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Telomeres and Telomerase in Normal and Malignant Haematopoietic Cells

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The normal haematopoietic system harbours telomerase-competent cells with a capacity to upregulate the activity to notable levels in a telomere length-independent manner. Strong telomerase activity is found in progenitor stem cells and activated lymphocytes *in vitro* as well as *in vivo*, indicating that cells with high growth requirements can readily upregulate telomerase. Despite detection of telomerase activity, a gradual telomere erosion occurs in stem cells and lymphocytes, with significantly shortened telomeres at higher ages, a phenomenon that might be of importance for developing immunosenescence and exhausted haematopoiesis. In malignant haematopoietic disorders, telomerase activity is a general finding with large differences in activity levels. The strongest telomerase expression has been shown in acute leukaemias and non-Hodgkin's lymphomas, especially high grade cases. There are indications that the level of activity might parallel tumour progression and be of prognostic relevance, but studies of larger patient materials are needed. An association between the cell cycle and telomerase activity exists, especially for normal haematopoietic cells, and induction of a differentiation programme in immortalised cell lines downregulates telomerase activity. The expression of telomerase activity seems to be regulated at different levels, since for immature bone marrow cells the level of activity seemed to parallel better the phenotype than the proliferation state. The frequent expression of telomerase in leukaemias and lymphomas makes these disorders interesting targets for future anti-telomerase therapy.

Key words: telomere, telomerase, haematopoiesis, stem cell, lymphocyte, leukaemia, lymphoma

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TELOMERES AND TELOMERASE IN NORMAL HAEMATOPOIETIC CELLS

HAEMATOPOIESIS, WITH lifelong, continuous production of blood cells of multiple lineages, requires self-renewal of haematopoietic stem cells, progenitor cell expansion, proliferation of committed cells, differentiation to mature cells and, for lymphocytes, the potential to expand clonally upon antigen challenge. The total process demands an extraordinary proliferative capacity and for certain cell types, especially within the immune system, a large number of accumulated cell cycle rounds can be expected. These features of the haematopoietic system implicate specific needs for telomere maintenance and telomerase activation.

Telomere restriction fragment (TRF) length of bone marrow, peripheral blood and lymphoid cells

There is an association between TRF length and age for both immature and mature haematopoietic cells, indicating a gradual telomere erosion for normal haematopoiesis. The TRF length of nucleated haematopoietic cells shorten from approximately 12–16 kbp in fetal liver and cord blood to 8–10 kbp in adult bone marrow [1, 2]. A number of studies have demonstrated that peripheral leucocytes have a TRF length of around 10 kbp or just below and shortening is regularly found with increasing age [2–6]. In very old individuals, the mean TRF length decreases to approximately 5 kbp [3, 4]. Interestingly, in peripheral lymphocytes the frequency of dicentric chromosomes is significantly associated with age [7]. Recently, TRF measurements on specific cell subpopulations have been performed that could give insights into their replicative history during differentiation and clonal expansion *in vivo*. One example is the finding that primitive stem cells lose telomeric repeats *in vivo* and thus seem to possess a telomeric 'clock' [1]. Furthermore, a significant difference of 1.4 kbp in TRF lengths has been found comparing naive with memory-helper-T-cells, and a similar difference has been demonstrated for CD8 + /CD28+ and CD8 + /CD28– cells indicating a minimum of 14–28 div-

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isions during cellular expansion [5, 8]. CD28 expression is decreased in older individuals and is downregulated upon cessation of proliferation in long-term T-cell cultures and could serve as a marker of replicative senescense [9, 10]. In HIV-infected individuals, CD8 + /CD28– cells have shorter TRFs than in normal persons, with a mean TRF of 5–7 kbp, which is similar to that of the aged and senescent T-cells in culture [3, 4, 11]. Thus, replicative exhaustion of CD8+ cells could be associated with disease progression.

Given that 100 bp of telomeric arrays are lost at each cell division, this would provide stem cells, starting with around 12 kbp of TRF length in the newborn, a division potential of approximately 100 divisions, including 2 kbp of subtelomeric DNA in the TRF estimation. The theoretical number of cells produced would easily satisfy the total life supply, which has been estimated to be around 1.8×10^{16} cells. From this point of view, the haematopoietic system would be fully functional throughout life with no need for telomere stabilisation. However, the above given estimate can be questioned due to several observations. One is the fact that subtelomeric DNA is included in the TRF after cutting with 4-base cutters, which means that the actual telomeric arrays are 2–4 kbp shorter than the TRF value obtained [12]. There is evidence from studies on yeast that uncapping of a single chromosome is enough to signal cell cycle arrest and, together with the observed heterogeneity of the number of telomere repeats at individual chromosome ends, these two factors will act to decrease the actual division potential [13, 14]. There might also be a cell type-specific minimum number of repeat arrays necessary for a telomere to be functional and allow further proliferation.

