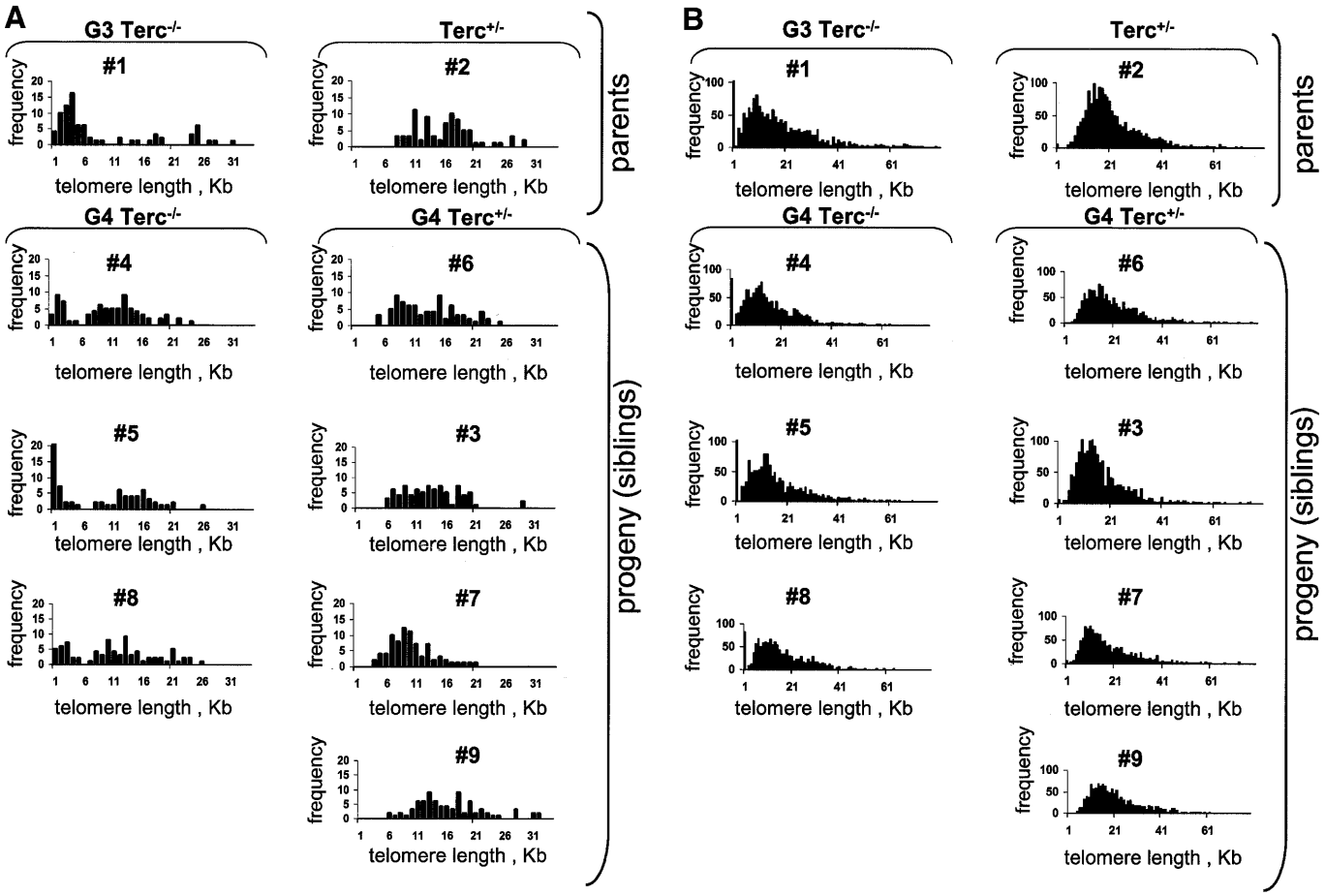

Fig. 3. (**A**) Telomere length distribution of chr2-telomeres in primary splenocytes from the parents and the G4 progeny as determined by Q-FISH. The histogram depicts a population of critically short chr2-telomeres in the G3 father and in the G4 Terc$^{-/-}$ littermates. In contrast, the G4 Terc$^{+/-}$ littermates show disappearance of the peak corresponding to short telomeres. (**B**) Telomere length distribution of all chromosome telomeres in primary splenocytes from the parents and the G4 progeny as determined by Q-FISH. The histogram depicts a population of critically short telomeres (≤1 kb) in the G3 father and in the G4 Terc$^{-/-}$ littermates. The G4 Terc$^{+/-}$ littermates show disappearance of ≤1 kb telomeres.

