Pergamon

European Journal of Cancer Vol. 33, No. 5, pp. 716 726, 1997

Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(97)00064-6

Proliferative Lifespan Checkpoints: Cell-type Specificity and Influence on Tumour Biology

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Lifespan checkpoints are viewed here as intrinsic mechanisms which desensitise cells to external growth signals as a programmed response to proliferative age, as distinct from externally-triggered differentiation. This review focuses on the role of tumour suppressor gene products as essential mediators of cell cycle arrest at lifespan checkpoints, concentrating in particular on p53. Although drawing inevitably on fibroblast senescence and telomere erosion paradigms, other lifespan clocks and signal pathways are discussed. Particular emphasis is placed on cell-type diversity in the nature, number and timing of lifespan checkpoints and its importance for tumour biology. Breast and thyroid cancer are used to illustrate the concept that the "choice" of checkpoint(s) in a given normal cell may have a determining influence on the mutational spectrum and clinical behaviour of its tumours. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: senescence, immortalisation, p53, pRb, tumour suppressor genes

Eur J Cancer, Vol. 33, No. 5, pp. 716–726, 1997

INTRODUCTION

IT IS now a widely accepted principle that, with the exception of germ cells and primitive embryonic lineages, normal mammalian cell populations are capable of only a finite number of doublings. However, the exact way in which this limitation of proliferative lifespan is achieved varies widely in different situations.

The simplest scenario concerns the so-called "conditional renewal" populations [1] typified by the human fibroblast. In the adult, the balance of inhibitory and stimulatory extrinsic controls (e.g. growth factors, cytokines and extracellular matrix ligands) acting on these cells is such that their proliferation rate is normally extremely low, but can be dramatically increased given the appropriate stimuli. If such stimulation is continued, then after a given number of cell divisions (which varies widely with cell type), the proliferative response becomes progressively downregulated due to the operation of cell-intrinsic inhibitory controls. In the classic fibroblast model, this desensitisation occurs after a lifespan with a mean of many tens of population doublings (PD) and a wide spread [2], and results in the state of proliferative quiescence described variously as "senescence" or "mortality stage M1" [3, 4]. As discussed elsewhere in this Special Issue, it is now widely believed that the underlying biological clock which signals this checkpoint is linked to the progressive erosion of chromosome telomeres with each successive round of replication in normal telomerase-negative cells [5, 6].

In other, less well-characterised conditional-renewal cell populations, normal proliferation terminates much earlier and more synchronously. Human thyroid epithelial cells, for example, which in other ways resemble fibroblasts in their response to extrinsic growth stimulation, are capable of only a handful of PD before entering a state of normally irreversible quiescence [7]. The kinetics and morphological features associated with this lifespan checkpoint suggest that it is distinct from conventional "senescence" and may even be mediated by a different "clock" (see below). Indeed, an alternative view of populations such as thyroid epithelium is that they are effectively transit populations, derived from stem-type precursors, which in the adult have reached a stage of reversible arrest a few PD short of final terminal differentiation.

The relationship between terminal differentiation and proliferative ageing is particularly relevant to "renewing" (stem cell) populations [1, 8], in which the nature of lifespan checkpoints is much less clear. Here there are two issues: (i) are stem cells really immortal? and (ii) is growth arrest in the differentiating compartment (terminal differentiation) mediated by a fundamentally different mechanism from cellular senescence?

Although stem cells may appear to have infinite proliferative capacity, the situation is of course not comparable with that of the fibroblast model, since it is only one daughter cell from each mitosis which retains this ability, and the number of stem cells does not normally increase. Only if

forced to undergo true exponential expansion would stem cells be expected to display a senescence-type lifespan limitation (*in vitro* behaviour suggests this is the case, at least for some). The behaviour of these populations may perhaps best be thought of as an asymmetric apportionment of lifespan to the daughter cells, one ageing little if at all, the other being committed to early growth arrest. Whether the underlying mechanism reflects differential retention of telomerase activity [9, 10] or asymmetric segregation of DNA strands [11] remains to be elucidated.

Classically, growth arrest in terminally differentiating daughter cells takes place in a structured micro-environment (typified by intestinal crypts) and can be adequately explained as the response of the daughter cell to external cues, having been presensitised by asymmetric division of the stem cell. This, together with other phenotypic and kinetic differences, suggests that growth arrest associated with terminal differentiation in renewing tissues is mechanistically distinct from senescence and may not require any cell-intrinsic "timer".