Telomerase activity in bone marrow, peripheral blood and lymphoid cells

Telomerase activity determinations using the sensitive TRAP assay have demonstrated low-activity levels in nucleated bone marrow cells [2, 15–17]. More detailed characterisation of stem cell subpopulations showed that primitive stem cells expressed low telomerase activity whereas early progenitors were found to exhibit a significant upregulation of the enzyme activity [15, 18]. More mature CD34– cells appeared to have a downregulation of telomerase to levels expressed by primitive stem cells [15]. Interestingly, when these cell fractions were activated *in vitro* using cytokines, strong telomerase activity followed the early progenitor cell phenotype. The regulation of telomerase did not strictly correlate to proliferation since CD34– cultures had low telomerase activity despite exponential growth [15].

Similar to bone marrow cells, low levels of telomerase have been found in peripheral blood cells [2, 16–18]. Subfractionation experiments revealed activity in both T-cells and monocyte/B-cell fractions [17, 18]. Helper (CD4+) and supressor/cytotoxic (CD8+) T-cells from peripheral blood expressed low activity, which was upregulated in corresponding fractions of tonsil T-cells [19]. The highest *in vivo* telomerase expression in normal T-cells seems to be present in the thymus [19]. Highly purified normal peripheral B-lymphocytes were almost completely telomerase-negative (K.-F. Norrback, Umeå University, Sweden) whereas significant activity was associated with the B-cell fractions in lymph nodes and tonsils [16]. In benign lymphoid tissues a correlation was found between S-phase fractions and telomerase activity levels, strongly indicating a relationship between cell cycle progress and telomerase expression. In tonsil extracts, very strong telomerase activity has been demonstrated at levels even comparable to established cell lines, and the activity could be attributed to germinal centre cells [16]. Thus, it seems that mature T- and B-cells *in vivo* express low to intermediate telomerase activity and the activity in germinal centre B-cells clearly shows that strong upregulation can occur *in vivo* upon antigen challenge.

Activation of T-cells *in vitro* by a multitude of signals can upregulate telomerase, as shown after stimulation by PHA, anti-CD3, anti-CD3/CD28, PMA/ionomycin or PDB/Ca [18–21]. The upregulation was abrogated by the presence of protein or RNA synthesis inhibitors [19]. The timing of the upregulation correlated with an increase of S-phase DNA content in the T-cells [19, 21]. PHA-activated T-cells, blocked at the G1/S transition by rapamycin, showed no induction of telomerase, in contrast to an S-phase block induced by hydroxyurea [21]. Thus, telomerase is upregulated in the transition from G1 to S-phase. One study, using CD3/CD28 activation, CD4+ cells and cell sorting, found no significant difference in telomerase activity during cell cycle progression [19]. One explanation is that the reported long half-life of telomerase makes it improbable that there are significant changes during the cell cycle in growing cells [22]. B-lymphocytes seem to respond similarly to T-cells upon stimulation *in vitro* as demonstrated by PWM activation [18] or IgM activation (K.-F. Norrback, Umeå University, Sweden), in agreement with the *in vivo* data described above. Thus, specific stem cells in the bone marrow and T- and B-cells can be described as competent of telomerase upregulation. These telomerase-competent cells are transiently telomerase-positive and, during acquisition of cell type-specific properties, the induction signals and regulation of telomerase might diverge. There is a strong correlation between cell cycle entry and telomerase upregulation, but the above given data on stem cell cultures indicate that other levels of regulation must also be considered.