Rescue of chromosomal instability in G4 *Terc*$^{+/-}$ siblings

Critically short telomeres in late generation *Terc*$^{-/-}$ mice in two different genetic backgrounds result in telomere dysfunction and increased chromosomal instability, mostly consisting of end-to-end fusions (Robertsonian-like fusions and dicentrics) (Blasco *et al.*, 1997; Herrera *et al.*, 1999b). Here, we studied whether re-introduction of the *Terc* gene in these mice, hence of telomerase activity, is able to rescue chromosomal instability. For this, we scored chromosomal aberrations on primary splenocytes using Q-FISH. Table II shows the frequencies of different chromosomal aberrations in the parents and the G4 progeny. All G4 siblings that received the null allelle, G4 *Terc*$^{-/-}$ (numbers 4, 5 and 8 in Table II), showed increased end-to-end fusions, as well as chromosome breaks and fragments compared with the G4 *Terc*$^{+/-}$ littermates (numbers 3, 6, 7 and 9 in Table II) and with the *Terc*$^{+/-}$ mother (number 2 in Table II). As an exception, G4 *Terc*$^{+/-}$ mouse number 9 showed a low frequency of RT fusions; however, it did not show chromosome ends without detectable TTAGGG repeats (Table II), suggesting that these fusions were not due to critically short telomeres and may have been inherited directly from the father. The G4 *Terc*$^{-/-}$ progeny also showed a higher chromosomal instability than the G3 *Terc*$^{-/-}$ father (number 1 in Table II), in agreement with further telomere shortening in the absence of telomerase (see above).

Telomerase rescues premature aging phenotypes in G4 *Terc*$^{+/-}$ mice

To study whether telomerase-mediated rescue of short telomeres and of chromosomal instability also prevented the occurrence of severe proliferative phenotypes in the *Terc*$^{-/-}$ mice, we did a complete histopathological analysis of the parents and the G4 progeny 21 days post-partum (Table III). All G4 *Terc*$^{-/-}$ siblings (numbers 4, 5 and 8) showed bone marrow aplasia, spleens dramatically reduced in size (not shown), as well as different degrees of intestinal atrophy (see Figure 5 for example). As an exception, G4 *Terc*$^{+/-}$ mouse number 3 also showed reduced

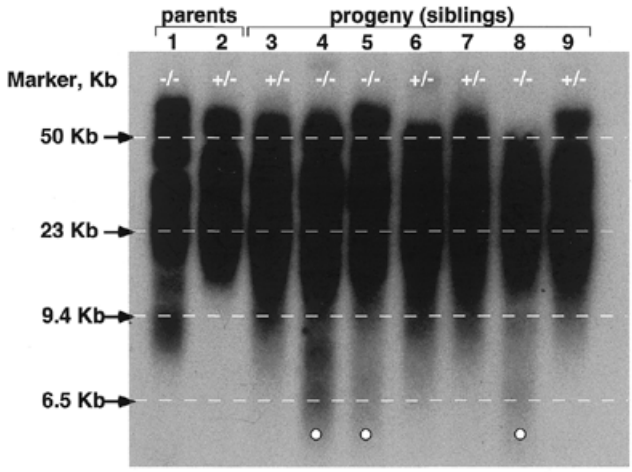

Fig. 4. Measurement of terminal restriction fragments (TRFs) in bone marrow cells from both parents and the G4 progeny. Decrease in telomere length is visualized by the appearance of TRF bands below the 6.5 kb marker in the G4 *Terc*$^{-/-}$ mice that are not present in the G4 *Terc*$^{+/-}$ littermates (indicated by open circles). +/–, *Terc*$^{+/-}$ (numbers 2, 3, 6, 7 and 9); –/–, *Terc*$^{-/-}$ (numbers 1, 4, 5 and 8).

spleen size; however, this could be due to the fact that the G4 mice were only 21 days old at the time of the analysis and there could be variability in spleen size at early ages. One of the G4 *Terc*$^{-/-}$ males, number 4, showed a very severe testicular atrophy (Figure 5). These disease states are typical of late generation telomerase knockout mice in a C57Bl6 genetic background and have been associated with premature aging in these mice (Herrera *et al.*, 1999b). In marked contrast, G4 *Terc*$^{+/-}$ littermates showed no pathologies, with the exception of a slight villi atrophy in mice numbers 3 and 6 (Table III). Figure 5 shows a comparison of three different tissues in two G4 littermates that carry, or not, a copy of the *Terc* gene (mice numbers 6 and 4, respectively). The rescue of severe premature aging phenotypes in the G4 progeny that received the *Terc* wild-type allele is in agreement with the previous data, which showed that all telomeres were restored to their normal length and chromosomal aberrations were prevented in these mice. However, those G4 mice that received the null *Terc* allele showed the characteristic severe disease states associated with short telomeres, in agreement with the fact that they carried a set of telomeres that were critically short (see Tables I and II, as well as Figure 3).

