FEBS Letters 583 (2009) 3076–3080

FEBS Letters

journal homepage: www.FEBSLetters.org

Short telomeres are preferentially elongated by telomerase in human cells

Bethan Britt-Compton, Rebecca Capper, Jan Rowson, Duncan M. Baird *

Department of Pathology, School of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, UK

ARTICLE INFO

Article history:
Received 22 April 2009
Revised 20 August 2009
Accepted 23 August 2009
Available online 29 August 2009

Edited by Varda Rotter

Keywords:
Telomere
Telomerase
Senescence
Replicative lifespan
Cancer

ABSTRACT

Short telomeres have been shown to be preferentially elongated in both yeast and mouse models. We examined this in human cells, by utilising cells with large allelic telomere length differentials and observing the relative rates of elongation following the expression of hTERT. We observed that short telomeres are gradually elongated in the first 26 PDs of growth, whereas the longer telomeres displayed limited elongation in this period. Telomeres coalesced at similar lengths irrespective of their length prior to the expression of hTERT. These data indicate that short telomeres are marked for gradual elongation to a cell strain specific length threshold.

1. Introduction

In the absence of telomerase, telomere length determines the replicative capacity of many cell types. The repression of telomerase in human somatic cells coupled with a limiting telomere length, is considered to confer a stringent tumour suppressive mechanism in long lived species such as humans [1]. Telomerase is expressed in stem cell populations; this is considered to slow, but not prevent, the rate of erosion and provides for additional replicative capacity to maintain tissue homeostasis [2]. Telomerase is also expressed in majority of cancers thereby circumventing the telomere driven tumour suppressive mechanism. Thus from the standpoints of both ageing and cancer biology there is a requirement to understand the mode of action of telomerase.

The original biochemical characterisation of telomerase activity in vitro indicated that human telomerase is comparatively processive, synthesising up to 390–420 nt (65–70 repeats) [3]. However, in vitro processivity is not directly related to telomeric extension in vivo [4]. Telomerase mediated lengthening has been most extensively studied in yeast where it has been shown that telomere elongation is inversely proportional to telomere length [5]. Telomerase is less processive on telomeres greater than 125 bp, with a 2–3 times increase in the processivity on telomeres <125 bp in length. This represents an increase in the rate of extension from 44 nt to over 100 nts, a significant proportion of the overall length of the telomere [4,5], indeed some telomeres may be extended by several multiples of their original length. These observations are consistent with data from mouse models that indicate that the shortest telomeres in a cell are preferentially elongated [6]. Studies of telomerase mediated lengthening in human cells indicate that telomerase levels must be limiting in order to maintain a telomere length homeostasis [7]. In these studies when the catalytic subunit of telomerase (hTERT) was expressed alone in telomerase negative fibroblast cells telomeres were elongated at a rate of 62 bp/PD, however if both hTERT and the telomerase RNA component (hTR) are expressed together the rate of elongation increases dramatically to 783 bp/PD [7]. The preferential elongation of short telomeres has not been demonstrated in human cells and indeed the rate of telomeric elongation as a function of the telomeric length is also unclear in human cells. Here we describe a simple experiment whereby we utilised a clonal fibroblast population that displays large allelic telomere length differentials, and we observed the relative rates of telomere elongation of individual alleles following the ectopic expression of hTERT. We observed that short telomeres are elongated at a rate of up to 544 bp/PD, whereas at a longer telomere no significant elongation was observed in the first 14 PDs growth. Elongation was accompanied by a commensurate increase in the heterogeneity of the telomere length profiles. These telomere dynamics resulted in a loss of the allelic and chromosome-specific telomere length differentials. We also observed that the telomere of 17p in a subset of cells was refractory to lengthening. These data are consistent with the presence of

* Corresponding author. Fax: +44 0 29 2068 7343.
E-mail address: bairddm@cardiff.ac.uk (D.M. Baird).

0014-5793/$36.00 © 2009 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2009.08.029

cis-acting determinants that regulate telomeric elongation in human cells.

2. Materials and methods

2.1. Cells

MRC5 cells were obtained from the Coriell Cell Repository and cultured at 37 °C in a low oxygen (3%) environment created using an oxygen sensor, regulator and nitrogen source. The cells were cultured in four parts Dulbecco's modified Eagle's medium to one part Medium 199 supplemented with 15% (v/v) foetal calf serum (Imperial Laboratories, London) as described previously [8]. Clones were taken from the parental culture using cloning rings.

