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Telomere Dynamics and Telomerase Activity In *In Vitro* Immortalised Human Cells

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This article reviews the current understanding of the involvement of telomerase in *in vitro* immortalisation of human cells. *In vitro* immortalisation with DNA tumour viruses or chemicals usually occurs in two phases. The first stage is an extension of lifespan beyond that at which cells would normally senesce, after which the culture enters a period of crisis. The second stage involves the escape from crisis of a rare cell in the culture, which goes on to proliferate indefinitely. The hypothesis that telomere shortening acts as a signal for senescence and crisis, and that cells need to activate telomerase to survive these states, gained support from early studies examining telomere behaviour and telomerase activity in immortalised cell lines. In many cases, telomeres were found to continue to shorten during the phase of extended lifespan, and no telomerase was detectable. Cells which survived crisis had activated telomerase and had stable or lengthened telomeres. However, it is now clear that this model does not apply to all cell lines. Approximately a quarter of *in vitro* immortalised cell lines so far examined have no detectable telomerase activity, yet have very long and heterogeneous telomeres. These cell lines have acquired a novel mechanism for lengthening their telomeres, named ALT (Alternative Lengthening of Telomeres). The nature of ALT is not yet understood, but may involve non-reciprocal recombination between telomeres. ALT is not merely a phenomenon of *in vitro* immortalised cell lines, but has also been found in tumours and tumour-derived cell lines. Furthermore, there are a number of cell lines which have been shown to have low levels of telomerase prior to crisis while telomere shortening is still occurring, and the function of these low levels of telomerase activity is unknown.

Key words: immortalisation, senescence, telomeres, telomerase

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INTRODUCTION

NORMAL HUMAN cells will only grow for a limited number of generations *in vitro* before they undergo senescence, a state in which they remain metabolically active but cease dividing [1]. In contrast, tumour cells often divide indefinitely *in vitro* and are termed immortal. Cellular senescence may be overcome by transformation with DNA tumour viruses such as Simian virus 40 (SV40), human papillomavirus (HPV) and adenovirus, chemical carcinogens and irradiation. In most cases, the transformed cell culture acquires an extended lifespan. The tumour viruses contain proteins which bind to and inactivate the cellular tumour suppressor proteins p53 and $p110^{RB}$, and there is good evidence that lifespan extension in virally-transformed cells is dependent on the inactivation of these molecules (reviewed in [2]). Many non-virally immortalised cell lines also lack wild-type p53 and $p110^{RB}$, due to mutation or gene deletions. In addition, inactivation of the $p16^{INK4}$ protein, which inhibits the phosphorylation and hence inactivation of $p110^{RB}$ [3], may play a role in extension of cellular lifespan. Non-virally immortalised cell lines that contain wild-type $p110^{RB}$ have lost expression of $p16^{INK4}$ [4–12], indicating that loss of $p110^{RB}$ and loss of $p16^{INK4}$ may be functionally equivalent. However, the loss of p53 and either $p110^{RB}$ or $p16^{INK4}$, while contributing to an extended lifespan, is insufficient for complete immortalisation [13].

Following this period of extended lifespan, the cells usually enter 'crisis', a state in which cell division is balanced by cell death [14]. A rare cell in this crisis culture may eventually recommence proliferation and become immortal. For SV40-induced immortalisation, the frequency of this event ranges from approximately 1 in 10^5 [15] to 1 in 10^9 (E.L. Duncan and R.R. Reddel, Children's Medical Research Institute, New South Wales, Australia) crisis cells, which is consistent with it being due to a small number of mutations in the cellular genome.

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The molecular basis of senescence is poorly understood, but recent evidence suggests that it may involve changes in the ends of chromosomes, i.e. telomeres. Telomeric DNA is highly conserved throughout evolution and consists of a large number of tandem repeats of short G-rich sequences, TTAGGG in humans and other vertebrates (reviewed in [16], and see the article in this Special Issue by R.J. Wellinger and D. Sen, pages 735–749). The amount and length of telomeric DNA decreases with passage of normal human fibroblasts *in vitro* [17] and with ageing *in vivo* [17–20], presumably due to the inability of DNA polymerases to replicate fully the ends of linear DNA molecules [21]. This has been demonstrated both with Southern blot analysis of terminal restriction fragments (TRFs) and more recently by fluorescence *in situ* hybridisation (FISH) on interphase or metaphase chromosomes [22, 23]. There is a good correlation between initial telomere length of normal somatic cells, either clones or mass cultures, and their replicative capacity [24, 25]. It was first proposed in 1971 by Olovnikov [21] that a progressive shortening of the chromosomes in the somatic cells of multicellular organisms may act as the 'molecular clock' that leads to cell cycle exit, and the above observations lend support to such a view.