Conclusions

Here we show that re-introduction of telomerase activity in late generation *Terc*$^{-/-}$ mice is sufficient to elongate short telomeres, rescue chromosomal instability and prevent to a large extent the severe phenotypes associated with telomere shortening in these mice. These observations have important implications for the future design of telomerase-based gene therapy of age-related diseases. In particular, we show here that (i) telomerase can elongate short telomeres in a mammalian organism and that (ii) this restoration of short telomeres by telomerase is sufficient to rescue chromosomal instability and to prevent the severe premature aging phenotypes associated with critically short telomeres. From these studies one can speculate that diseases which relate to defects in telomerase function such as Dyskeratosis congenita (Mitchell *et al.*, 1999) might be treatable by restoring functional telomerase in stem cells of the affected individuals. Telomerase-based therapy can also be useful in patients who suffer from diseases characterized by a faster rate of telomere shortening, such as Werner syndrome patients, whose fibroblasts have recently been shown to recover proliferative capacity upon Tert over-expression (Wyllie *et al.*, 2000). Furthermore, from the results presented here one might extrapolate that a putative anti-cancer therapy based on transient telomerase inhibition in cancer patients should not lead to irreversible side-effects in highly proliferative tissues, since the recovery of telomerase activity in these tissues will rescue critically short telomeres and prevent chromosomal damage and loss of proliferative capacity.

METHODS

Generation and genotyping of G4 *Terc*$^{-/-}$ and G4 *Terc*$^{+/-}$ littermates. *Terc*$^{-/-}$ mice in the C57BL6 background (Bl6) were described

Table III. Phenotypes in littermate mice generated by *Terc*$^{+/-}$ × G3 *Terc*$^{-/-}$ (C57Bl6) intercrosses

	Parents		Progeny						
Generation	G3	G3	G4	G4[a]	G4	G4[a]	G4	G4	G4
Genotype	–/–	+/–	+/–	–/–	–/–	+/–	+/–	–/–	+/–
Mouse number/sex	1 (xy)	2 (xx)	3 (xy)	4 (xy)	5 (xy)	6 (xy)	7 (xy)	8 (xy)	9 (xx)
Testis	normal	–	normal	severe testicular atrophy	normal	normal	normal	normal	–
GI tract	slight villi atrophy in small intestine	normal	slight villi atrophy in small intestine	severe atrophy in small intestine	moderate atrophy in small intestine	slight villi atrophy in small intestine	normal	normal	normal
Bone marrow	normal	normal	normal	severe aplasia lymphoid cells	aplasia	normal	normal	aplasia	normal
Spleen	normal	normal	reduced size	reduced size	reduced size	normal	normal	reduced size	normal

[a]Images of the pathologies observed in these mice are shown in Figure 5.