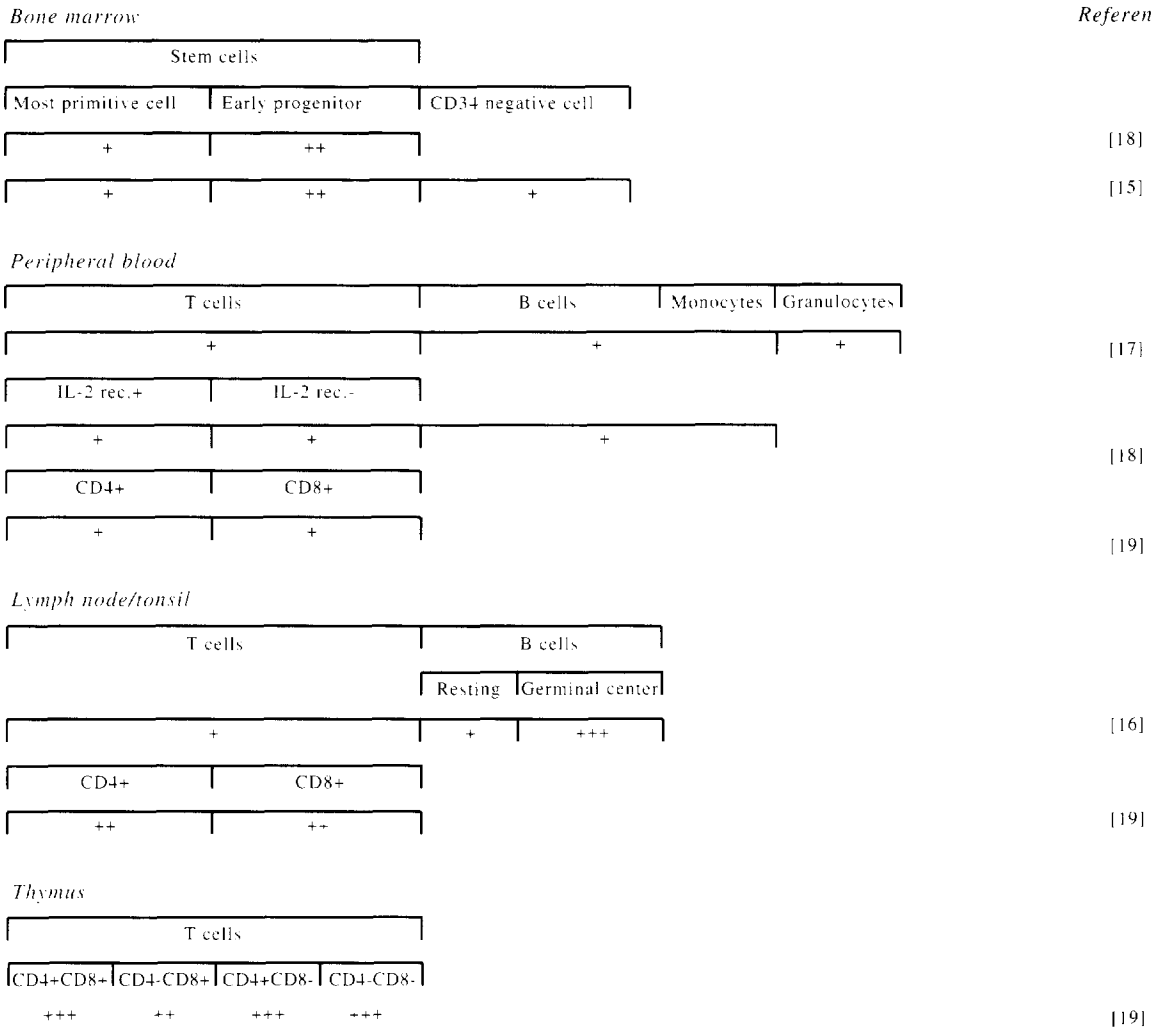
Regarding other haematopoietic cell types, telomerase activity has been detected in granulocytes [17], which is surprising for a terminally differentiated cell type and seems to be in contrast to data obtained from differentiation experiments on established cell lines [22–26]. The telomerase status of monocytes-macrophages and natural killer cells has yet to be determined.

In Table 1 telomerase expression in different normal haematopoietic cell subpopulations is summarised.