Telomere length is also reduced in blood cells relative to sperm [26–28] suggesting that cells of the germ line have a mechanism for avoiding the telomeric shortening characteristic of somatic cells. This is thought to be due to the actions of the enzyme telomerase, which is responsible for the addition of DNA to telomeres [29]. It has further been proposed that the immortalisation of human cells requires the reactivation of telomerase [30, 31]. Human telomerase activity was first identified in the immortal cervical carcinoma cell line HeLa [32]. While it is indeed the case that immortalisation does often coincide with telomerase activation, it is now clear that there may be at least one other mechanism in human cells for maintenance of telomere length. This article will review what is known about this mechanism and the dynamics of telomere maintenance in *in vitro* immortalised human cells.

ASSOCIATION BETWEEN IMMORTALISATION AND TELOMERASE ACTIVITY

According to the telomere hypothesis of senescence, loss of telomere repeats from chromosomes somehow triggers the senescence growth arrest. The mechanism of this trigger is unknown, but it may be that shortened telomeres resemble a DNA break and activate a p53-dependent cell-cycle arrest pathway [33]. Alternative models propose that senescence is triggered by the inactivation of essential subtelomeric genes by telomere length-dependent changes in heterochromatin configuration [34] or the inability of shortened telomeres to associate with the nuclear envelope [30]. However, at senescence the telomeres are not yet short enough to be incompatible with cell division since transformation by SV40 or other tumour viruses is able to override this block without causing telomere lengthening.

The telomere hypothesis of senescence was supported by early studies of telomerase in SV40-transformed embryonic kidney cells [35]. During the phase of extended lifespan prior to entry into crisis, telomeric sequences were lost at a similar rate in all transformed cell populations and telomerase activity was not detected. The mean telomeric TTAGGG length at crisis was estimated to be approximately 1.5 kb. The authors of this study speculated that since human telomeres are very heterogeneous in size, one or more chromosomes in the crisis culture may lack telomere repeats altogether and that some cells may have unprotected telomeres. This would lead to increased chromosome instability and aberrations that may become lethal. Indeed, it was observed that the frequency of dicentric chromosomes in these cells increased dramatically just prior to crisis [35]. In this study, one population emerged from crisis and became immortal, and the resulting cell line demonstrated stabilised TRF lengths and telomerase activity. The adenovirus-transformed human embryonic kidney cell line 293 also had stable telomeres and telomerase activity following immortalisation [35]. Similar results were obtained with Epstein–Barr virus immortalised B lymphocytes [36] and HPV-immortalised epithelial cells [37].

The above studies were carried out using an *in vitro* assay for telomerase activity that involves the extension of an oligonucleotide primer by telomerase and visualisation of the extended products. This method required at least 10^7–10^8 cells to observe telomerase activity reliably [32, 35]. An improved assay was recently developed, in which the extended telomerase products are amplified by polymerase chain reaction (PCR), resulting in an increase in sensitivity by at least a factor of 10^4 [38]. This assay has now been

Table 1. Telomerase status of in vitro *immortalised human cell lines*

Cell type	Telomerase+	Telomerase–	Reference
Breast epithelium	21	0	39, 40, 41
Bronchial epithelium	9	1	42
Retinal epithelium	1	0	38
Embryonic kidney	2	0	35*, 38, 43
B lymphocytes	2	0	36*, 43
T lymphocytes	1	0	44*
Mesothelium	3	1	42
Fibroblasts†	28	21	12, 38, 39, 42, 45
Skin keratinocytes	6	0	46
Cervical keratinocytes	1	0	47
Total	74	23	

* Telomerase assay carried out using the conventional method [32]; in all other cases the PCR-based TRAP assay was used [38]. † Includes breast, bronchial, jejunal, liver, lung, prostatic and skin fibroblasts.

used to examine a number of *in vitro* immortalised cell-lines, of which approximately 3/4 have detectable telomerase activity (see Table 1 and [39–47]).