We will leave aside the issue of terminal differentiation here and concentrate on those lifespan checkpoints where it is likely that growth arrest is triggered by an underlying intrinsic "clock". We will also not consider the final barrier to immortalisation in most cells — crisis or mortality stage 2 [3, 4] — since although almost certainly determined by a "clock" (telomere erosion), this is essentially a breakdown of normal structure and function rather than a regulated control mechanism.

TUMOUR SUPPRESSOR GENE PRODUCTS AS MEDIATORS OF LIFESPAN CHECKPOINT CONTROL: THE FIBROBLAST MODEL

p53 as a "guardian of cellular senescence"

The classic state of "senescence" or "mortality stage 1 (M1)" in normal fibroblasts represents a phenotype in which, although proliferatively arrested, cells remain biochemically active and viable for long periods [12]. This is programmed ageing *par excellence*, its active regulatory nature being evidenced, for example, by the induction of specific genes known to inhibit cell proliferation, notably $p21^{WAF1}$ [13] and $p16^{INK4a}$ [14], and its abrogation by many DNA tumour virus gene products which target specific cellular regulatory proteins, in particular the tumour suppressor gene products p53 and pRb [15].

Initial gene transfer experiments, using DNA tumour viral oncogenes (HPV E6 and E7) to target p53 and pRb separately, indicated that escape from normal M1 required that the function of *both* proteins be abrogated [16]. However, this work was based on the transfection of young fibroblasts, which exhibit a wide range of variability in remaining lifespan, potentially sufficient to blur the effect of any genetic manipulation.

We reanalysed this question [17] exploiting the ability of amphotropic retroviral vectors to target a near-senescent cell population, in which most of this lifespan variability had been removed by the synchronising effect of growth to within a few PD of M1. In this way, it could be shown conclusively that expression of a dominant-negative *TP53* mutant was able to extend the normal lifespan of human diploid fibroblasts (HDF) by an average of 17, and in some cases over 25 PD [17]. The conclusion that wild-type p53 function is essential for normal entry into M1 senescence is now also supported by complementary findings in fibroblasts from Li–Fraumeni syndrome (LFS) patients, which again indicate that loss of wild-type p53 delays entry into senescence by at least 20–30 PD [18]. An extension of this sort of magnitude may not seem great in comparison with normal fibroblast lifespan, and indeed further escape to generate an immortal line is exceedingly rare in LFS (and was never observed in our experiments). Nevertheless, 20–30 PD is more than sufficient to represent a strong selective pressure for loss of p53 in a tumour clone which is reaching senescence, and is of course enough to allow a high probability of additional mutation leading to yet further extension of the proliferative lifespan.

Mechanism of action of p53 in senescence

Given that the presence of functional wild-type p53 is necessary (if not sufficient) for the normal operation of the M1 lifespan checkpoint, it is important to determine whether it is acting as a direct "switch" or whether it is simply needed at a constant level as a "permissive" factor to enable some other inducer to operate. To support the former role, it is necessary to show that an alteration in one of the biochemical functions of p53 normally occurs at M1.

At least three such functions are potentially relevant: the ability of p53 to act as a transcription factor [19, 20]; its ability to inhibit transcription from some promoters [21, 22]; and finally, a direct inhibitory effect on DNA replication [23]. We have addressed the first of these, which is by far the most well-characterised.

The transactivation activity of p53 was assessed in a subclone of normal human diploid fibroblasts stably expressing a β-galactosidase (β-gal) reporter construct (RGCΔfosLacZ) [24]. As cells approached senescence, we observed a dramatic increase in p53-driven β-gal expression, which showed an almost perfect inverse correlation to the rate of proliferation as assessed by bromodeoxyuridine incorporation. The level of β-gal induction was comparable to the maximum achievable by the well-established p53 activator, UV radiation [25]. These data provide strong, albeit correlative, evidence that activation of this function of p53 is an essential step for normal entry into senescence (although of course not formally excluding a requirement for one of its additional functions).