2.2. Retroviral gene transfer

The catalytic subunit of telomerase, hTERT cloned into pBABE puro, was transduced into MRC5 cells using amphotropic retroviral vectors as previously described [8,9], and the empty vector used as a control. For infection, near senescent MRC5 clone 1 PD 23 were plated in 60 mm dishes at a density of 10^5 cells/dish and the following day exposed to retrovirus-containing medium from near-confluent producer cells, containing Polybrene at a concentration of 8 μg/ml. Two days later cells were passaged and maintained in medium containing puromycin at 2.5 μg/ml. Following puromycin selection the parental clonal cell culture was maintained for over 80 PDs. Clones were also picked from the parental hTERT culture.

2.3. DNA extraction and telomere length analysis

DNA extractions and STELA reactions at the 17p and XpYp telomeres were carried out as previously described [10]. DNA was extracted using standard proteinase K, RNase A, phenol/chloroform protocols, solubilized by digestion with EcoRI, and quantified it in triplicate by Hoechst 33258 fluorometry (Bio-Rad). The genomic DNA was diluted to 10 ng/μl in 10 mM Tris–HCl, pH 7.5. Ten nanograms of DNA was further diluted to 250 pg/μl in a volume of 40 μl containing 1 μM Telorette2 linker and 1 mM Tris–HCl, pH 7.5. Multiple PCRs (typically 6 reactions per sample) were carried out for each test DNA in 10 μl volumes containing 250 pg of diluted DNA, 0.5 μM of the telomere-adjacent and Teltail primers, 75 mM Tris–HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1.5 mM $MgCl_2$, and 0.5 U of a 10:1 mixture of Taq (ABGene) and Pwo polymerase (Roche). The reactions were cycled with an MJ PTC-225 thermocycler (MJ research) as described previously [10] with an extension at 68 °C for 10 mins. The DNA fragments were resolved by 0.5% TAE agarose gel electrophoresis, and detected by two separate Southern hybridizations with a random-primed α-^{33}P labeled (GE Healthcare) telomere repeat containing probe and telomere-adjacent probe together with a probe to detect the 1 kb (Stratagene) and 2.5 kb (Bio-Rad) molecular weight markers. The hybridized fragments were detected by phosphor imaging with a Molecular Dynamics Storm 860 phosphorimager (GE Healthcare). The molecular weights of the DNA fragments were calculated using the Phoretix 1D quantifier (Nonlinear Dynamics).

2.4. TRAP assay

For the TRAP assay, protein extracts were prepared from trypsinized cells and diluted to 5000 cell equivalents/reaction, where an oligonucleotide substrate was extended by telomerase, amplified by PCR, and products separated on 10% polyacrylamide gels as described previously [11].

3. Results

We choose to study telomerase mediated telomere elongation in MRC5 cells because this telomerase negative primary fibroblast strain displays a specific telomere length profile that is conducive for this work. The XpYp telomere-adjacent DNA of MRC5 contains multiple heterozygous single nucleotide polymorphic positions, these can be utilised to undertake allele-specific single telomere length analysis (STELA), which has the key advantage of being able to detect very short telomeres which are not represented in other assays [12]; in MRC5 the two XpYp telomeric alleles are widely divergent in length with a difference of over 5 kb [13]. The 17p telomeric sequence used for STELA is only present on one of the 17p alleles in MRC5, thus a single 17p telomeric allele can be analysed in isolation [14]. Telomere length of parental primary cell strains can be very heterogeneous which can confound the interpretation of telomere dynamics. We therefore picked single cell clones of MRC5 that exhibit homogeneous telomere length distributions, thus providing a more precise definition of the telomere length prior to the introduction of hTERT. We chose one clone that displayed the following telomere length profile at PD 23, 10 PD prior to the onset of senescence; 17p 1.52 kb ± 0.23 kb (mean ± S.D.), XpYp short allele 2.37 kb ± 0.39 kb and XpYp long allele 7.67 kb ± 0.52 kb).

We transfected the MRC5 clone at PD23 with either hTERT or an empty vector control, following puromycin selection the control culture underwent senescence 10.7 PDs after transfection, whereas the hTERT expressing culture continued to proliferate for a further 80 PDs until the experiment was discontinued (Fig. 1), telomerase activity was confirmed using the TRAP assay (data not shown).