The telomerase-positive cell lines include those that have been immortalised with SV40, HPV, adenovirus, EBV, cytomegalovirus and spontaneously immortalised cell lines. Of the cell lines that have also been examined for telomerase activity pre-crisis, all are negative [12, 35, 36, 39, 40, 42], with the exception of HPV E6-transformed cervical keratinocytes, foreskin keratinocytes and mammary epithelial cells and EBV-transformed B lymphocytes which have weak telomerase activity prior to crisis [43, 47]. This may be at least partly explained by the fact that normal keratinocytes in culture and *in vivo* already possess a low level of telomerase activity [46]. In fact, an increasing number of normal human somatic tissues are now known to have low levels of telomerase, although continued telomere shortening in these cells raises questions about the biological significance of the telomerase activity [48–50]. It is possible that some somatic tissues and cells have less stringent controls on telomerase expression than others. Hence, although the levels of telomerase are not high enough to maintain telomere length, it may be relatively easy to breach the remaining barriers to telomerase expression and raise it to functional levels. It is also possible that these "telomerase competent" cells and tissues may become immortal at a higher frequency than other cells.

IMMORTAL CELL LINES WITH NO DETECTABLE TELOMERASE

Approximately one in four *in vitro* immortalised cell lines have no detectable telomerase activity using either the conventional or the PCR-based telomerase assays (Table 1 and Figure 1). Surprisingly, when the TRFs of these cell lines were examined, they were found to be very long and heterogeneous, consisting of a smear extending from <1 kb to >50 kb with a mean of 20–25 kb ([12, 42, 45] and Figure 2). The very large TRFs shown in Figure 2 were shown to be telomeric by digestion with the exonuclease Bal31, which digests only from the ends of linear DNA molecules [42]. This resulted in a decrease in intensity and size of the TRF smear in DNA from the telomerase-negative cell lines. These cell lines had mean TRF sizes of <12 kb prior to crisis, and were clonally derived, indicating that telomere elongation had occurred [42]. All the telomerase-negative cell lines so far examined have exhibited very similar TRF patterns, distinct from that of telomerase-positive lines. It is thus apparent that telomeres may be maintained by a novel mechanism, which we have named ALT (Alternative Lengthening of Telomeres). These data suggest that it is necessary for telomeres to be maintained or lengthened either by telomerase or ALT if a cell is to become immortal. This supports the hypothesis that telomere shortening leads to senescence of human cells.

The telomere lengths of two cell lines with ALT have also been examined using FISH on interphase nuclei with a telomeric probe [23]. As in Southern blot analysis, the telomeres appeared very heterogeneous in size, with some telomeres being much larger than those in the normal cells from which the cell lines were derived.

ALT does not correlate with the method of immortalisation of the cell line. There are examples of telomerase-negative cell lines among those immortalised with SV40, HPV and chemical carcinogens, and in spontaneously immortalised lines. There is also no correlation between telomerase activity and cells of a particular cell type; there are both telomerase-negative and -positive fibroblast, epithelial and mesothelial cell lines [42]. A large proportion of the telomerase-negative cell lines, however, are fibroblasts, indicating that activation of ALT occurs more frequently in the immortalisation of fibroblasts than other cell types. This may be because other cell types, e.g. epithelial cells and keratinocytes, are 'telomerase competent' prior to immortalisation and hence are more likely to activate telomerase than ALT during immortalisation.

Cells of the same type and from the same individual may also be either telomerase or ALT-positive; of a set of seven SV40-immortalised jejunal fibroblast lines from one individual, six had telomerase activity and one displayed ALT (T.M. Bryan, P. Bonnefin, A. Englezou, J.P. Murnane and R.R. Reddel, Children's Medical Research Institute, New South Wales, Australia). ALT and telomerase activity also

Figure 1. Telomerase activity in immortal cell lines. CHAPS cell extracts were prepared from the cell lines indicated and analysed using the PCR-based TRAP assay, in which telomerase activity is indicated by a 6 bp ladder on an acrylamide gel [38]. The cell lines were derived from the following sources: EJ, bladder carcinoma; HT1080, fibrosarcoma; GM639, SV40-immortalised skin fibroblasts; BEAS-2B/R1, SV40-immortalised bronchial epithelial cells; SUSM-1, chemically immortalised liver fibroblasts; 143BTK-, osteosarcoma; A1698, bladder carcinoma; HeLaCOT, cervical carcinoma. LB is a lysis buffer negative control. 10, 100 and 1000 cells are HeLa cells, a positive control.