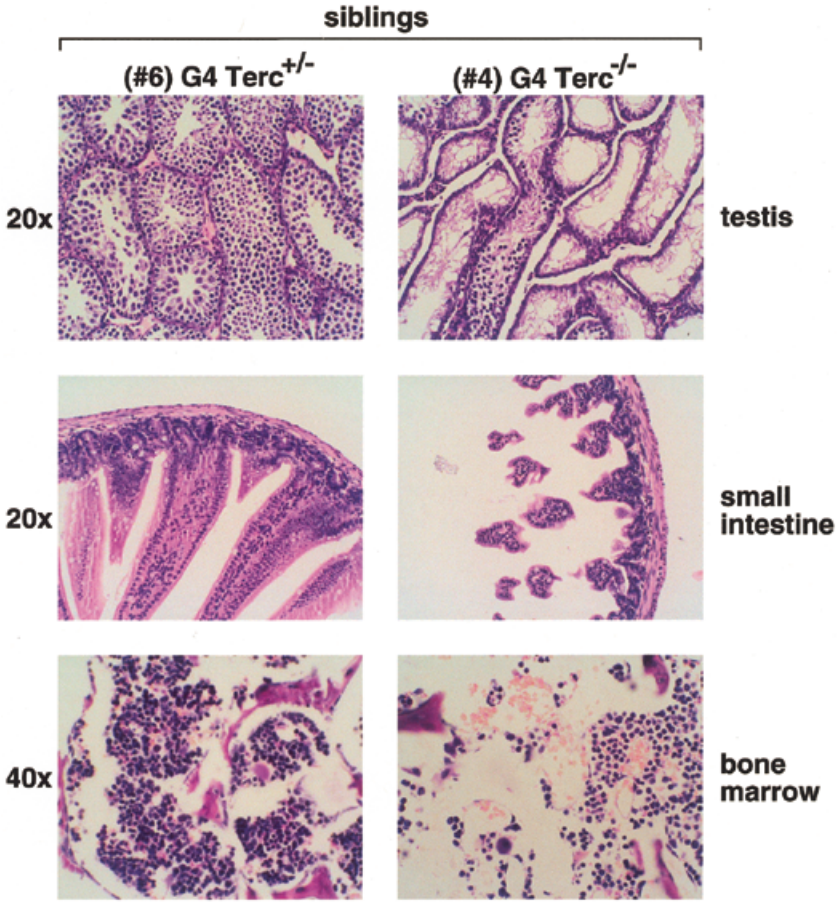

Fig. 5. Histology of testis, small intestine and bone marrow from littermate 21-day-old G4 *Terc*$^{-/-}$ and G4 *Terc*$^{+/-}$ siblings (numbers 4 and 6, respectively). Magnifications were 20 and 40×, as indicated.

elsewhere (Herrera *et al.*, 1999b). A 3-month-old *Terc*$^{-/-}$ male was crossed with a 6-month-old *Terc*$^{+/-}$ female to derive a litter of seven pups that were killed at 21 days post-partum due to the ill health of some of the littermates. Both parents were killed in parallel with the seven pups. A tail necropsy from all animals was taken to extract DNA for genotyping purposes. Germline identification of wild-type and null *Terc* alleles was carried out using both Southern blot genotyping (Blasco *et al.*, 1997) and PCR genotyping (Argilla and Hanahan, unpublished data).

Mice handling. Mice were housed at our barrier area, where pathogen-free procedures are employed in all mouse rooms. Quarterly health monitoring reports have been negative for all pathogens in accordance with FELASA recommendations (Federation of European Laboratory Animal Science Associations).

Histopathological analyses. Testis, bone marrow and small intestine sections from the parents and the progeny (21 days post-partum) were fixed in 10% buffered formalin and hematoxylin-eosin stained. Images were captured with an Olympus-Vanox microscope at 20 or 40× magnification, as indicated.

Telomere length measurements. Q-FISH spleens were removed, washed in sterile phosphate-buffered saline (PBS) and ground between two pieces of nylon mesh to obtain fresh splenocytes. After lysis with 0.75% ammonium chloride, splenocytes were counted and cell cultures with 3–5 million cells were initiated in RPMI 1640 with 20% fetal calf serum, antibiotics, 20 µg/ml lipopolysaccharide and 5 µg/ml Con A. After 70 h in culture, 0.1 µg/ml colcemid was added for 2 h and metaphase cells were prepared by standard methods. Splenocyte metaphases were hybridized with a PNA-tel probe, and telomere length was determined as described (Zijlmans *et al.*, 1997; Samper *et al.*, 2000) using the TFL-TELO program (gift of Dr Lansdorp, Vancouver, Canada). The location of the metaphases was recorded for further analysis with a chromosome 2 painting probe (see below). After capturing the images for Q-FISH analysis, the coverslips were removed with acetone and the slides were washed in PBS for 10 min. After increasing ethanol series, slides were air-dried and denatured in 70% formamide, 2× SSC at 65°C for 2 min. Following denaturation, slides were quenched in ice-cold 70% ethanol, dehydrated in increasing ethanol series and air-dried. The Cy3-labeled chromosome 2 painting probe was obtained from Cambio Ltd (UK). Fifteen microliters of painting probe per slide were denatured for 10 min at 65°C and re-annealed at 37°C for 1.5 h, and then hybridized to the metaphases at 42°C overnight. Subsequently, slides were

washed twice with 50% formamide in 2× SSC at 45°C for 5 min, and in 0.1× SSC at 45°C for 5 min. Finally, slides were mounted in Vectashield medium (Vector labs) with DAPI. Those same metaphases that were previously recorded for Q-FISH analysis were relocated, and the chromosome 2-hybridized metaphases were captured again. Fifteen different metaphases were analyzed in each case. For chromosomal instability determinations, between 25 and 31 metaphases were analyzed.

The chromosome Y was identified by reverse DAPI banding in the original image taken for Q-FISH.

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