We tracked telomere elongation at the short 17p allele, and the XpYp long and short alleles. Both the short alleles were rapidly elongated; during the first 26 PDs the rate of extension was 305 and 274 bp/PD for the 17p and XpYp short alleles, respectively (Fig. 2a and b). In contrast the long XpYp allele was not elongated during the first 14 PDs (Fig. 2a and b). Following this point there was a gradual increase in the mean telomere length at both XpYp alleles which was associated with an increase in the heterogeneity of the telomere length distribution (Fig. 2c).

In order to remove the confounding effects of the multiple different hTERT expressing cells in the bulk population we examined the telomere dynamics of a clonal population picked following transfection with hTERT. The earliest PD point that provided sufficient cells for analysis was PD 19, by this point all the telomeric alleles had been elongated to almost identical lengths irrespective of the starting telomere length, 11.6 kb ± 0.23 (mean ± S.D., Fig. 3a and b). As expected the telomere length heterogeneity was less in the clonal population (mean S.D. = 2.95 kb) compared to the

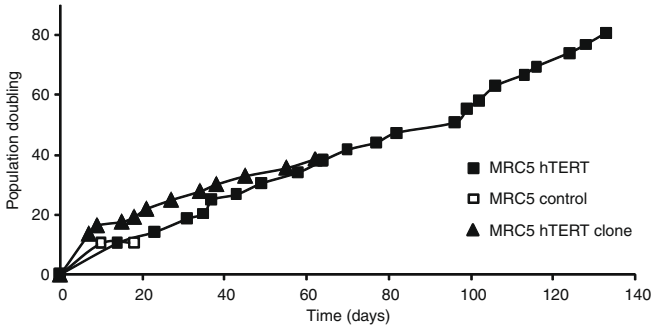

Fig. 1. Cell growth data of telomerase expressing MRC5 cells (filled markers) and empty vector control cells (unfilled markers). Population doublings and time are taken from the point of hTERT transfection.