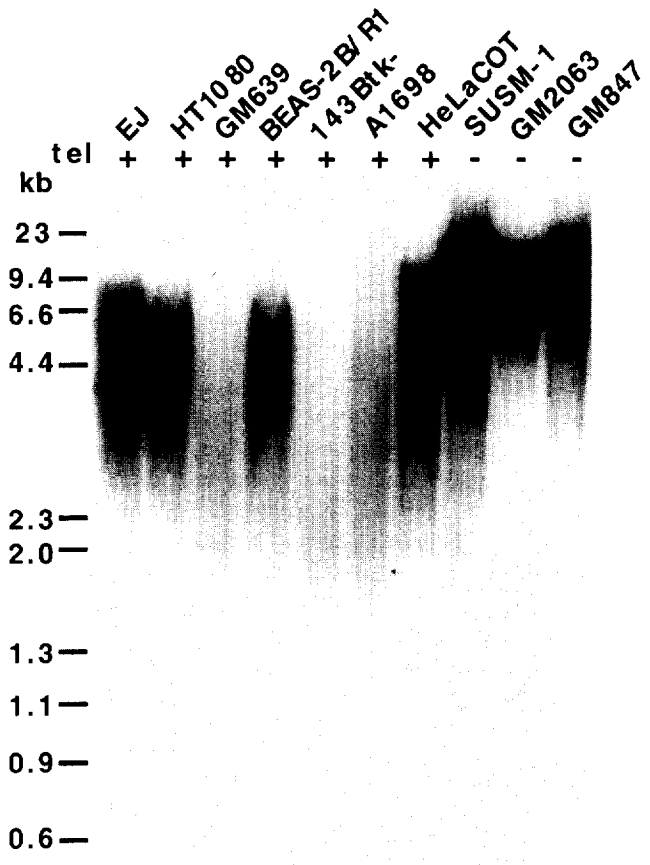

Figure 2. Telomere length analysis of immortal cell lines. Genomic DNA from the cell lines indicated was digested with restriction enzymes *Hin*F1 and *Rsa*1, electrophoresed on a 0.8% agarose gel and probed with a $(TTAGGG)_3$ probe [35]. GM847 are SV40-immortalised skin fibroblasts, and GM2063 is the cell strain from which GM847 was derived. All other cell lines are as in the legend to Figure 1. Tel, telomerase status of cell line

do not correlate with the four complementation groups that have been identified for immortalisation [10, 51].

The mechanism of telomere lengthening in ALT is currently unknown. However, it does not appear to be due to a burst of telomerase which is subsequently switched off; a gradual increase in telomere length in the telomerase-negative spontaneously immortalised IIICF/c cell line occurs in the absence of telomerase activity [12]. Similarly, the intensity of the TRF smear in the cell line IIICF-T/A6 gradually increases by 3-fold during the 30 population doublings (PDs) following crisis [42]. This demonstrates that there has been an increase in TTAGGG-hybridising DNA in the absence of telomerase.

It is possible that ALT is due to non-reciprocal recombination between telomere repeats, resulting in a gain of repeats by short telomeres. The yeast *Saccharomyces cerevisiae* has the ability to utilise recombination as a backup mechanism of telomere repair. *S. cerevisiae* cells lacking a functional *EST1* gene show progressive shortening of telomeres and the majority of cells in the culture die [52]. However, a minor subpopulation of cells survives as a result of the acquisition of subtelomeric repeats by a RAD52-dependent recombination mechanism [53]. Recombination between actual telomere repeats has also been demonstrated as a means of telomere acquisition in *S. cerevisiae*, although this differed from the aforementioned phenomenon by its RAD52-independence [54, 55]. Evidence in favour of ALT being a recombination mechanism includes the rapid gains and losses of telomeres in clones of such cell lines ([45]; see below), and the fact that ALT is independent of the RNA subunit of telomerase, hTR (data not shown). A recombination mechanism of telomere maintenance has also recently been described in the yeast *Kluyveromyces lactis* [56]. Survivors of the senescence that results from deletion of the *K. lactis* telomerase RNA gene show elongated telomeres in a RAD52-dependent manner. This raises the possibility that telomere–telomere recombination may occur in other eukaryotes.