Interestingly, transcriptional activation by p53 in senescent fibroblasts does not seem to be accompanied by the marked stabilisation of the protein which follows exposure to some activating stimuli, notably UV-induced DNA damage. Although one group [26] reported a modest elevation (2–3-fold when normalised to protein content) most [27, 28] have failed to demonstrate a reproducible increase in p53 protein content in senescent fibroblasts. This is perhaps not unexpected, since it is now becoming clear that stabilisation is not a prerequisite for activation of p53 [25].

Downstream targets

The above result further increases the likelihood that the p53 growth arrest signal will be mediated via a p53-inducible gene with growth inhibitory properties for which the cyclin kinase inhibitor $p21^{WAF1}$ is a major candidate. Smith and colleagues [13] first showed that p21 was strongly induced in senescent cells (indeed it was on this basis that it was first cloned). We therefore examined the expression of p21 in fibroblasts rescued from senescence by our mutant

p53 retrovirus vector [29]. Surprisingly, we could detect no apparent reduction in p21 protein expression (as assessed by immunocytochemistry) in even the earliest clones examined, which were proliferating at a rate comparable with young fibroblasts in which p21 is virtually absent.

The first conclusion that follows from this result is that p21 expression in senescent cells is not dependent on wild-type p53. This is now not without precedent since many examples of p53-independent WAF1 expression have been observed, mainly in relation to terminal differentiation [30], and in fibroblasts Tahara and associates [31] showed that much of the elevation of p21 in senescence is not abrogated by the expression of SV40T.

The second implication, which in many ways is more surprising, is that p21 overexpression is not *sufficient* to induce senescence, a second (unknown) signal ("X" in Figure 1a,b) being also required, which *is* strictly dependent on p53. On the basis of the evidence presented so far, this could most simply be another cyclin kinase inhibitor acting ultimately through pRb. However, as described below, p53 is able to bring about growth arrest in cells in which the pRb pathway has been inactivated (e.g. by HPV E7), indicating therefore the existence of a truly *RB*-independent pathway (Z in Figure 1).

Probably the most likely possibility is that X is indeed a CDKN, but one with an action on CDK(s) which targets a pRb-independent regulator of G1–S transition. The existence of such targets is clearly indicated by the retention of G1/S arrest in *RB*-deficient cells in response to several (although not all) situations, e.g. mitogen deprivation [32]. CDK2 is the obvious candidate for Z in this pathway, either in complex with cyclin E, and/or perhaps cyclin A. For example, growth arrest in anchorage-deprived cells correlates with loss of E/CDK2 activity without any change in *RB*-kinase activity [33]. Furthermore, G1–S transition in *RB*-minus cells shows an absolute requirement for E/CDK2 [34]. CDK2/cyclin A is also a potential target since its phosphorylation of E2F/DP1 is required to permit progression through the S phase [35].

Alternatively, the *RB*-independent signal pathway may by-pass the CDK system altogether. At least one p53-inducible gene product—GADD45—is potentially capable of such an action [36].

The contribution of p53-mediated *apoptosis* (as opposed to growth arrest) also needs to be clarified in this context. While this has been observed in some situations where *RB* function is selectively eliminated [37, 38], in our hands, E7-expressing fibroblasts in late M1 appear to show growth arrest rather than cell death (J.A. Bond, University of Wales College of Medicine, U.K.).

"Back-up" pathways: p105 Rb

Just as selective knock-out of *TP53* function leads to extension of lifespan and temporary escape from M1 in HDF, so too does selective abrogation of the pRb pathway, for example, by HPV E7. The *RB* pathway therefore represents a second pathway required for normal functioning of the M1 checkpoint in these cells. This is not unexpected given that the pRb protein stands at a key "gateway" in cell cycle progression. Normal G1–S phase cell-cycle transition is dependent on inactivation of its growth-repressor activity through phosphorylation [39], and a well-established biochemical feature of senescent fibroblasts is their failure to

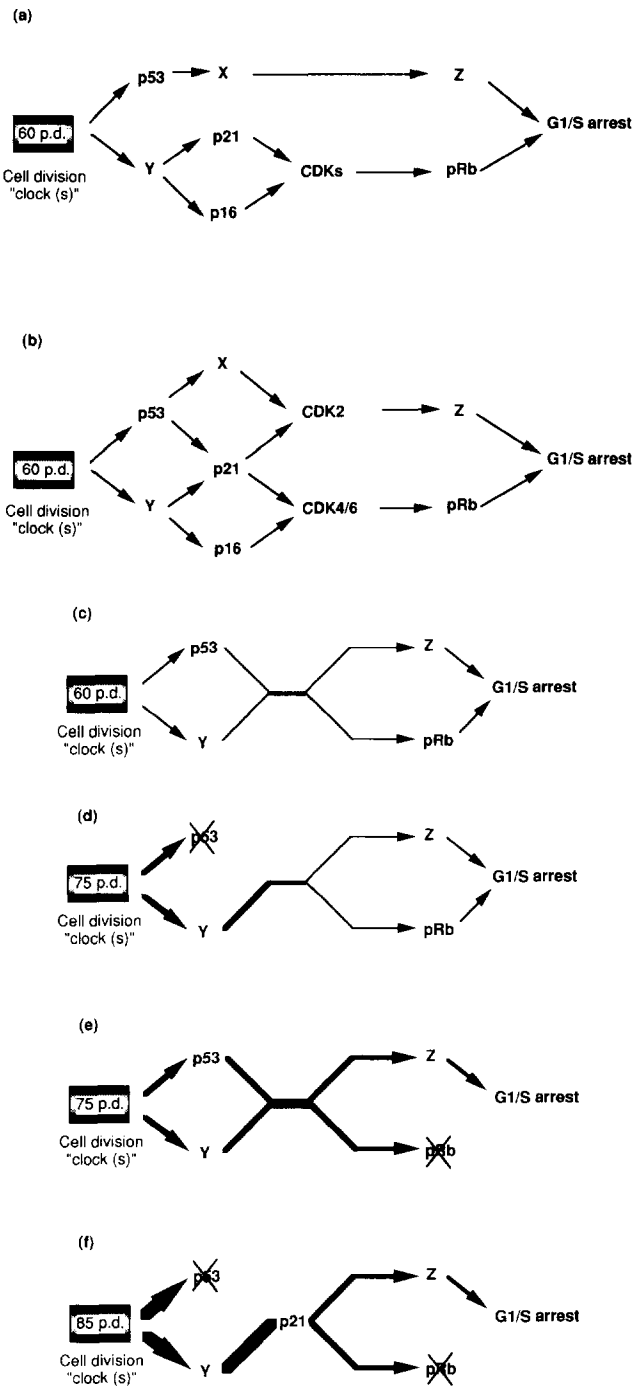

Figure 1. Potential signal pathways mediating G1/S arrest in senescing human fibroblasts. In all models, growth arrest is normally achieved by a cooperative interaction between *TP53* and *RB*-dependent signals. In the "parallel" model (a) these are assumed to be quite separate pathways acting on distinct cell-cycle regulators (Z and pRb, respectively). In the "cross-over" model (b), there are common elements, of which currently the most convincing is the cyclin-kinase inhibitor $p21^{WAF1}$. (c)–(e) illustrate, using a simplified form of model (b), the way in which such a network can maintain finite proliferative lifespan despite loss of individual components: (c) normal M1 arrest achieved by additive effect of both pathways; (d) compensation for loss of *TP53* by increased activity of the p53-independent pathway driven by Y; (e) compensation for loss of *RB* (or $p16^{INK4a}$) by increased activity of pathway Z. The model makes the interesting prediction (f), consistent with experimental evidence, that compensation via $p21^{WAF1}$ should still be possible even in cells which have lost both functional *RB* and *TP53*.

phosphorylate pRb, despite the presence of adequate extracellular growth signals [40]. The most likely explanation for this is the overactivity in senescent cells of inhibitors of *RB* kinases, an obvious candidate being $p21^{WAF1}$ which we have already seen is elevated in senescence by largely p53-independent mechanisms. Another major candidate is the cyclin kinase inhibitor $p16^{INK4a}$ [41], which is also markedly induced in senescence [14] and is now known to be a frequent target for mutational loss in tumours and immortal cell lines [42].