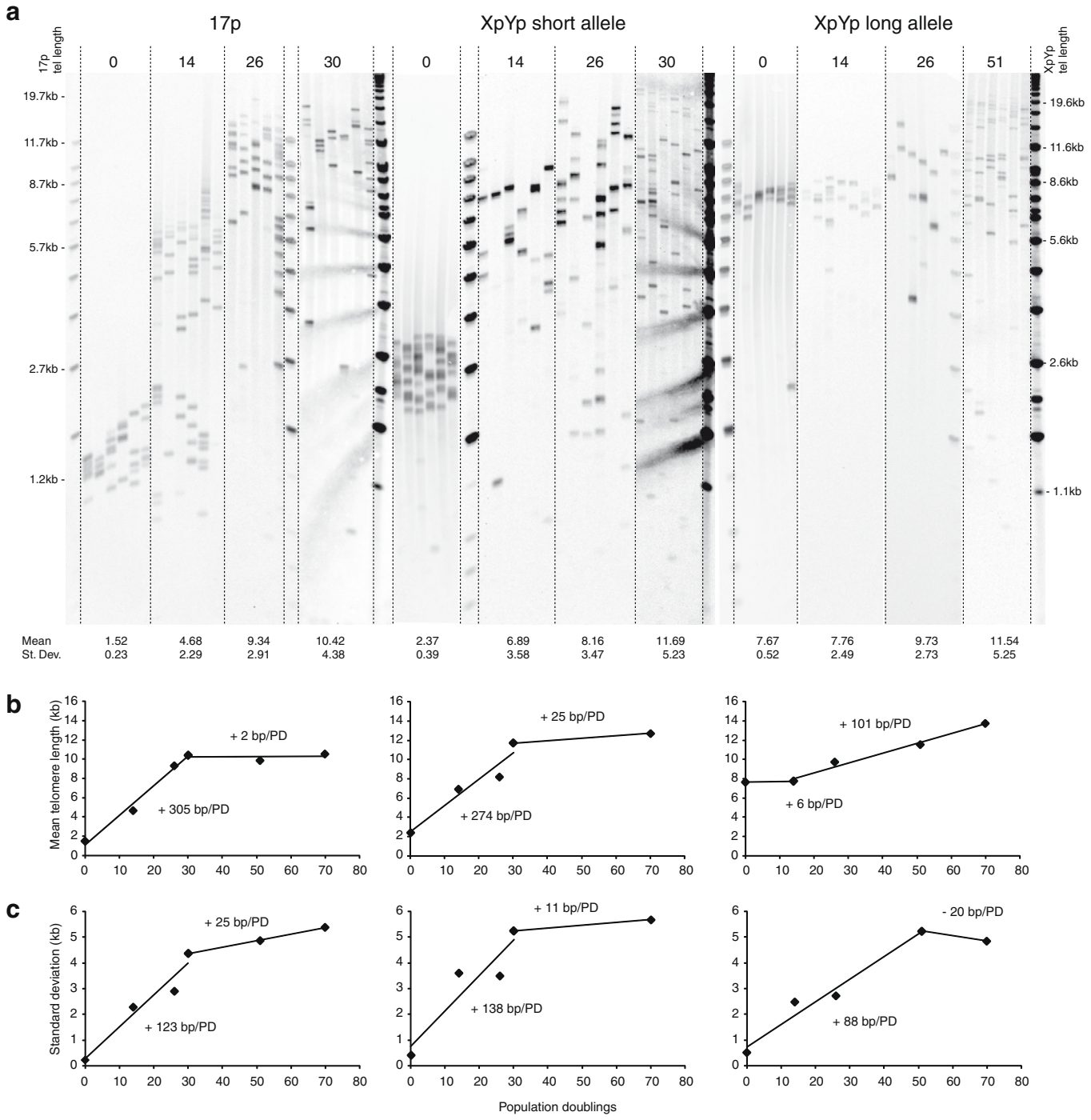

Fig. 2. STELA of a clonal population of MRC5 cells transfected with hTERT (a) 17p and XpYp allele-specific STELA gel. The number of population doublings (PD) from the point of hTERT transfection is detailed above each set of six STELA reactions with the mean and S.D. displayed below. (b) Mean 17p or XpYp data plotted as a function of PD, for the telomeric alleles as detailed at the top of the figure, rates of telomeric elongation calculated from the slop of the regression lines are detailed. (c) Displaying telomere length heterogeneity as S.D., plotted as a function of PD, rate of change in S.D. calculated from the slope of the regression lines are detailed.

bulk population (mean S.D. = 5.09). In both the bulk and clonal cell populations elongation was coupled with an increase in the heterogeneity of the telomere length distribution in the bulk population (Figs. 2c and 3c). This increase appeared to stabilise once the telomeres had been fully lengthened.

In addition, whilst the bulk of telomeres at 17p were rapidly elongated, in both the parental and clonal populations there was a subset of 17p of telomeres that were not elongated, this represented an estimated 15% of the 17p telomeres (PD 14 in Fig. 2a and PD 19 in Fig. 3a).

4. Discussion

By taking advantage of telomeric alleles with a large length differential, here we provide clear evidence of the preferential elongation of short telomeres compared to longer telomeres in human cells. This phenomenon has been demonstrated in both yeast and mouse models [5,6,15] but not directly in human cells. Data concerning the kinetics of telomere elongation in yeast indicate that in a single extension reaction telomere length can be extended by several times the length of the originating telomere, indeed a

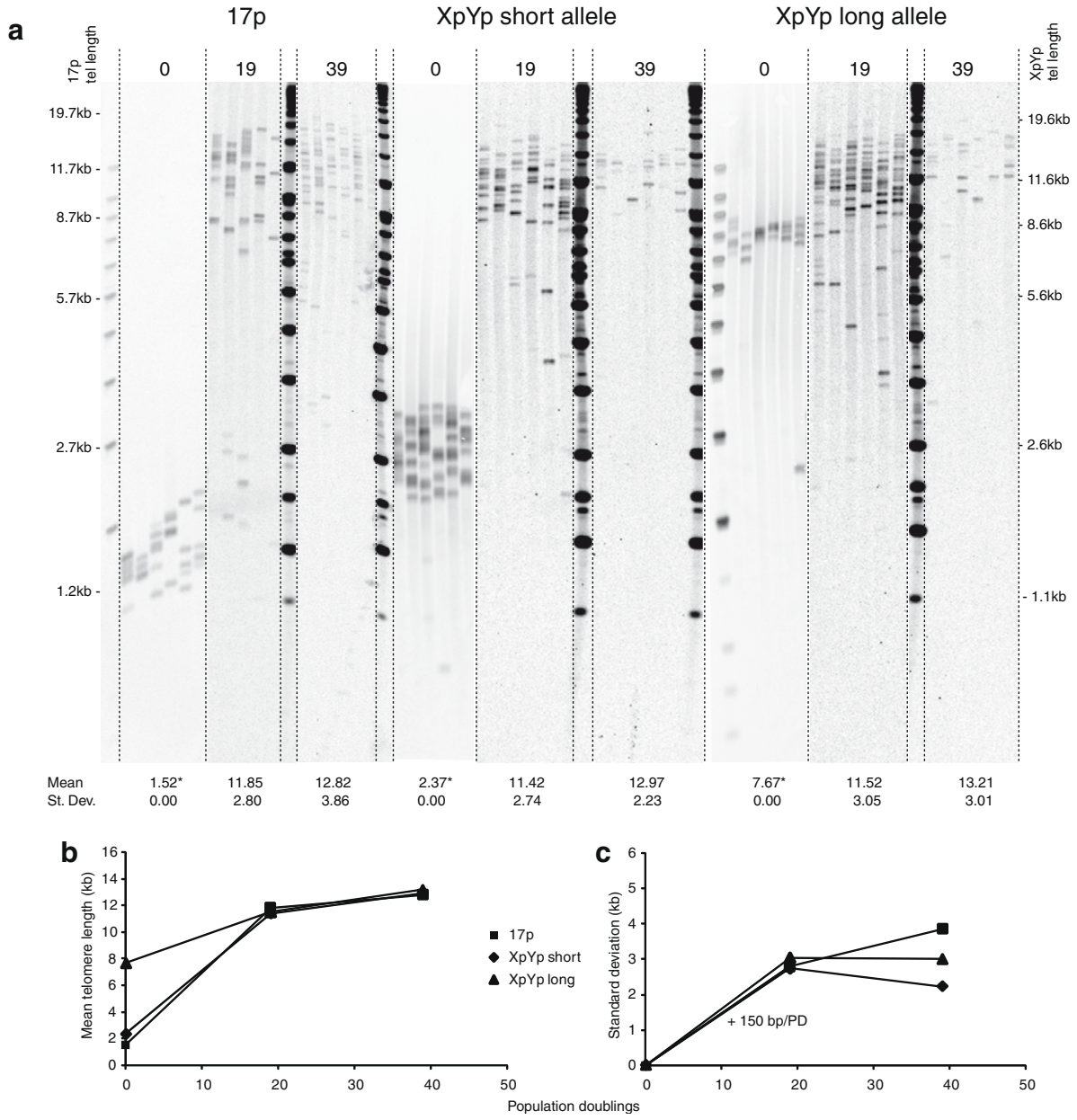

Fig. 3. STELA of a clone derived from a clonal population of MRC5 cells transfected with hTERT. (a) 17p and XpYp allele-specific STELA gel. The number of population doublings (PD) from the point of hTERT transfection is detailed above each set of six STELA reactions with the mean and S.D. displayed below. ∗denotes the mean telomere of the clonal population from which the telomerase expressing clone was derived. (b) Mean 17p or XpYp data plotted as a function of PD, for alleles as detailed in the legend. (c) Displaying telomere length heterogeneity as S.D., plotted as a function of PD, rate of change in S.D. calculated from the slope of the regression lines are detailed.

short telomere can be restored to the length of the longest telomere in cell. In contrast our data from human cells indicate that telomere elongation is gradual; we saw no evidence that, in a single cell division, telomeres could be extended by multiples of their own length or elongated to the maximum length; instead we observed a gradual increase in the mean and S.D. of the distributions. These data are consistent with the view that telomerase in human cells adds a regulated amount of telomere repeats every cell division. The maximum rate of elongation was observed at the shortest telomeric allele at 17p (305 bp/PD). In the absence of telomerase we have previously determined a rate of erosion of 75 bp/PD at 17p in MRC5 cells [14]; thus taking to account erosion rates telomerase can elongate telomeres at rates of up to 380 bp/PD. Our data from the clonal population indicate that there is a telomere length at which all the alleles coalesce, irrespective of their length prior to the expression of hTERT. This was not so apparent in the parental culture that contained multiple different telomerase expressing clones and thus displayed considerable telomere length heterogeneity. The gradual elongation, together with a length threshold indicates that the shorter telomeres are marked epigenetically for elongation; they are then extended until they reach the specific length threshold.

In both the parental and clonal populations, subsets of cells were observed at PD 14 that displayed no elongation at the 17p telomere, whereas the short XpYp allele was elongated. These cells were not detected in the subsequent PD points and thus it is not clear whether these cells underwent senescence, or immortalisation following 17p elongation. This implies that these cells are competent for telomeric elongation and express telomerase, but some specific aspect of the 17p telomeric structure in these cells prevented immediate elongation. During first 14 PDs following the transduction of hTERT, the shorter telomeric alleles were

elongated, whereas the longer allele at XpYp displayed no evidence of elongation, instead the length of this allele was maintained, indicating that telomerase was counteracting end-replication losses. Together these dynamics appear to be consistent with the view that telomeres can exist in two states, one extendable and one not [5], and this is dependent upon the length of the telomere to be elongated.

Acknowledgements

This work was funded by Cancer Research UK. D.M.B. is a Cancer Research UK senior fellow.

References

[1] Deng, Y., Chan, S.S. and Chang, S. (2008) Telomere dysfunction and tumour suppression: the senescence connection. Nat. Rev. Cancer 8, 450–458.
[2] Hills, M., Lucke, K., Chavez, E.A., Eaves, C.J. and Lansdorp, P.M. (2009) Probing the mitotic history and developmental stage of hematopoietic cells using single telomere length analysis (STELA). Blood.
[3] Morin, G.B. (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 59, 521–529.
[4] Chang, M., Arneric, M. and Lingner, J. (2007) Telomerase repeat addition processivity is increased at critically short telomeres in a Tel1-dependent manner in *Saccharomyces cerevisiae*. Genes Dev. 21, 2485–2494.
[5] Teixeira, M.T., Arneric, M., Sperisen, P. and Lingner, J. (2004) Telomere length homeostasis is achieved via a switch between telomerase-extendible and -nonextendible states. Cell 117, 323–335.
[6] Hemann, M.T., Strong, M.A., Hao, L.Y. and Greider, C.W. (2001) The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell 107, 67–77.
[7] Cristofari, G. and Lingner, J. (2006) Telomere length homeostasis requires that telomerase levels are limiting. EMBO J. 25, 565–574.
[8] Britt-Compton, B., Wyllie, F., Rowson, J., Capper, R., Jones, R.E. and Baird, D.M. (2009) Telomere dynamics during replicative senescence are not directly modulated by conditions of oxidative stress in IMR90 fibroblast cells. Biogerontology.
[9] McSharry, B.P., Jones, C.J., Skinner, J.W., Kipling, D. and Wilkinson, G.W. (2001) Human telomerase reverse transcriptase-immortalized MRC-5 and HCA2 human fibroblasts are fully permissive for human cytomegalovirus. J. Gen. Virol. 82, 855–863.
[10] Capper, R., Britt-Compton, B., Tankimanova, M., Rowson, J., Letsolo, B., Man, S., Haughton, M. and Baird, D.M. (2007) The nature of telomere fusion and a definition of the critical telomere length in human cells. Genes Dev. 21, 2495–2508.
[11] Jones, C.J. et al. (1998) Dissociation of telomere dynamics from telomerase activity in human thyroid cancer cells. Exp. Cell Res. 240, 333–339.
[12] Baird, D.M. (2005) New developments in telomere length analysis. Exp. Gerontol. 40, 363–368.
[13] Baird, D.M., Rowson, J., Wynford-Thomas, D. and Kipling, D. (2003) Extensive allelic variation and ultrashort telomeres in senescent human cells. Nat. Genet. 33, 203–207.
[14] Britt-Compton, B., Rowson, J., Locke, M., Mackenzie, I., Kipling, D. and Baird, D.M. (2006) Structural stability and chromosome-specific telomere length is governed by cis-acting determinants in humans. Hum. Mol. Genet. 15, 725–733.
[15] Zhu, L., Hathcock, K.S., Hande, P., Lansdorp, P.M., Seldin, M.F. and Hodes, R.J. (1998) Telomere length regulation in mice is linked to a novel chromosome locus. Proc. Natl. Acad. Sci. USA 95, 8648–8653.