An alternative model is that ALT cell lines possess telomerase that is active *in vivo*, but mutant in at least one of its subunits. Presumably the mutation has two effects: (i) a dysregulation of telomerase activity *in vivo*, resulting in abnormal lengthening of telomeres, and (ii) the inability of telomerase to use an oligonucleotide as an *in vitro* substrate. This possibility is under investigation.

It initially seemed possible that ALT was unique to *in vitro* derived cell lines, since all 110 tumour-derived cell lines had telomerase activity [57]. However, we have recently assayed a further 54 cell lines derived from a variety of tumours, and have found three cell lines that lack telomerase activity and have the long telomeres characteristic of ALT (T.M. Bryan, A. Englezou and R.R. Reddel, Children's Medical Research Institute, New South Wales, Australia). Furthermore, we have found very long telomeres together with a lack of telomerase activity in a small number of human tumours (data not shown). Others have reported similar results for renal cell carcinoma [58]. Thus it is possible that ALT functions as an alternative to telomerase in human tumour development, albeit in a very small minority of tumours. It is not known why ALT is much more frequent in *in vitro* derived cell lines than tumour-derived ones, but if ALT is indeed a recombination mechanism, it is possible that SV40 and other transforming agents inactivate a negative regulator of this pathway (a possible example being p53) and make it more likely that immortalisation will occur via ALT activation. Alternatively, the very long telomeres characteristic of ALT may be tolerated by cells growing *in vitro* but not *in vivo*.

Nevertheless, the occurrence of ALT in human tumours has implications for antitelomerase therapies for human cancer. If ALT can act as a back-up mechanism for telomere lengthening in the absence of telomerase, it may make such therapies ineffective.

TELOMERE DYNAMICS

Telomere dynamics in telomerase-negative cell lines

The telomere dynamics of KB319, an SV40-immortalised cell line with ALT, and many subclones derived from it have been extensively studied [45, 59]. Fortuitously, this cell line contains an integrated plasmid at the truncated telomere of chromosome 13 [60], making it possible to follow the dynamics of a single chromosome by Southern blotting with a probe from this plasmid. First-generation and second-generation subclones of this cell line showed a remarkable degree of heterogeneity in telomere length at chromosome 13 considering that they all descended from a single founder [59]. When such subclones were analysed with a probe that detects all telomeres, the pattern of hy-

bridisation resembled that of the cell lines shown in Figure 2, i.e. there was a high degree of heterogeneity, with the telomeres on some chromosomes being very long [45]. Thus the heterogeneity of telomere length is probably not restricted to chromosome 13.

Subsequent generations of subclones demonstrated several types of changes in telomere length [45]. Clones with telomere repeat lengths of >1 kb produced subclones with bands of a similar size, but slightly shorter. When one of these clones was followed over time in culture, it became apparent that these bands were decreasing gradually, at a rate of approximately 52 bp per cell division. This rate is consistent with that seen in telomerase-negative mortal cells [18].

Subclones of clones with discrete telomeric bands also often demonstrated lighter bands that were much more diffuse and heterogeneous in size, extending from approximately 4.5 kb to >23 kb. These dramatic length changes had occurred within the 25 PDs between clone generations. This rapid elongation always occurred in those clones whose telomeres were approaching a length corresponding to a total lack of telomere repeat sequences. Telomeric DNA therefore appears to be added when the size of the telomeres is reduced beyond a critical limit, although the amount added to chromosomes in different cells in a population varies widely.

A third type of telomere length change seen in these clones was rapid increases and decreases in size on both long and short telomeres, resulting in extremely diffuse bands. It was suggested that these changes may be due to a different process than the rapid elongation of short telomeres [45].

Non-reciprocal recombination could conceivably result in the rapid addition of long sequences. However, this does not rule out other mechanisms, such as a telomerase-like enzyme that has the capability of adding many repeats in a single cell division.