What is currently not so clear is the nature of the detector (Y in Figure 1) which could link the telomere clock to expression of such genes, independently of p53. Perhaps this pathway is activated, not by a "DNA damage" related mechanism as postulated for p53 [17, 43, 44], but by the alternative telomere-silencing related signal based on altered expression of subtelomeric genes [3], although direct support for this in human cells has so far been lacking [45]. More provocatively, of course, a non-telomere-dependent clock for this pathway can still not be formally excluded! Indeed, this would explain the worrying anomaly that fibroblasts from the laboratory mouse (*Mus musculus*) senesce more rapidly than human cells, despite having enormously long telomeres [46, 47]. Clearly, further analysis of the transcriptional and post-transcriptional control of p21 and p16 expression in senescence will be of major importance in this area.

Interaction between pathways: "dual-circuit braking"

Although abrogation of p53 function clearly leads to a failure to enter M1 after the normal number of divisions, the extension of lifespan is finite and indeed is significantly less than that produced by a dual knockout of both *RB* and *TP53* ([16]; and J.A. Bond, University of Wales College of Medicine, Cardiff, U.K.). Similarly, abrogation of pRb by viral oncoproteins which leave p53 function intact does not supplant the need to also abrogate p53. HPV E7, for example, fails to mimic the full effect of E6 plus E7 (or SV40T), as evidenced by the timing of growth arrest and the subsequent immortalisation competence.

This indicates that the two pathways, one dependent on *TP53*, the other on *RB*, normally cooperate to bring about normal M1, but that if one is eliminated, hyperinduction of the remaining pathway as cells continue to proliferate past the normal M1 limit, can eventually lead to compensation and effective, if delayed, growth arrest.

A "parallel" pathway model (Figure 1a) is conceptually the simplest explanation for this behaviour, with two quite separate signals, converging only through the additive interaction between their respective targets in the cell cycle machinery (Z and pRb in Figure 1a). From the arguments presented above, however, a more complex model (Figure 1b) must be considered, in which there is a "crossover" of pathways at the level of cyclin/CDKs. Although both primary signals (Y and p53) can affect both ultimate targets (pRb and Z) in this model, as illustrated in Figure 1c–e, it does nevertheless adequately accommodate the features of additivity and compensatability. For example, if p53 function alone is lost, there is initial escape from M1 due to loss of the p53-dependent component of the senescence signal. With further proliferation, however, increasing activity of the p53-independent pathway (mediated by p21 and/or p16) eventually compensates, acting thereby as a "second line of defence" against escape from senescence. Although our findings [29] suggest that p21 elevation at the *normal* point of entry into M1 is insufficient alone to produce growth arrest, this does not exclude the possibility that its level may increase further in postsenescent cells, to the point where it could eventually mediate such a compensatory inhibition. Indeed, this is consistent with serial observations in postsenescent, precrisis fibroblasts expressing wild-type [48] or temperature-sensitive [31] SV40 T. p16 may also behave similarly [14].

Other pathways

Although reviewed extensively elsewhere in this Special Issue, for completeness it must be pointed out here that somatic cell hybridisation experiments indicate the existence of at least one more independent pathway capable of inducing senescence in fibroblasts. Immortal lines which have lost both *TP53* and *RB* function (e.g. through SV40 T expression) generate limited-lifespan hybrids following fusion with normal fibroblasts [49] and with functionally *RB*/*TP53* defective lines from other complementation groups [50]. Furthermore, such hybrids can still be telomerase-positive [51], excluding telomerase repression as a mechanism. Although there is not a simple relationship between the immortalisation complementation groups defined by cell fusion data and the cell lineage of the lines, it nevertheless appears that most fibroblast lines fall into one group (Group A) [49, 52] and may therefore need to abrogate the same additional lifespan checkpoint pathway. Chromosome transfer experiments suggest that a key component of this pathway may be encoded by a gene, termed SEN-6, localised to the long arm of chromosome 6 [53].

An interesting prediction regarding this "third line of defence" against immortalisation can be made from the model presented in Figure 1. This signal diagram highlights the key nodal position occupied by $p21^{WAF1}$ and its non-*RB*-dependent targets. As illustrated in Figure 1f, even in *TP53* and *RB*-deficient cells, growth arrest should still be possible if the induction of p21 by Y (SEN-6?) can eventually reach a threshold sufficient to block cell cycle progression through a pRb-independent target. This may again operate via inhibition of CDK2–cyclin complexes, or alternative p21 targets may be involved, such as direct inhibition of DNA synthesis by binding to PCNA [54] (although this is still controversial [55, 56]), or direct inhibition of E2F transcriptional activity [57]. The progressive elevation of p21 expression up to crisis in cells expressing SV40, its fall in postcrisis derivatives [48], and the intolerance of a wide variety of immortal lines to p21 expression [58], are all consistent with such a "last-ditch" role for p21 in maintaining cellular mortality.

One point which is often overlooked in cell fusion work is the need to distinguish limited lifespan in hybrids due to crisis from that due to true restoration of senescence. If telomerase is suppressed in the hybrid, as recently demonstrated, for example, in fusions of normal with SV40-transformed fibroblasts [59], then lifespan would be expected to be limited eventually by the onset of crisis (after a number of PD, depending on the prevailing telomere length in the immortal parent), irrespective of any other senescence pathway. Evidence for the restoration of a true senescence pathway depends on showing growth arrest in an M1-like state *ahead of* the onset of crisis, such as pre-

sumably occurs in those hybrids between different immortal lines which remain telomerase-positive [51].

One final puzzle is why there should be such a long *delay* (sometimes amounting to many tens of PD [49]) before the onset of senescence is observed in limited-lifespan hybrids formed between immortal *RB-* and *TP53*-deficient fibroblasts and other *RB-/TP53-* lines from a different complementation group. Given that both parent cells have proliferated beyond M1 and M2, it might be expected that restoration of an M1 lifespan checkpoint would result in rapid growth arrest, as indeed is the case for restoration of p53 and pRb function, as seen following switch-off of SV40 T expression in immortalised human fibroblasts [60]. A general explanation is reactivation of a senescence clock which had not only been stopped but also "wound back" in the immortal parents (akin to telomere elongation in immortal telomerase-positive cells), the delay representing the number of PD required to return to the critical checkpoint. However, the difference in kinetics of arrest in these hybrid experiments from that seen following restoration of pRb/p53 function, together with the evidence that this holds true even in hybrids which retain telomerase activity [51], points to the existence of a second clock, distinct from telomere erosion.

BEYOND THE FIBROBLAST PARADIGM: CELL-SPECIFIC DIVERSITY IN LIFESPAN CHECKPOINTS AND THEIR SIGNAL PATHWAYS

While control by multiple pathways may also be the rule in many epithelial cell types, e.g. keratinocytes [61–63] (see below), in some the picture appears to be surprisingly simpler, with only one major pathway apparently playing a role. Although, unfortunately, only a few cell types have been analysed in sufficient detail, examples of each of these "extreme" cases can now be cited.

Control by a p53-dependent pathway only

Band and associates [64] first made the observation that primary cultures derived from normal (mammoplasty) breast tissue contained an epithelial population which could surprisingly be immortalised efficiently by the introduction of just a single DNA tumour virus oncogene (HPV E6) which was known to target *TP53* without affecting the function of *RB*. Subsequently, Shay and colleagues extended this result by showing [16] that in sharp contrast to fibroblasts, in these breast epithelial cells, expression of E6 not only extends lifespan but also confers immortalisation competence (and with a surprisingly high frequency). This result has now also been reproduced with vectors expressing mutant *TP53* (as opposed to viral genes), albeit with only one of a series of mutants [65]. Additional expression of HPV E7 (thereby abrogating *RB*) in breast cells expressing E6 conferred no extra proliferative capacity at any stage, and when expressed alone E7 has no effect on their lifespan, both results again contrasting with fibroblast observations.

Taken together these data strongly suggest that in this population of breast cells, loss of *TP53* function is sufficient for cells to escape M1 fully and argues that the putative *RB*-dependent pathway (Y in Figure 1) is either absent or incapable of generating a significant inhibitory signal even in post-M1 cultures; this is consistent with the phosphorylation state of pRb in E6-expressing cells [66]. It also emphasises the importance of the *RB*-independent signal pathway (Z) in mediating the action of p53.

It can readily be imagined that the apparent dependence of these cells on just a single control mechanism should make them more vulnerable than fibroblasts to spontaneous escape from senescence [67]. This, together with their apparently higher intrinsic ability to escape M2 [68], may well contribute to their greater tumorigenic potential *in vivo*. At the very least, it would predict a major selective advantage for loss of *TP53* function in an emerging tumour clone.

Control by a p53-independent pathway only

It is now becoming clear that the cells analysed in the above studies are not representative of the major component of breast epithelium *in vivo*, but represent instead a small subpopulation which has been highly selected for by the *in vitro* conditions used. These cells show features intermediate between that of myo-epithelial and luminal cells [69], characterised by expression of "basal" markers vimentin and cytokeratin 14, together with luminal markers 8 and 18, and lack expression of oestrogen receptor (ER). They are likely to correspond to the "basal" phenotype postulated by Taylor-Papadimitriou and colleagues [70] to represent a stem cell population, which importantly appears to give rise to a more aggressive subset of breast cancers (see below).

The major *in vivo* populations, including the classic luminal phenotype (positive for cytokeratin 8, 18 and 19 and ER; negative for vimentin) have been less adequately investigated, due to greater technical difficulties in cell culture. Recent studies [66, 71] are now pointing to a remarkable difference in the control of senescence between these and the "stem cell" population.

Epithelial cells isolated from early passage mammoplasty samples undergo growth arrest which resembles M1 senescence in morphology but occurs after a much smaller number (10–15) of PDs than in fibroblasts or "stem-type" breast cells, and without elevation of $p21^{WAF1}$, leading some workers to distinguish it as an M_0 state [71]. Abrogation of *TP53* function, e.g. by expression of HPVE6, is strikingly without effect on the timing of this arrest in these cells. In contrast, abrogation of pRb is apparently sufficient alone to allow cells to escape fully from M_0. Escape from senescence by loss of *RB* function clearly occurs despite the presence of wild-type p53, and indeed levels of the protein are increased in such cells [71] suggesting that the p53 pathway is being partially activated, but is in some way prevented from achieving growth arrest. Control of senescence in these cells therefore appears to be the inverse of that in "stem" cells i.e. p53-independent but pRb-dependent.

A somewhat similar situation has been observed by our laboratory in thyroid follicular epithelial cells [72]. These also show a limited proliferative potential in culture entering a state of viable quiescence from which they can be rescued by expression of HPVE7 alone, but not by HPVE6 or mutant p53 [73], to which they are totally indifferent. Also in striking similarity to breast cells, thyroid epithelial cells growing in response to HPVE7 express high levels of wild-type p53, to which they apparently fail to respond. Since, in these primary culture experiments, there is insufficient "opportunity" for additional spontaneous genetic events to have occurred, it must be concluded that wild-type p53,

although clearly modified sufficiently to be stabilised, is either not fully activated or else its activity is blocked by an endogenous inhibitor such as mdm2 [74].

In contrast to breast, however, the initial growth arrest in thyrocytes occurs even earlier—after just 2–3 PD of proliferation—following stimulation by either its physiological mitogen, TSH (thyroid stimulating hormone), or serum growth factors [7]. The rapidity and synchrony of this arrest, together with the lack of any morphological features of senescence, suggest that it represents a downregulation of responsiveness triggered by mitogen stimulation, i.e. a differentiation switch rather than a "timed" senescence mechanism. Although the desensitisation to extracellular mitogens is irreversible, activation of intracellular signalling pathways by mutant *RAS* or *RET* oncogenes [75] leads to a much longer period of proliferation (up to 25 PD) which ends in a phenotype apparently closely similar to the M_0 state described in early passage breast cells.

Both this delayed "M_0" and the "normal" checkpoint are regulated by an *RB*-dependent, *TP53*-independent pathway. Thyroid cells which have escaped this block by expression of E7 (or E7/E6) subsequently enter a further state of viable arrest, perhaps equivalent to M1 in fibroblasts (Figure 2). Interestingly, though, escape from this checkpoint in thyroid cells is extremely rare [72] and is not influenced by simultaneous abrogation of *TP53* by E6 or SV40 T (unless a switch in differentiation supervenes—see below). This therefore represents an *RB*- and *TP53*-independent lifespan checkpoint, the "tightness" of which suggests that multiple events are required to overcome it and may partly explain the surprisingly limited proliferative capacity of many thyroid tumours. Analysis of cyclin/CDK expression and activity is currently underway to attempt to elucidate its mechanism. pRb-dependent breast cells may escape this fate either because they do not exhibit this checkpoint, or because the relatively high probability of spontaneous telomerase activation already noted in the breast [67, 68] may allow most post-M_0 cells to stop the lifespan clock before reaching it.

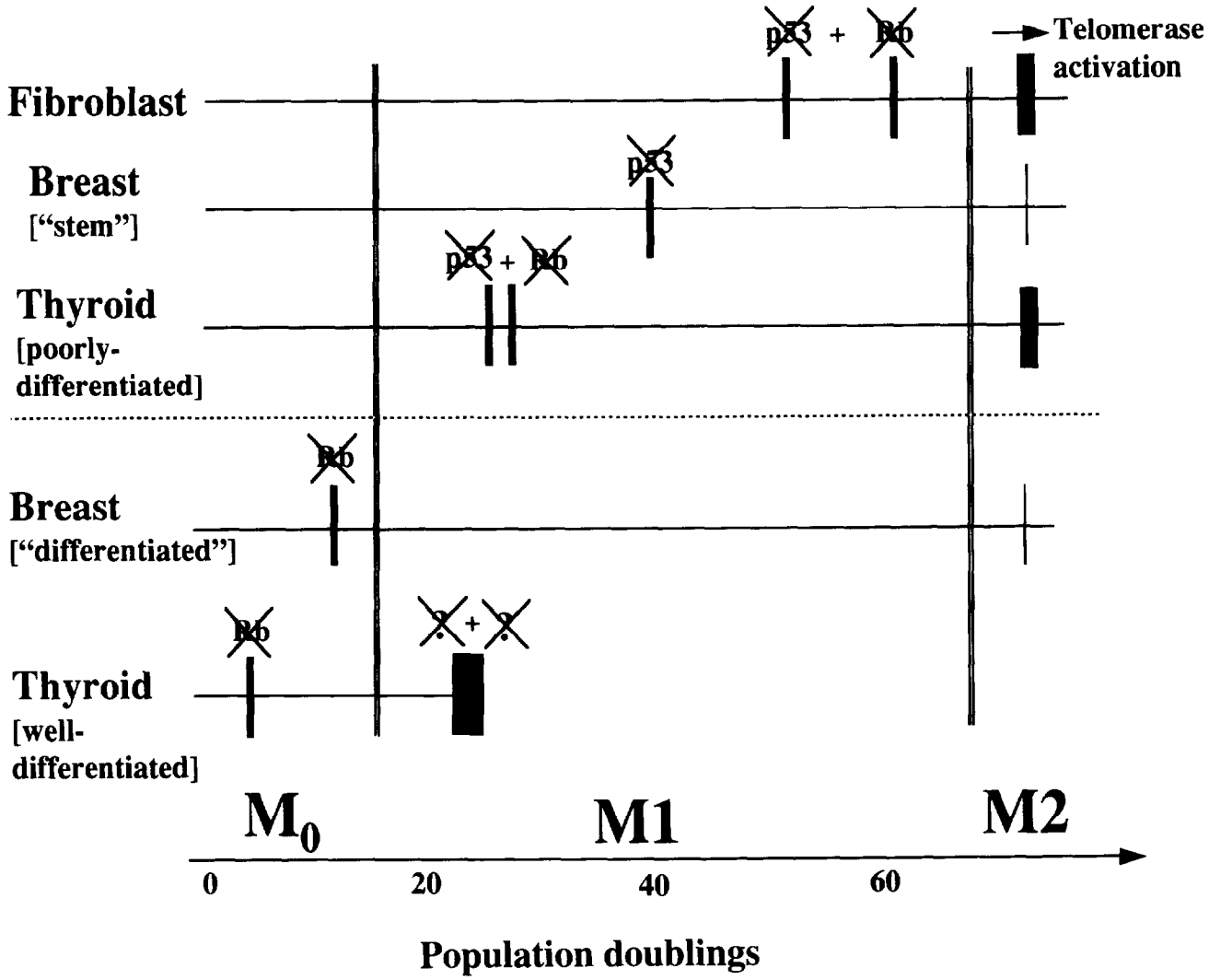

Figure 2. Cell-type diversity in lifespan checkpoints. For each cell type, the vertical bars indicate the approximate timing of checkpoints (based on population doublings observed in tissue culture). For M_0/M1, the cell cycle regulator(s) whose loss is sufficient to allow escape are shown (for the thyroid, the "tightness" of the M1 block suggests two separate controls). For M2 escape, it is assumed that telomerase activation will be common to all. The width of the bars indicates the probability that escape will occur (thickest = least likely). