JOURNAL OF PATHOLOGY
J. Pathol. **187**: 100–111 (1999)

CELLULAR SENESCENCE AND CANCER

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SUMMARY

The proliferative lifespan of normal mammalian cells is limited by intrinsic controls, which desensitize the cell-cycle machinery to extrinsic stimulation after a given number of cell divisions. One underlying clock driving this process of 'replicative senescence' is the progressive erosion of chromosome telomeres, which occurs with each round of DNA replication. This appears to trigger growth inhibition via activation of the tumour suppressor gene (TSG) product, p53, and the consequent up-regulation of the cell-cycle inhibitor $p21^{WAF1}$. Other inhibitory pathways are also activated (possibly by additional clocks), including the TSG $p16^{INK4a}$ and the less well-defined complementation group genes. Loss of one pathway can be compensated, after a limited extension of lifespan, by further up-regulation of the others, so that to escape mortality a developing tumour must overcome multiple 'proliferative lifespan barriers' (PLBs) by successive genetic events, each conferring a new wave of clonal expansion. This provides one explanation for the existence of *multiple* genetic abnormalities in human cancers; furthermore, the diversity in the nature and timing of these PLBs between different cell types may explain the variation in the *spectrum* of abnormalities observed between the corresponding cancers. Even if all senescence pathways are inactivated, immortalization can only be achieved if erosion of telomeres is halted, before their end-protecting function is lost. This usually requires either activation of telomerase during tumour development, if the cell of origin is telomerase-negative, or up-regulation if the normal cell already has some activity, but not enough to prevent erosion. In either case, cancers often maintain near-critical telomere lengths; hence pharmacological inhibition of telomerase remains an attractive approach to the selective killing of tumour cells. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—**senescence; cancer; immortalization; cell cycle; p53; cyclin; tumour suppressor gene; telomeres; telomerase; breast cancer**

THE BIOLOGY OF REPLICATIVE SENESCENCE

Over 30 years ago, the pioneering observations of Hayflick[1] established that human diploid fibroblasts show a spontaneous decline in growth rate on continuous culture, related not to elapsed time but to an increasing number of population doublings (PD), eventually terminating (after 50–70 PD) in a quiescent but viable state, now termed 'replicative senescence'. Similar behaviour has since been observed in a wide variety of normal cells, both mesenchymal and epithelial (although for technical reasons this list still unfortunately lacks the cells of origin of many common cancers), and there is now widespread[2,3] (although not universal[4]) agreement that normal human somatic cells have an intrinsically limited proliferative lifespan even in ideal growth conditions.

The kinetics of replicative senescence (Fig. 1) show not an abrupt arrest of the whole population, but a gradual decline in the proportion of dividing cells,[5] the exact timing of which varies both between cell types and between sister clones. This behaviour is best explained as the result of (i) an intrinsic control mechanism linked to elapsed cell divisions—the 'replicometer'—which progressively desensitizes the cell-cycle machinery to growth factor stimulation (Fig. 2), together with (ii) a stochastic component probably having the same (still unknown) basis as that observed in immortal cells under conditions of growth factor restriction.[6]

On purely theoretical grounds, Olovnikov[7] originally postulated that the biological clock which counts cell division events is based on the progressive erosion of chromosome telomeres with each cell cycle, which occurs in somatic cells due to incomplete replication of one end of each DNA strand.[8,9] The resulting shortening of telomeres may generate a signal either by *cis*-acting effects on sub-telomeric genes, by the release of *trans*-acting factors, and/or by loss of binding proteins resulting in the exposed end being seen as a 'DNA damage' signal.[10–13] In addition to a large volume of correlative data, this hypothesis has now been convincingly proven—at least for fibroblasts—by the essential 'interventional' evidence[14] that prevention of telomere erosion by forced expression of the enzyme telomerase prevents the onset of replicative senescence. Although this has also been shown in several other cell types, its universality nevertheless remains to be tested. It is almost certainly not the rate-limiting clock in many rodent cells which have short lifespans despite extremely long telomeres,[15] and moreover, candidate human cell types (e.g. thyroid follicular cells,[16] some oral keratinocytes,[17] and uroepithelial cells[18]) are emerging in which the kinetics of growth arrest and/or telomere behaviour are inconsistent.

Other potential 'clocks' include the progressive decrease in DNA methylation observed in senescing cultures[19] which may switch-on intrinsic inhibitory regulators, notably $p16^{INK4a}$, by promoter demethylation (although how this might be linked to elapsed divisions is less clear). Alternatively, accumulated random DNA damage[20] rather than a 'clock'-type mechanism is still a possibility, although it is more likely that

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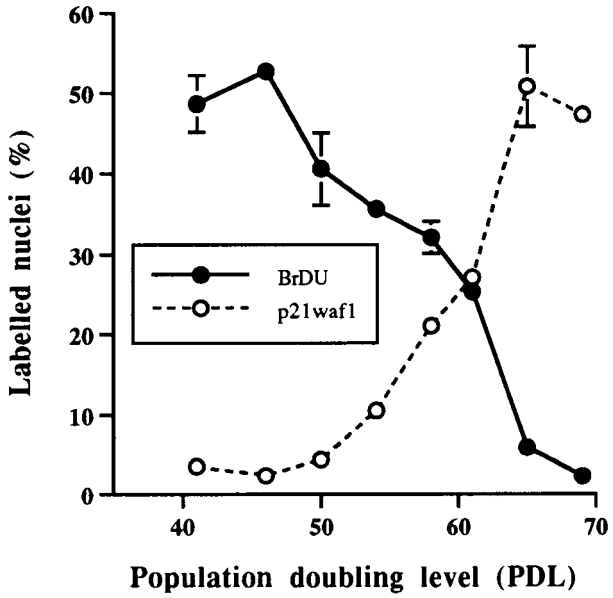

Fig. 1—Kinetics of replicative senescence and cell-cycle inhibitor expression in human diploid fibroblasts. A sub-clone of the normal fibroblast strain HCA2 was analysed from its first isolation (corresponding to ~40 PD of the original culture) up to senescence (~70 PD). The percentage of cells undergoing DNA synthesis is shown as the percentage of nuclei incorporating BrdU after a 1 h labelling period (detected by immunocytochemistry). In parallel cultures, the proportion of nuclei expressing detectable levels of the cyclin/CDK inhibitor $p21^{WAF1}$ was also determined by immunocytochemistry. After an initial lag, there is a progressive fall in the BrdU labelling index which is almost perfectly correlated with a corresponding increase in the p21 labelling index (F. S. Wyllie and M. Haughton, unpublished data)

the observed effects of oxidative damage on lifespan operate through modulation of the rate of telomere erosion.

Given the existence of cellular 'clocks', what are the effector pathways by which they control senescence? One important player is the p53 tumour suppressor. Although it originally appeared from early studies using DNA tumour virus genes that escape from fibroblast senescence required loss of both the p53 and the pRb tumour suppressor genes, our laboratory and others[21,22] have subsequently shown that loss of wild-type p53 function is sufficient for escape, i.e. that p53 forms part of an essential effector pathway. Consistent with this, senescence is associated with a switch-on of the transactivation function of p53, as shown both by reporter construct experiments and by DNA binding,[23–25] although interestingly not accompanied by a corresponding increase in protein content. Such a response resembles that seen following DNA damage (e.g. by ionizing radiation) and hence supports the notion[11,13] that telomere erosion acts by generating a 'DNA damage' signal. The details of the mechanism by which p53 is activated, however, remain to be elucidated, though formally at least three possibilities exist: (i) direct post-translational modification, most obviously by phosphorylation; (ii) up-regulation of a transcriptional cofactor, for which $p33^{ING1}$ is currently a strong candidate;[26] and (iii) down-regulation of an inhibitor. The latter possibility is supported by the finding that the product of another gene up-regulated in senescence—$p19^{ARF}$—can release p53 from inhibition by mdm2,[27] which, as we have recently shown, is sufficient to cause growth arrest in young fibroblasts.[28] Against this, however, this mechanism should lead not only to activation, but also to stabilization of p53, for which there is little evidence in senescent fibroblasts.[29]

Activation of p53 is in turn associated with the up-regulation of the cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1}$, which has a direct inhibitory action on the cell-cycle machinery,[30] is essential for senescence,[31] and correlates well with the declining growth rate in senescing cultures (Fig. 1). Although p21 induction initially appeared to be independent of p53, this now appears to be explained by the use of mutant p53 and SV40T constructs which only partially abrogated p53 function.[32,33] Subsequently, use of micro-injected anti-p53 antibodies[29] has convincingly demonstrated that loss of p53 function abolishes p21 expression and that this reverses growth arrest even in fibroblasts which have already senesced. Nevertheless, the early experiments fortuitously revealed that a partial loss of p53 function, insufficient to turn off p21, is enough to prevent the onset of senescence, hence pointing to the existence of essential downstream p53-dependent signals additional to p21, a current candidate being IGFBP-3.[25,34,35]

Senescence is also tightly correlated with the up-regulation of another CDK inhibitor, $p16^{INK4a}$[36,37] (which, interestingly, is encoded by a locus which overlaps that of $p19^{ARF}$). This provides a p53-independent pathway to cell-cycle inhibition which, like p21, converges on Rb (Fig. 3), although it remains to be shown whether p16 function is also essential for normal fibroblast senescence. The upstream controls of p16 expression are still obscure, although the finding that it can be induced by DNA damage[38] suggests that it, too, may be activated by telomere erosion. Alternatively, the decrease in DNA methylation in senescence referred to above may be involved, particularly given the known importance of promoter methylation for regulation of p16 expression.[19,39]

BEYOND SENESCENCE: MULTIPLE LIFESPAN BARRIERS

Expression of the DNA tumour virus genes SV40T or HPVE6/E7 prevents human fibroblasts from entering senescence and confers an extended lifespan of around 30–40 PD, after which, however, net growth again ceases in a state variously termed 'crisis' or 'mortality stage 2 (M2)'.[40] This differs fundamentally from senescence [mortality stage 1 (M1)] in that growth arrest is due not so much to a decrease in proliferation as to an increase in cell death rate. Escape from M2 can occur rarely as a spontaneous event (in human fibroblasts, at a frequency of around 1 in 10^7 cell divisions) giving rise to an immortal sub-clone.

Crisis now appears to be a direct result of the progressive erosion of telomeres which continues unabated once cells have bypassed the growth arrest signals of

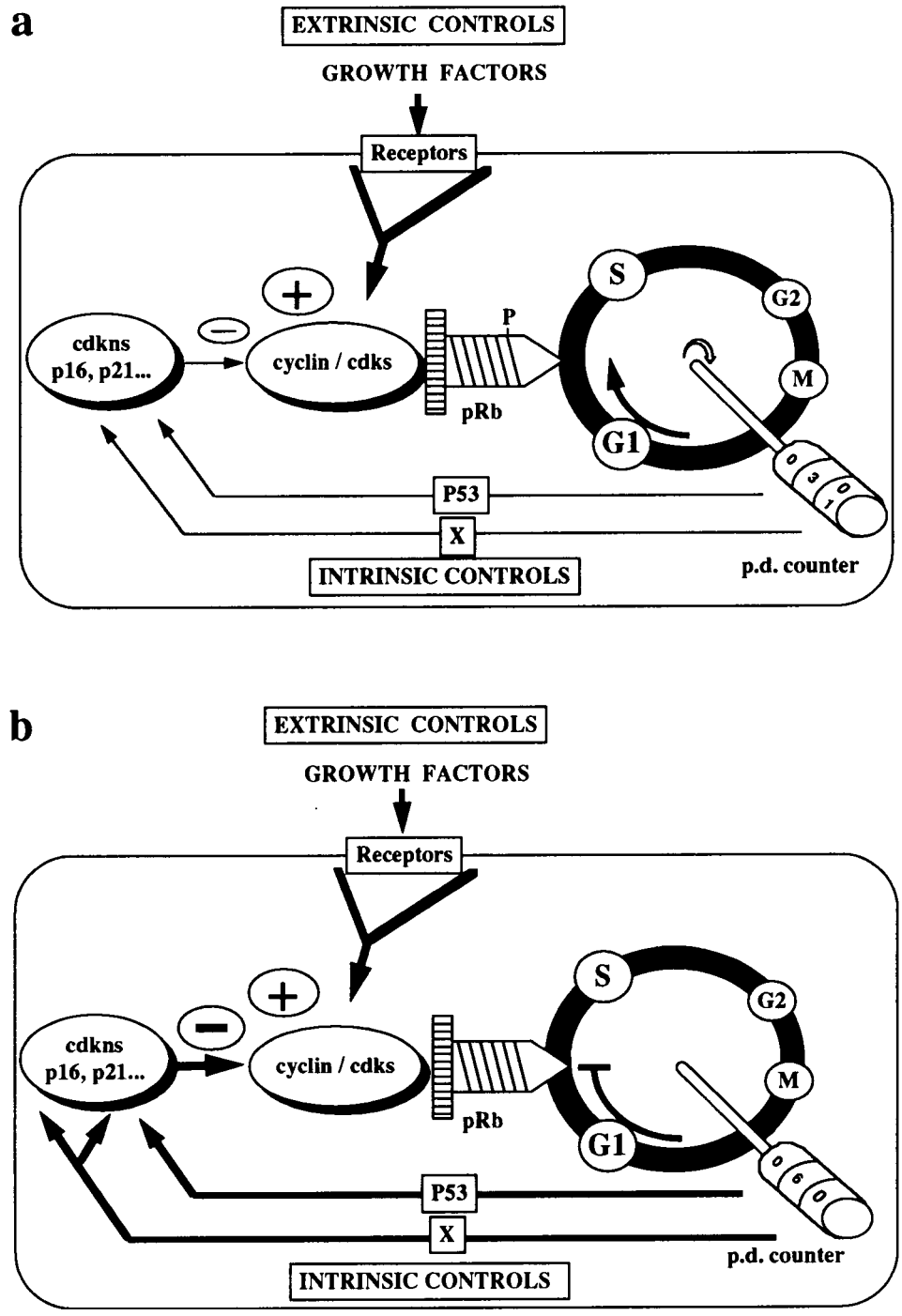

Fig. 2—Diagrams illustrating the underlying mechanisms of cell-cycle arrest in replicative senescence. For simplicity, the cell cycle is shown here as regulated solely by pRb, which acts as a 'valve' controlling G1→S progression depending on its state of phosphorylation by cyclin-dependent kinases (CDKs). These act effectively as an integrator of (i) stimulatory signal pathways activated by extrinsic growth factors and (ii) intrinsic inhibitory signals activated by a cell-division counter. One such pathway operates via p53 to up-regulate the CDK inhibitor $p21^{WAF1}$; at least one other pathway (shown here as X) is also involved, a strong candidate being the CDK inhibitor $p16^{INK4a}$. In the young fibroblast (a) growing in an optimum mix of growth factors, the stimulatory signals predominate, resulting in Rb phosphorylation and cell-cycle traverse. With increasing replicative age (PD), however, there is a progressive activation of the inhibitory controls, until the stimulatory signals are counter-balanced, Rb phosphorylation is prevented, and cell-cycle progression arrested. This state of replicative senescence (b) is reached in human fibroblasts after around 60 PD but may occur much sooner in other cell types

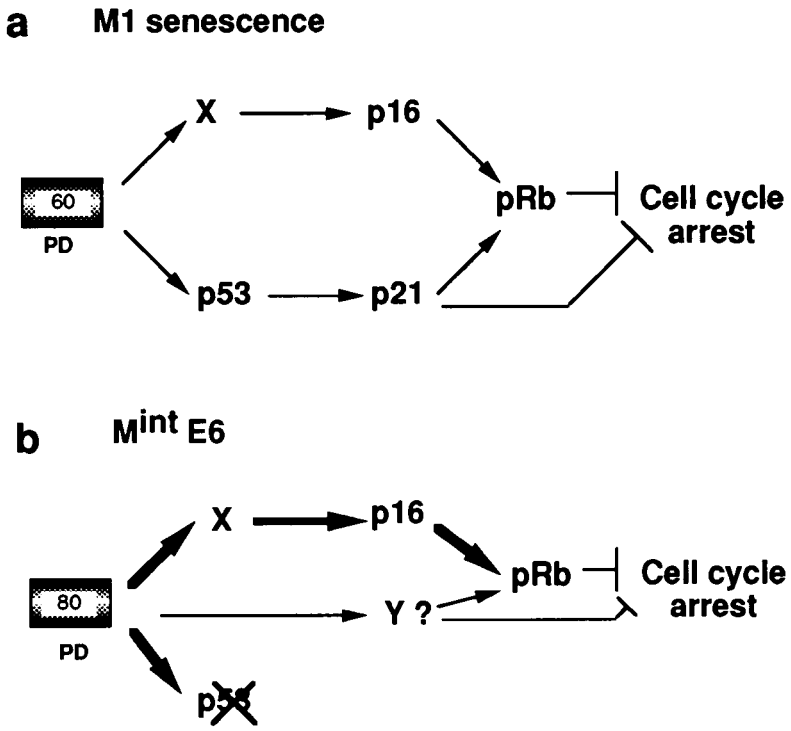

Fig. 3—Multiple signal pathways regulating proliferative lifespan. (a) Senescence (M1) in normal human fibroblasts is associated with the up-regulation of at least two growth inhibitory pathways: one acting via p53 and p21, and the other (less well characterized) acting through p16. This inhibits cell-cycle progression by preventing phosphorylation of Rb and, in the case of p21, possibly additional downstream targets of cyclin E/CDK2. (b) Loss of one pathway, illustrated here by p53 mutation, allows a temporary escape from senescence, following which cell-cycle arrest is reinstated $M^{int}E6$. This correlates with, and is almost certainly in part due to, a further up-regulation of the remaining intact p16 pathway, restoring the inhibition of Rb phosphorylation. However, the requirement for an additional pathway (Y) and the involvement of targets other than Rb cannot be excluded at this stage

senescence,[41] and eventually results in loss of the end-protecting function of telomeres and their associated binding proteins (notably TRF2).[42] The resulting end-to-end chromosome fusions probably lead via bridge-breakage cycles to cell death. Consistent with this, spontaneous escape from crisis is associated with the stabilization of telomeres either by reactivation of the enzyme telomerase[41] (see below) or by a still poorly understood alternative mechanism (ALT),[43] probably recombinational in nature.

More recently, studies using dominant-negative p53 mutants or individual HPV genes, which produce more restricted signal pathway defects, have revealed the existence of intermediate lifespan barriers between M1 and M2. This is most clearly seen in fibroblasts having a specific loss of p53 function but an intact p16/Rb pathway. Using HPV16E6 or mp53, for example, we have shown[44] that although such cells escape senescence, they subsequently undergo growth arrest after significantly fewer population doublings (PD) than an M2 culture ($\sim$20 vs. 30–40). Furthermore, this intermediate arrest state (which we have termed $M^{int}E6$) resembles much more closely M1 than M2 and indeed is indistinguishable from M1 in terms of morphology and cell kinetics. An equivalent state has been observed in fibroblasts cultured from Li–Fraumeni syndrome (LFS) patients bearing a germ-line p53 mutation, following spontaneous loss of the remaining wild-type p53 allele.[22]

Analysis of CDK inhibitors confirms that $M^{int}E6$ cells arrest despite the absence of p21 up-regulation (due to lack of p53 function). There is, however, a further increase in p16 protein levels over and above that seen at M1 which, given the presence of functional Rb, provides a plausible explanation for the restoration of growth arrest (Fig. 3). This is consistent with the ability of high-level, experimentally-induced p16 expression to induce a senescent-like state in young fibroblasts[45] or immortal LFS cells,[46] without the co-operation of p21, and also the observation that loss of p16 appears to be necessary for immortalization of LFS fibroblasts.[22]

As shown in Fig. 3, therefore, the p53 and p16 pathways may act as a biological analogue of dual-circuit braking, loss of one being compensatable by up-regulation of the other. The reverse scenario, i.e. selective loss of the p16 pathway, has proven more difficult to examine, due to technical dificulty in specifically targeting p16, and the uncertain interpretation of results with HPVE7 given that by targeting Rb (and indeed probably also p21) it affects both pathways. Nevertheless, even with E7 an M^{int} state is observed occurring after approximately the same number of

PD as M^{int}E6 but more closely resembling M2 in its phenotype and resulting, at least in part, from p53-dependent cell death.[44]

In addition to the above two pathways, there is longstanding evidence from somatic cell fusion data that at least one additional pathway needs to be lost before many cell types can escape mortality. Immortal lines which have lost both p53 and Rb function (e.g. through SV40 large T antigen expression) generate limited-lifespan hybrids following fusion with normal fibroblasts[47] and with functionally Rb- and p53-defective lines from other complementation groups.[48] Loss of heterozygosity (LOH) and microcell fusion data also reveal a consistent loss of the putative senescence genes corresponding to these complementation groups in specific cell and tumour types. For example, loss of a gene on chromosome 6q termed SEN-6 appears to be necessary for fibroblast immortalization,[49] while keratinocytes need to lose a senescence gene on 4q32 or 7q31 (the complementation group B and D genes, respectively).[50] These genes do not seem to function as telomerase suppressors as shown, for example, by the keratinocyte line BICR7, which has lost the D-group locus but is still telomerase-negative,[50] and by the finding that mortal hybrids between different complementation groups can still be telomerase-positive.[51] Elucidation of the nature of this third 'braking circuit' awaits cloning of the respective genes.

Cell-type diversity in lifespan regulation

Although only a few cell lineages have been analysed in sufficient detail, major differences in the rate-limiting controls on proliferative lifespan are already becoming apparent (Fig. 4), with both p53-dependent and p53-independent pathways existing.

When primary cultures of breast are maintained in the appropriate medium, a long-lived sub-population grows out which is capable of 30–40 population doublings (PD) before entering a typical M1 senescence-like state. In striking contrast to fibroblasts, however, full escape from senescence in these so-called 'post-selection' mammary cells can be conferred by abrogation of p53 function alone, e.g. by HPVE6, following which cells continue to proliferate until they reach crisis.[52] This apparent dependence on just a single pathway, together with a greater spontaneous frequency of escape from M2, has been suggested to contribute to the greater tumourigenic potential of breast epithelium compared with fibroblasts.[52]

While operationally these observations remain valid, it has subsequently emerged that the lack of a p16/pRb 'back-up' pathway is not due to failure to activate it, but simply that it has already been suppressed early in the life history of these cells by methylation of the p16 promoter.[53,54] What is still not clear is whether this event occurs during the selection process in culture, or whether it is a constitutive feature of these cells even *in vivo*. It is also not clear why E7 does not extend lifespan in these cells, since its 'central' action on Rb (and p21) should also compromise at least part of the p53 pathway. p53-induced cell death does not appear to be the explanation (J. Shay, personal communication), suggesting that in these cells p53-mediated senescence can be maintained via a p21- and pRb-independent effector pathway.

The nature of the clock which activates p53-dependent senescence in these breast cells is almost certainly telomere erosion. Like fibroblasts, they are telomerase-negative, undergo progressive shortening of telomeres up to and beyond senescence, and enter crisis unless telomerase becomes spontaneously reactivated.[52]

The 'post-selection' breast cells referred to above express a phenotype intermediate between that of myoepithelial cells and luminal cells, characterized by 'basal' cell features such as vimentin, cytokeratin (CK) 14, and epidermal growth factor (EGF) receptor expression and lack of oestrogen receptor (ER), together with luminal cell features, notably expression of CK8 and CK18.[55] These cells may represent a stem cell pool in the normal gland and as discussed below, are probably the progenitor of a sub-set of aggressive, ER-negative breast cancers. The majority of epithelial cells initially present in primary cultures of normal breast exhibit a shorter proliferative lifespan in culture (up to 20–25 PD depending on the medium used), after which they enter a senescence-like state sometimes termed M_0.[56] These 'pre-selection' cells show a strikingly reciprocal pattern of lifespan control compared with their post-selection counterparts. Expression of HPVE7 overcomes M_0, leading to an extension of lifespan, whereas E6 is without effect.[53,56–58] Similar data have been obtained from luminal breast cells cultured from milk,[57,58] in which E7 conferred up to 60 PD of additional lifespan.

Again, therefore, these cell types appear to differ from the fibroblast paradigm in that their normal lifespan is regulated by a sub-set of pathways, in this case p53 being the 'unimportant' component. Recent work[53,54] has shown that, as predicted, M_0 is associated with an elevation in p16 expression, consistent with the p16/Rb pathway being the limiting control. Again, though, the difference from fibroblasts may be more apparent than real, since if passaged for long enough, breast cells which have bypassed M_0 through expression of E7 eventually enter an M1-like state which is now p53-dependent.[53] In summary, therefore, an apparent dependence on a single pathway for lifespan regulation occurs either because the alternative pathway is already inactivated (post-selection breast cells) or because the timing of its activation is greatly delayed (pre-selection cells) (Fig. 4).

'p53-independent' senescence may in fact be quite widespread, other examples including at least some sub-populations of keratinocytes,[17] thyroid epithelial,[59–61] and uroepithelial[62] cells. Two features which suggest that the senescence clock in such cases is not telomere erosion are firstly the more rapid onset of senescence and secondly the lack of involvement of p53. Furthermore, keratinocytes[17] and uroepithelial cells[18] exhibiting this behaviour have recently been shown to be constitutively telomerase-positive and to senesce without detectable telomere shortening.

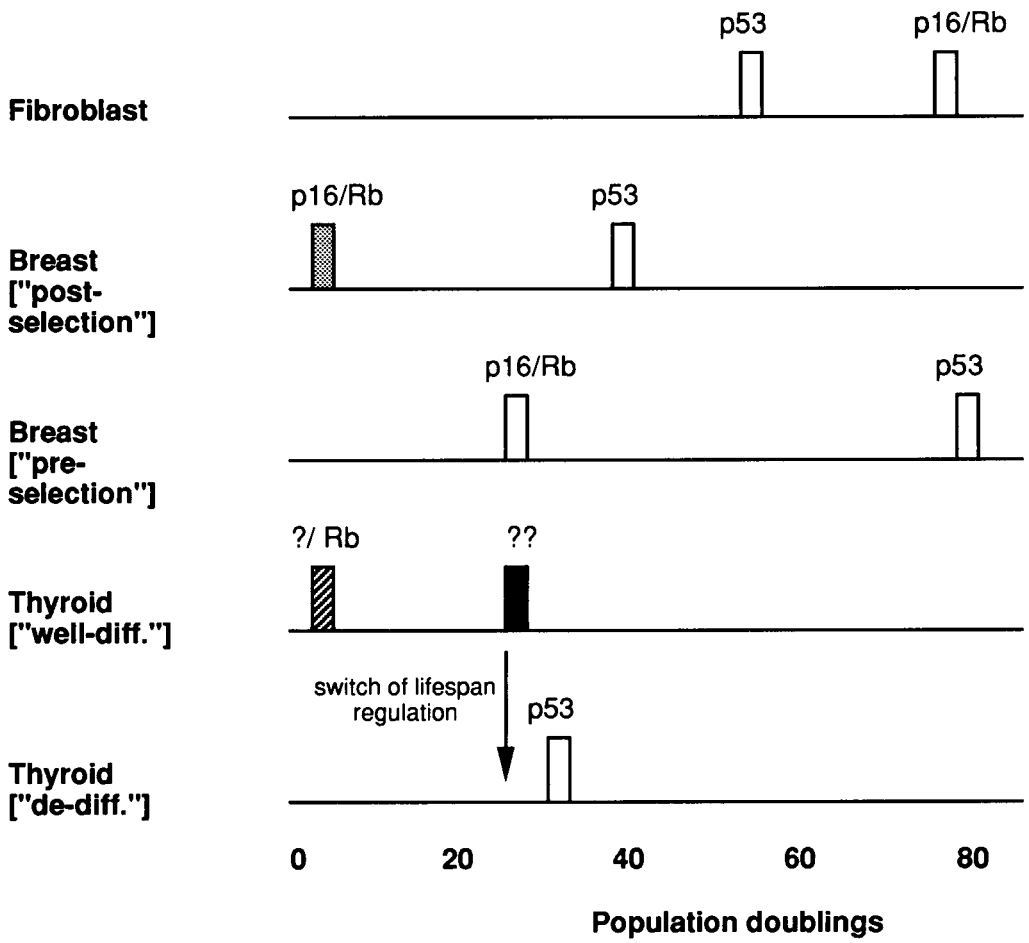

Fig. 4—Cell-type diversity in proliferative lifespan control. A compendium of data derived from cell culture studies illustrating the variation in the nature and timing of proliferative lifespan barriers (PLBs) between different cell types. Vertical bars show the approximate replicative age at which PLBs come into operation and the corresponding tumour suppressor genes whose loss allows the barrier to be circumvented. 'Post-selection' breast epithelial cells resemble fibroblasts in the timing of their p53-dependent PLB but do not exhibit a second PLB because the p16 pathway has already been turned off by methylation (shaded bar) by the time the cultures are first isolated. Conversely, 'pre-selection' breast epithelial cultures are regulated primarily by a p16-dependent PLB and although escape from this is followed eventually by a p53-mediated PLB, this is delayed to such an extent that it may not be a significant barrier to cancer progression. Thyroid follicular cells show a very early PLB (which may be mechanistically distinct from senescence) which is Rb-dependent but not p16-dependent (hatched bar). Escape from this is followed by a second, unusually stringent PLB, the nature of which is still obscure (solid bar). Escape from this can, however, occur indirectly by spontaneous dedifferentiation, which generates a cell with PLBs resembling those of the fibroblast

RELEVANCE OF SENESCENCE TO HUMAN CANCER

Senescence as a natural tumour suppressor mechanism

Depending on the cell kinetics of the tissue of origin, tumour development can be initiated by genetic events causing either a block in terminal differentiation (hence leading to inappropriate retention of daughter cells) or/and inappropriate activation of growth stimulatory signal pathways. The net result in both cases is the generation of a clone of cells which should in theory be capable of infinite expansion (provided that it is not constrained by physical barriers or lack of blood supply). In practice, however, it is clear from the multiplicity of genetic abnormalities observed in nearly all cancers that progression to a clinically relevant mass requires additional clonal selection steps. While almost certainly not the only hurdle which a developing tumour has to overcome, one potential selection pressure driving this clonal evolution is the need to escape proliferative lifespan ('senescence') barriers.

The notion that replicative senescence could provide an innate tumour suppressor mechanism has been criticized on the basis that a proliferative lifespan of even 50 PD, as seen in fibroblasts, would be sufficient to generate a tumour mass several orders of magnitude greater than that required for lethality, before senescence was reached. However, this argument fails to take account of at least two factors which may greatly reduce the number of cells generated for a given number of PD: firstly, the existence of ongoing cell death and differentiation within a tumour, which effectively 'wastes' PD, and, secondly, the occurrence of clonal selection driven by barriers unrelated to senescence. Finally, as noted

above, the proliferative potential of many clinically relevant cell types is probably far less than that of the archetypal fibroblast.

Given, then, that senescence is a plausible mechanism, what is the evidence that it occurs in real tumours? *In vivo* data are necessarily limited, but are consistent with senescence being the factor which limits the growth of many early-stage tumours, including a large number of benign connective tissue and epithelial tumours, adenomas of the colon[63] and thyroid[64] being particularly good candidates. The indolent behaviour of some cancers may also be explained in this way, notably occult carcinomas of the thyroid and prostate.

In vitro studies can potentially provide more direct evidence. In some cases, e.g. colon[63] and squamous carcinomas of the head and neck,[65] the proliferative potential of cells cultured directly from varying stages has been consistent with the earlier lesions being composed of senescing cells. Moreover, where early stages of tumourigenesis have been reconstructed *in vitro* by transfer of the appropriate mutant oncogene into normal human cells,[66] the resulting clones exhibit a limited lifespan (<25 PD), ending in a state consistent with replicative senescence.

Genetic evidence is also strongly supportive. Most clinical cancers exhibit losses of multiple genes associated with lifespan regulation, including p53, p16, and the complementation group genes.[50,67] Furthermore, although still mainly based on tumour-derived cell lines, replacement of these genes individually by transfection[68] or micro-cell fusion[69] has been shown to induce senescence in immortal cancer cells.

It should be noted, though, that while most cancers probably contain immortal cells, some may simply be at a stage of *extended* lifespan. This was well illustrated by an analysis of the proliferative potential of cells cultured directly from a series of head and neck cancers, which revealed tumours which had undergone some but not all of the losses of lifespan regulatory controls needed for immortalization.[70] Finally, these studies also emphasized the caveat that it is possible for even a clinically significant cancer to be composed of entirely mortal, pre-senescent cells if the cell of origin has sufficient proliferative lifespan and the tumour develops with sufficiently few successive clonal expansion steps and/or a sufficiently low cell death rate. Even in these examples, however, senescence may, of course, still become a significant barrier to the *recurrence* of tumours from the small numbers of residual cells following therapy.

Proliferative lifespan barriers as the selection pressure for tumour suppressor gene mutation

As discussed above, the relative timing and functional importance of different lifespan regulating signal pathways differ significantly between cell types. Such differences in the rate-limiting controls on proliferative lifespan represent an obvious basis for differential selection pressure for mutations interrupting these pathways, and indeed might represent a major determinant of the observed patterns of tumour suppressor gene mutation in human cancers. Although the available data are still scanty, there is growing evidence in support of this concept, in particular with regard to p53.

p53 mutation is, overall, by far the most frequent somatic genetic abnormality in human cancer, yet in some common tumour types, it is seen only in a phenotypically-distinct sub-set of cases.[61] In breast, for example, approximately 30 per cent of invasive ductal carcinomas exhibit p53 mutation, which correlates with absence of ER expression, high EGF receptor expression and poor prognosis. These tumours probably also correspond to a separately identified ER-negative, poor prognosis sub-group defined by CK14 and vimentin expression. These phenotypic features strongly suggest that this sub-set of tumours is derived from the minority cell type which grows out in culture to form the 'post-selection' population referred to above[56,57] and which notably exhibits a strictly p53-dependent regulation of senescence. In contrast, the majority of breast cancers retain wild-type p53 and have a phenotype corresponding to a sub-population of breast cells *in vitro* (luminal cells) which shows a p53-independent regulation of senescence[57,58] and (once this is overcome) is capable of a large number of population doublings before a p53-dependent barrier finally comes into operation.

Taken together, these data suggest that the observed frequency of p53 mutation in the different sub-sets of breast cancer directly reflects the differing selection pressures operating on their respective cells of origin.

Most reviews of this field make the tacit assumption that the existence of a more aggressive sub-set within a given cancer type is somehow driven by these cases having acquired a p53 mutation, i.e. that different phenotypes arise from a common cell of origin depending on the chance nature of the genetic events undergone. If the above interpretation is correct, however, both the genotype and the phenotype of the tumour are essentially predetermined by the phenotype of the cell of origin. Viewed in this way, it is not that the tumour is aggressive and less differentiated *because* it has acquired a p53 mutation, but that the p53 mutation is simply an inevitable reflection of the normal controls operating in the cell of origin. Perhaps the major implication of this idea is that the correlation between a given gene mutation and a particular clinico-pathological behaviour, in this case mutant p53 with poor prognosis, may be an indirect one, both being determined by the underlying cell context. In such cases, it follows that the search for a direct link between mutation and tumour behaviour will be potentially fruitless (although in the case of p53, secondary effects of mutation might be expected through loss of genomic stability). It is not surprising, therefore, that suggestions as to how p53 mutation might cause increased breast cancer aggressiveness have been unconvincing!

The need to overcome *multiple* lifespan barriers, as illustrated by the fibroblast model, also provides one explanation for the occurrence of *multiple* tumour suppressor gene losses in most cancers. Although direct support for this concept in human tumours is difficult to obtain from clinical samples, cell culture studies have identified the predicted existence of intermediate stages in which a sub-set of senescence genes has been lost. For example, in the head and neck cancer series referred to

above, one tumour had lost p16 expression but still retained wild-type p53 and gave rise, as expected, to cultures which were still mortal.[50,70]

Again, this is an area where cell-type diversity is likely to be important. For example if the successive lifespan barriers are separated by a sufficiently large number of population doublings, a cell which has escaped senescence may be able to generate a clinically significant tumour without ever reaching the second barrier. A good candidate for this type of behaviour is the sub-set of breast epithelial cells described earlier which display an initial p53-independent senescence followed many tens of population doublings later by a p53-dependent barrier (Fig. 4).

Finally, a more subtle way in which lifespan barriers may be involved in tumour progression is suggested by *in vitro* studies of thyroid tumourigenesis.[59,61] In this model, well-differentiated thyroid epithelial cells which have escaped senescence are ultimately arrested by an Rb- and p53-independent proliferation barrier, the nature of which is, at present, unclear but which appears to be extremely stringent. However, such cells can undergo a spontaneous switch in differentiation to a more mesenchymal phenotype, whereupon the lifespan barrier of the thyroid cell appears to be replaced by a p53-dependent control, escape from which can then potentially occur at a measurable frequency by p53 mutation. An otherwise insurmountable lifespan barrier can therefore be effectively side-stepped by a change in differentiation to a cell type which employs a less stringent control (Fig. 4).

Such de- or trans-differentiation events are a widespread feature of tumour progression and frequently accompany more aggressive behaviour. A particularly clear-cut example is the phenotypic switch which occasionally occurs in thyroid cancers, from the well-differentiated sub-types with limited proliferative potential and excellent prognosis to the exceptionally aggressive undifferentiated (anaplastic) form.[71] Conventionally, such changes in tumour differentiation, while useful for diagnosis, have tended to be regarded as epiphenomena, secondary to the underlying mutationally driven progression of tumour growth. The parallels between the above *in vitro* model and the conversion of well- to un-differentiated thyroid cancer *in vivo* suggest, however, that at least in this example of progression, increased proliferative potential may be dependent on epigenetic as well as mutational mechanisms and may result from a synergism between the differentiation switch discussed here and the occurrence of p53 mutation, which is a hall-mark of undifferentiated thyroid cancer.[72] The rarity with which the transition to undifferentiated cancer is seen clinically in thyroid can then be readily explained by the need for the differentiation switch and the p53 mutation to arise independently in the same cell before any selective advantage is obtained.

TELOMERASE AND CANCER: A GROWING CONTROVERSY

The above discussion has centred on lifespan barriers based on cell-cycle inhibition by specific signal pathways. However, even when such 'regulatory' mechanisms are overcome, clonal expansion can only continue indefinitely if tumour cells can avoid the 'structural' barrier ultimately imposed by telomere erosion. Telomere erosion can be prevented or reversed by expression of an RNA-dependent DNA polymerase—telomerase—which uses an RNA template to add TTAGGG repeats to the 3′ ends of DNA molecules,[9] hence compensating for sequence 'lost' at each round of cell division through the end-replication problem[7] and C-strand degradation.[73] Telomerase activity is constitutively present in germ cells and early embryos but was originally thought to be switched off in all somatic cells from an early stage in embryogenesis. Clonal expansion from an adult cell, as occurs in a tumour, should therefore lead inevitably to telomere erosion, until or unless telomerase activity is restored.

The interest in telomerase was initially sparked by the demonstration[41] that in several tissue culture models, telomerase activity appeared to be 'turned on' as a spontaneous rare event, which correlated with stabilization of telomere length and escape from crisis (M2). This suggested that telomerase reactivation might also be a critical step in allowing a tumour cell population to avoid critical telomere shortening *in vivo*. This prediction initially appeared to be strikingly confirmed by surveys of human tumour samples[74] showing that over 90 per cent of cancers were telomerase-positive, whereas nearly all normal or benign tumour tissues were negative.

This classic telomerase reactivation model has, however, received two serious challenges in the last few years. The first came from the analysis of telomerase-null transgenic mice,[75] which not only appeared to develop normally, but whose cells seemed no less able to give rise to tumours or transformed immortal lines than those of its wild-type counterparts. These findings, however, were inherently misleading,[76] since this mouse species possesses telomeres which are very much larger than human telomeres, hence potentially delaying the onset of any phenotype related to telomere erosion. Indeed, now that several successive generations have been bred it is clear that once telomeres have shortened sufficiently, deleterious effects on proliferating tissues *are* seen[77] with, moreover, a corresponding impairment of tumourigenic capacity.[78]

The second problem, which is proving far more difficult to resolve, has come from the realization that one of the central tenets of the original model—the absence of telomerase activity in normal cells—is only partially true. It is now becoming clear that telomerase activity is present in a wide range of normal adult tissues but is confined, in each case, to a small sub-population of cells—which explains why it was overlooked in the initial surveys based on biochemical assay of whole-tissue homogenates. In many tissues, these sub-populations correspond almost certainly to stem cells, e.g. basal keratinocytes in epidermis;[79] in others, they represent cells which are required to undergo repeated clonal expansion, e.g. lymphocytes.[80] Furthermore, recent *in situ* analysis of the expression of the catalytic sub-unit of telomerase, hTERT,[81] currently believed to

be the rate-limiting determinant of activity, indicates that the list may be even longer, including some previously unsuspected cell types such as terminal ductal epithelium of the breast.

Whilst such findings provide a satisfying explanation for the avoidance (or at least delay) of telomere erosion in high-turnover tissues, they pose a major problem for the role of telomerase in cancer, since over 90 per cent of cancers arise from exactly those cell types which now seem to be telomerase-positive even in the normal state. Reports of absent telomerase activity in early-stage tumours, e.g. colonic adenomas,[74,82] do not help since this can also be explained as a false-negative because, like normal tissue, such lesions probably contain only a minor sub-population of cells in a 'stem-like' state.[83] Indeed, it is an unfortunate irony that the cell types (notably fibroblasts) used in the original *in vitro* studies may well turn out to be among the few which are genuinely telomerase-negative!

The most sceptical viewpoint, therefore, would be that most of the reported differences in biochemically-assayed telomerase activity between tumours and normal tissue merely reflect differences in tissue composition[83] and that the finding of higher telomerase activity in cancers does not imply any genetically-based selection event. One set of observations, however, keeps the telomerase model alive. This is the original finding by Hastie *et al.*,[84] subsequently widely confirmed,[85] that the majority of human cancers have much shorter telomeres than the corresponding normal tissue, in many cases approaching that associated with crisis. It follows, therefore, that even if the cell of origin and its progeny were telomerase-positive, the activity must have been insufficient to *prevent* telomere erosion, even though it may well have *slowed* the process. For such a tumour to continue to grow to a clinically significant size may therefore have required selection for an up-regulation of telomerase activity to stabilize telomere length, in which case there is no conceptual difference from the original reactivation model except that the activity starts from a finite level rather than zero.

REVISED MODELS

Clarification of the behaviour of telomere length and telomerase activity in real human tumours, as opposed to cell lines, is currently hampered by the difficulty of interpreting their respective biochemical assays (TRF and TRAP) performed on tissue homogenates and in most cases, at just a single time-point in tumour evolution. While we await the further application of *in situ* methods, however, it is useful to envisage the following three scenarios as a conceptual framework (Fig. 5).

Type 1

This is the classic model[41] revisited, in which the cell of origin is telomerase-negative and in which telomerase reactivation is directly selected in pre-crisis tumour cell populations, resulting in an immortal tumour with telomeres stabilized at or above the crisis threshold. The additional criterion now required is that the cell of origin must be shown to be effectively telomerase-negative, not just in its normal *in vivo* state but also when subject to 'excessive' growth stimulation. Many cell types which appear to be telomerase-negative *in vivo*, for example uroepithelium, may nevertheless be competent to express telomerase when subject to a sufficient proliferative stimulus.[18]

It now appears, therefore, that this model is applicable to a restricted range of tumour types. Connective tissue tumours remain the strongest candidates and at least for fibroblasts, the cell of origin is convincingly telomerase-negative,[14] even when rapidly proliferating, and gives rise to cancers which are telomerase-positive with short telomeres.[86,87] As regards the more common epithelial tumours, the situation is less clear. Some sub-populations of breast cells[52] and keratinocytes[50] are convincingly telomerase-negative, but here there is no formal proof that the telomerase-positive tumours observed in the corresponding tissues derive from these precursor cells, as opposed to other sub-populations within the same tissue which may already be constitutively telomerase-positive.

Type 2

This is conceptually the inverse of type 1, in which the cell of origin constitutively expresses telomerase at a level of activity sufficient to prevent telomere erosion throughout tumour development. From the above discussion, this scenario would also appear to be rare except possibly in tumours arising from strongly telomerase-positive germ cells, such as those of testis.[88,89]

One interesting non-germ cell candidate is breast cancer, many of which are telomerase-positive with a telomere length in the normal range.[90] This is consistent with their origin from terminal ductal cells, which were recently shown[81] to be strongly hTERT-positive (although as with all single time-point studies, the possibility of post-crisis re-elongation can never be excluded) (Fig. 5). Likewise, some sub-populations of oral keratinocytes[17] and uroepithelial cells[18] appear to be sufficiently telomerase-positive to maintain an undiminished telomere length throughout their normal proliferative lifespan (although not necessarily thereafter).

Type 3

This is the intermediate scenario, in which the tumour derives from a precursor cell which constitutively expresses telomerase at a level sufficient to *slow* but not *prevent* telomere erosion, and probably applies to the majority of common cancers, colon being perhaps the most well-studied example. The eventual decline in telomere length creates progressively increasing selection pressure for up-regulation of telomerase, which may occur as a single step through a primary genetic event, e.g. loss of a telomerase suppressor,[67,69,91] or more indirectly as a secondary consequence of genetic changes selected primarily for other reasons.

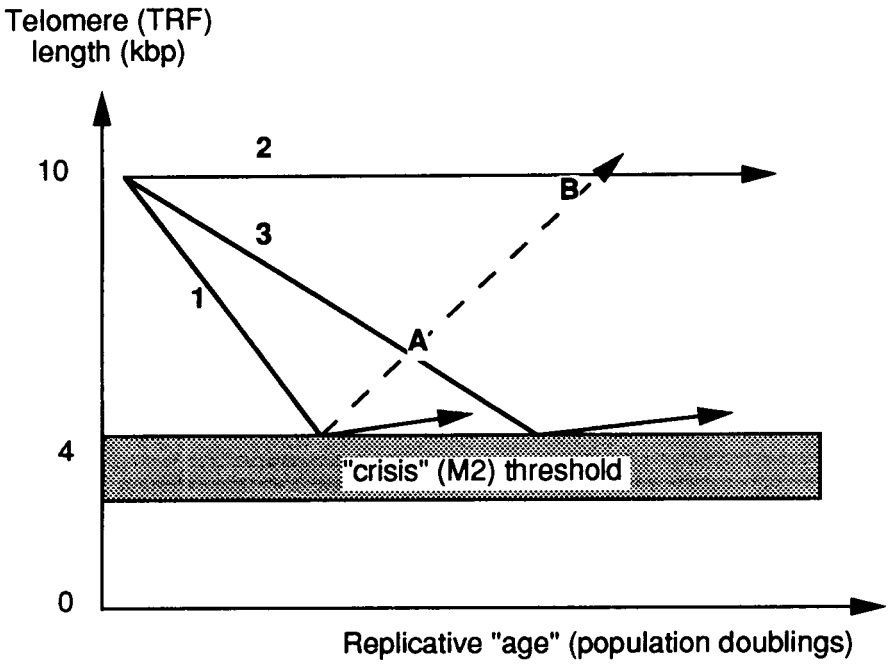

Fig. 5—Telomere dynamics in human cancer. (1) In the classic model, the cell of origin is telomerase-negative and telomere erosion proceeds to a critical threshold, escape from which is dependent on reactivation of telomerase. This is followed by stabilization of the telomere length (solid arrow) or re-elongation (dashed line). (2) In some cancers, the cell of origin may have sufficient constitutive telomerase activity to prevent significant telomere erosion. (3) In most common cancers, it now seems that an intermediate model applies, in which telomerase activity in the cell of origin is sufficient only to *slow* the rate of telomere erosion, escape from crisis still requiring up-regulation. The diagram illustrates the difficulties of interpreting telomere dynamics from 'snap-shot' analysis of tumour evolution. At point A, for example, it is impossible to distinguish between a telomerase-positive tumour of type 3 prior to crisis and a post-crisis tumour of type 1 in which telomerase reactivation has led to significant telomere elongation. Indeed, the telomere length may continue to increase to the extent that a type 1 tumour becomes indistinguishable even from type 2 (point B)

It might be argued that this is conceptually no different from type 1, but that the latter simply represents an extreme case in which the initial level of telomerase activity is below detection. However, it is likely that there is a fundamental biological distinction between the two, based on the tightness of telomerase regulation, i.e. that the quantitative increase in telomerase activity required for telomere stabilization in a cell of type 3 can occur much more readily than the *de novo* expression required in type 1. Indeed, this is indirectly supported by the fact that spontaneous immortalization is never observed in normal fibroblast cultures, despite the observation that it can be readily brought about simply by experimentally induced telomerase reactivation (by hTERT expression).[14]

Although up-regulation of telomerase is the most likely mechanism for eventual telomere length stabilization in these tumours, as a cautionary note a more subtle explanation needs to be borne in mind. This comes from the recognition that the control of telomere length involves a feedback loop which should favour the elongation of 'short' telomeres.[92] The possibility exists, therefore, that a level of telomerase activity insufficient to sustain telomeres at their normal length may nevertheless suffice once they have shortened sufficiently. Indeed, a longitudinal study of a telomerase-positive human cancer cell line has recently provided direct support for this sort of telomere dynamics.[93]

On the other hand, there is increasing genetic evidence that cancer cells regularly lose genes which code for suppressors of telomerase (probably in most cases of hTERT expression).[50,91] Furthermore, recent *in situ* surveys of hTERT expression also show up-regulation of expression on an individual cell basis in cancers compared with earlier tumour stages.[81] On balance, therefore, it is likely that up-regulation of telomerase activity is essential for most cancer cells to escape mortality.

CLINICAL IMPLICATIONS

From a diagnostic standpoint, the realization that telomerase activity and expression are not restricted to cancer cells is potentially a serious blow to its utility as a diagnostic marker. Indeed, from the above discussion it may well be that it is no better than previous markers, such as Ki67,[94] based merely on proliferative status. *In situ* analysis of hTERT expression,[81] however, offers hope that at least in some sites, such as colonic mucosa, it has a much more restricted distribution than proliferation *per se*. It remains to be determined whether this

feature, and/or quantitative differences between telomerase-positive normal and tumour cells, will turn out to be sufficiently useful in practice.

On the other hand, there are more definite grounds for optimism from the therapeutic standpoint. The above modifications to the original telomerase model do not substantially alter the fact that many cancers have mean telomere lengths well below normal and often close to the threshold for cell survival. It is this difference which provides the potential therapeutic 'window' through which future anti-telomerase therapies might be capable of selectively killing cancer cells.

ACKNOWLEDGEMENTS

Work in the author's laboratory is supported by the Cancer Research Campaign and the Medical Research Council. The editorial assistance of Theresa King is gratefully acknowledged.

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