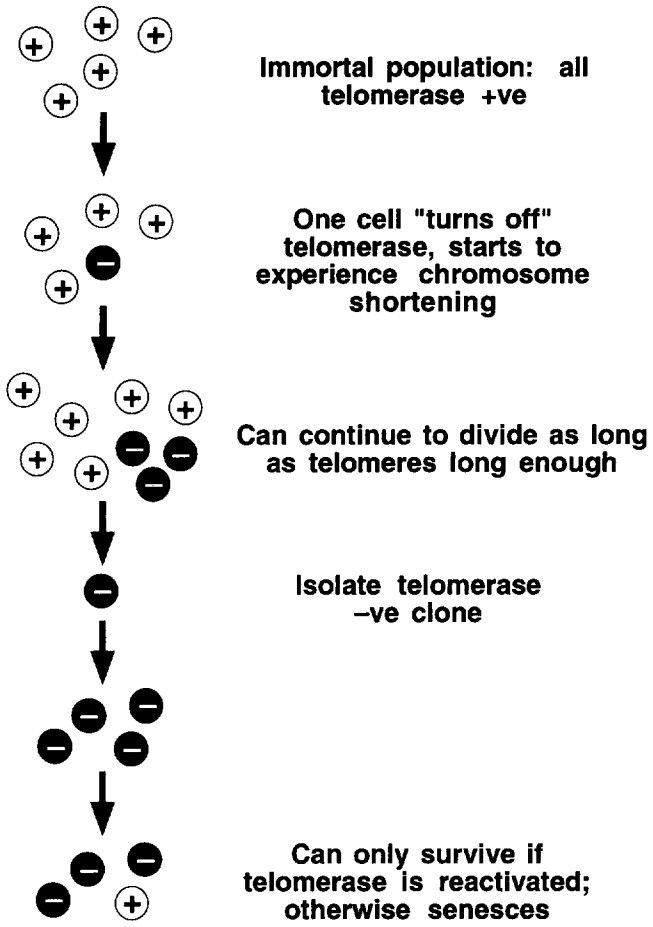

Figure 3. Model to explain variations in telomere lengths in clones of telomerase-positive immortal cell populations.

Telomere dynamics in telomerase-positive cell lines

The heterogeneity in telomere lengths in clones of ALT-positive cell lines leads to the question of whether a similar heterogeneity exists in telomerase-positive cell lines. We have recently addressed this question in the telomerase-positive cell lines 293 (adenovirus-immortalised embryonic kidney cells) and HeLa (cervical carcinoma line) (T.M. Bryan, A. Englezou and R.R. Reddel, Children's Medical Research Institute, New South Wales, Australia). There was a large range of mean telomere sizes in subclones of these cell lines. Furthermore, a TRF gel of the mass culture of 293 cells showed a band that decreased gradually in size over passage in culture. This led us to propose a model (Figure 3) in which some cells within a telomerase-positive cell line cease expression of telomerase and experience gradual telomere shortening. An alternative explanation would be that telomerase maintains telomeres to different extents in different cells over time.

A prediction of this model is that the lineages that lack telomerase activity will eventually senesce and be lost from the culture. In keeping with this, a small number of cells with the characteristic appearance of senescence are observed in both 293 and HeLa populations. Presumably, telomerase is reactivated in most clones, enabling them to survive. The mechanisms of inactivation and reactivation of telomerase in these clones, if it occurs, is currently unknown.

CONCLUSION

The basic model for the involvement of telomerase in *in vitro* immortalisation, i.e. that telomeres shorten until the cells enter crisis after which telomerase is activated and telomeres are stabilised, is supported by the evidence. The majority of immortalised human cells do express telomerase activity after crisis, and not before. However, additional layers of complexity are now unfolding. Firstly, even in those cell lines that do have active telomerase, the dogma that telomeres are stably maintained may be true on a mass population basis, but is being challenged by clonal studies. Secondly, it now seems that some pre-crisis transformed cells do have telomerase activity, consistent with recent findings of telomerase activity in an expanding number of normal somatic tissues. The function of these low levels of telomerase have yet to be established. Lastly, approximately one in four *in vitro* immortalised cell lines have no telomerase activity, but their telomeres are maintained by some other mechanism. The nature of this mechanism, and the extent of its involvement in immortalisation *in vivo*, are pressing questions, not least for their clinical implications.

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