Blood cells exhibit TRF reduction despite telomerase activity

As described above, different blood cell populations show telomere erosion *in vivo* despite detectable telomerase activity [1, 5, 8, 15, 19]. One further example is EBV-infected, proliferating B-cells that have low levels of telomerase and lose telomeres until crisis, whereupon surviving cells exhibit higher telomerase levels and stabilised telomere lengths [27, 28]. Also, CD3/CD28 activation of CD4+ cells results in telomerase induction as well as telomere erosion [5, 19]. This seemingly contradictory phenomenon can be interpreted differently. It is obvious that the PCR-based TRAP assay can detect positivity present in only a minor cell fraction whereas the major cell population could be negative and allow for a reduction of telomere repeats. There is also a possibility that the telomeric ends have been

Table 1. Telomerase activity in normal, haematopoietic cell populations: + – + + + = levels of telomerase activity, roughly estimated by the authors and based on data presented in the references given



rec., receptor.

made inaccessible to the telomerase complex or that the telomerase *in vivo* is complexed with blockers/regulators which are lost upon extraction. An interesting observation is that telomerase activity decreases with age in peripheral blood cells, possibly contributing to the association between age and TRF length-shortening [18]. Considering that telomerase activity is expressed in cells with an extensive need of proliferation, it seems reasonable to assume that telomerase has an active role in extending the cellular division potential. The activity level can however be insufficient to compensate fully for the telomere erosion. Thus, normal telomerase competent cells, such as stem cells and lymphocytes, can be characterised as 'telomerase-positive and mortal with an extended but limited proliferation capacity'. It has yet to be shown that telomere reduction in these cells is partly compensated. Some answers can be possibly found in studies of telomere dynamics using telomerase inhibitors. In one study on mono- and dizygotic twins, it was found that telomere length maintenance had a strong genetic, inborn component, which means that telomere dynamics seems to be regulated at different levels [3]. One interesting aspect of telomere erosion in haematopoietic cells is its possible implications for the development of immunosenescent states and decreased or exhausted haematopoiesis.

Premature ageing syndromes

Telomerase activity in stem cells and lymphocytes thus indicates a key role in supporting and regulating the division potential of these cells. This is further actualised in states with disturbed telomere maintenance and/or diminished replicative potential. Down's syndrome (DS) patients are characterised by immune dysfunction and premature age-related changes of immune cells [29, 30]. It has been shown that DS patients have an increased loss of telomeres per year in peripheral leucocytes compared with age-matched donors [4]. Also, older DS patients and normal aged persons exhibit similar TRF lengths around 5 kbp, which is similar to senescent T-cells in culture [3, 4]. The increased telomere loss *in vivo* could be attributed to lower levels of telomerase in these cells, a greater turnover of cells or loss of more telomeric repeats per cell division. Replicative

senescence could thus play a relevant role in both aged and Down's syndrome patients.

Ataxia telangiectasia (AT) is a syndrome with general genomic instability and increased frequency of chromosomal abnormalities including telomeric end associations in lymphocytes [31, 32]. AT is also characterised by immunodeficiency, premature ageing and predisposition to cancer development. It has been shown that AT blood lymphocytes have an increased loss of telomeres *in vivo* compared to lymphocytes from controls [33]. Fibroblasts from patients with Hutchingtons–Gilford progeria, a syndrome with the most severe premature ageing, have significantly shortened telomeres compared to controls [34]. Similarly, in the progeroid Werner's syndrome an increased telomere loss per population doubling compared with control cells has been demonstrated [35]. Thus, *in vitro* as well as *in vivo* studies indicate a close association between premature ageing and increased telomere loss, but the pathogenetic mechanisms for the accelerated telomere loss remain to be elucidated.

In summary, normal stem cells and lymphocytes are telomerase competent and mortal. Substantial, transient telomerase activation can occur at specific situations, exemplified by antigen stimulation of lymphocytes. The gradual telomere loss seen with increasing age and accelerated telomere erosion in specific conditions might contribute to immunosenescence, immunodeficiency, decreased haematopoiesis and increased likelihood of malignant transformation. One interesting clinical aspect of these observations is the future possibility of selectively upregulating telomerase and controlling telomere length in certain cell types in order to achieve a delayed induction of replicative senescence.

TELOMERES AND TELOMERASE IN MALIGNANT HAEMATOPOIETIC CELLS

Only a few studies have been published regarding telomere length and telomerase activity in neoplastic haematopoietic cells and the total number of patients in most diagnostic groups is small. Therefore, it is necessary to be cautious in interpreting these data, especially concerning the clinical significance of telomerase activity.

Lymphoid neoplasia

Telomeric associations were recognised early in haematological malignancies and permanent cell lines, a phenomenon that has been coupled to the shortening of telomeres [27, 36–41]. A general decrease in telomere length compared to normal haematological cells and tissues has also been observed in leukaemias [2, 42–44] and malignant lymphomas (K.-F. Norrback, Umeå University, Sweden).

Chronic lymphocytic leukaemia (CLL) cells in early-stage disease have been found to exhibit very low levels of telomerase showing a mean TRF of 7.9 kbp, whereas late-stage CLL has increased telomerase activity with levels comparable to control cells demonstrating short mean TRF of 4.4 kbp [2]. In both early- and late-stage CLL, specimens with very low or absent activity have been found and more data are needed in order to verify a possible connection between telomerase levels and CLL stage. CLL is characterised by a very low fraction of cycling cells, and if the expression of telomerase is linked to the cell cycle in the tumour cells, low activity levels would be expected.

Telomerase positivity was first described in non-Hodgkin's lymphoma using the conventional assay, but no activity was detected in acute leukaemias, arguing for higher expression levels in lymphomas than leukaemias [45]. There was no correlation between telomere shortening and telomerase expression, neither did the positive samples exhibit shorter telomeres than the negative samples and the existence of a TRF-independent activation pathway was proposed [45]. Later studies using the TRAP assay have detected telomerase activity in all lymphomas investigated [16, 46]. Purified normal germinal centre B-cells seem to have higher activity levels than lymphomas, and tonsils with a follicular hyperplasia exhibit telomerase levels comparable to lymphomas. High-grade malignant lymphomas have increased levels of telomerase in comparison with low-grade malignancies. Interestingly, in contrast to benign lymphoid tissues, no association between cell proliferation and telomerase activity has been found in the lymphomas, indicating a deregulation of telomerase activity in the neoplastic lymphocytes [16]. This aspect needs further and more detailed investigations.

Hodgkin's disease

No data on TRF length or telomerase expression have been published for Hodgkin's disease. We have recently analysed Hodgkin's lymphoma samples with respect to telomerase activity in a quantitative manner using an internal TRAP assay standard, and a minority of the tumours exhibited positivity (K.-F. Norrback, Umeå University, Sweden). The result is surprising in light of the fact that other haematological malignancies have a very high fraction of telomerase-positive cases. We have initiated more detailed studies to clarify if telomerase reactivation in Hodgkin's disease is associated with specific clinicopathological features.

Myelodysplastic syndrome (MDS) and myeloid leukaemias

Variable telomere length has been observed in bone marrow samples with myelodysplastic morphology [2, 47]. One study of 16 MDS patients found two groups with either long or short telomeres that remained at the same length at leukaemic transformation and a third group exhibiting shortening of telomeres with disease progression [47]. It was suggested that short telomeres at the time of MDS diagnosis is a bad prognostic feature and that critical telomere shortening resulting in genomic instability enabling additional mutations can be an initiator of leukaemic transformation [47]. Telomerase activity seems to be increased in MDS in comparison with normal bone marrow samples and is further upregulated in acute myeloid leukaemia (AML) [2]. The AML cases also have shorter telomeres than MDS patients and control samples. In a recent study of peripheral cells from AML patients telomerase positivity was demonstrated in 73% and the level of telomerase was suggested to be a prognostic indicator of chemoresistance [48]. Data on telomerase activity in chronic myeloid leukaemia are sparse, but in one study of 12 patients, no association between telomerase activity and stage of disease was found [17].

Acute leukaemias seem to have higher telomerase levels than chronic leukaemias, as demonstrated for the myeloid as well as the lymphoid lineages [2, 17]. This variance can possibly be attributed to differences in proliferation rate

and/or degree of differentiation. It has recently been found that induction of terminal differentiation and decreased proliferation downregulate telomerase activity in immortal cell lines of myeloid and erythroid origin ([22–26] and see the article in this Special Issue by S.E. Holt *et al.*, pages 761–766). It is currently not possible to discern whether induction of the differentiation programme or simply the subsequent decrease in proliferation downregulates the telomerase activity. The correlation between the cell cycle and telomerase activity in bone marrow stem cells and lymphocytes suggests that their tumour-derived progeny would express increasing levels of telomerase with an increasing fraction of cycling tumour cells, but this remains to be proven.

Table 2. Factors of potential importance for increased levels of telomerase in malignancies derived from telomerase competent cells

(1) High fraction of tumour cells in a sample
(2) Differentiation arrest; differentiation ⇒ downregulation of telomerase
(3) High fraction of cycling tumour cells resting cells have no or low telomerase activity
(4) Increased deregulation of telomerase due to tumour progression progression ⇒ genetic instability with acquisition of deregulated telomerase expression
(5) High level of telomerase in the normal counterpart retention of a telomerase-positive phenotype

Mechanisms of telomerase activation in haematopoietic malignancies

There are two principal ways, currently under debate, for a tumour to acquire telomerase activity [49–51]. One is reactivation of telomerase through a process induced by critical telomere shortening. The steps involved in this mechanism for activation have not been clarified, but inactivation of immortality repressors is probably important. The second pathway is through retention of activity in cells competent of telomerase upregulation, like bone marrow stem cells, lymphocytes and possibly stem cells from diverse tissues [52, 53].

Telomere length-dependent activation of telomerase is supported by cell culture studies and cell hybrid experiments indicating that mortality is a dominant phenotype and that loss of gene function renders a cell immortal [41, 54, 55]. *In vivo* observations pointing in the same direction are short telomere lengths in leukaemias and lymphomas. However, long telomeres and telomerase activity in early tumorigenesis would support a retention of activity in an already telomerase-competent cell. TRF profiles reflect the lengths of all chromosome ends in a cell population, and since the PCR-based telomerase assay is very sensitive and can detect minute fractions of positive cells, it is impossible to make definitive conclusions regarding the interrelationship between TRF length and telomerase activity. This is further complicated by the fact that most normal haematopoietic cells have active telomerase to some degree, and despite this activity, telomere repeats are lost. Thus, it cannot be discerned whether a telomerase-positive tumour with short telomeres has reactivated telomerase following critical telomere shortening or if the tumour has retained activity from a telomerase-competent cell and thereafter lost telomeric repeats.

The regulation of telomerase activity in bone marrow and peripheral lymphocytes is most likely TRF length-independent and coupled to the cell cycle, suggesting that many haematological malignancies could originate from a telomerase-competent cell that retains telomerase activity. We believe that this is the main pathway that cells acquire telomerase activity in leukaemias and lymphomas, and thus expect to find telomerase positivity early in tumorigenesis. This is also supported by the very high fraction of telomerase-positive haematological malignancies [2, 16, 17, 46, 48]. The TRF length-dependent pathway can, however, never be excluded and is likely to be of significance in specific situations, as demonstrated *in vitro* for EBV-transformed, lymphoblastoid B-cells [27, 28]. The lymphoblastoid cells exhibit low telomerase activity before crisis, which is upregulated in the immortalised cells. One interpretation is that critical telomere reduction induces increased levels of telomerase synergistically.

Several factors, some of which have been discussed above, are important to consider when interpreting telomerase activity levels in haematopoietic as well as in other types of malignancies as outlined in Table 2. These factors will not be discussed further. It is, however, necessary to stress that detection of telomerase in a tumour does not prove that it is immortalised, but simply that it fulfils one requirement for immortality.

CLINICAL IMPLICATIONS

Leukaemias and lymphomas are usually telomerase-positive and most cases seem to have shortened telomeres, features that make these diseases interesting targets for antitelomerase therapy. Standard chemotherapy protocols are effective in achieving remissions, but relapses often occur especially for lymphomas and leukaemias in adults. An attractive future scenario is a first-line treatment with cytotoxic drugs followed by antitelomerase drug(s), leading to progressive telomere erosion and hopefully activation of the senescence programme with subsequent cell death in the tumour cells. Antitelomerase 'therapy' has been successful *in vitro* using ddG or AZT to inhibit telomerase activity in two lymphoid cell lines [56]. Side-effects of antitelomerase drugs can be anticipated from organs with telomerase-positive cell populations, such as bone marrow, lymph nodes and skin. Since most stem cells are quiescent and only transiently cycling, side-effects might be relatively mild. Stem cells also have longer telomeres than most tumour cells and would probably not be affected by treatment over a limited time span. The TRF profile of expanding germinal centre B-cells is unknown and the high levels of telomerase in these cells might reflect a vital requirement for compensating telomere erosion during activation and subsequent clonal expansion. Thus, side-effects from the immune system cannot be excluded. One factor that might confer resistance to telomerase blockers is the theoretical presence of a telomerase-independent mechanism for telomere maintenance, as demonstrated in cultured cell lines [56, 57]. When studying progressive non-Hodgkin's lymphomas, we have found a heterogeneity of telomere length in relapsing subclones, which might be a complicating factor since at relapse a completely 'new' TRF profile demonstrating long telomeres can often emerge (K.-F. Norrback, Umeå University, Sweden). Despite these possible confounding factors, haematological malignancies can be considered attractive

targets for future therapeutic efforts using antitelomerase technology.

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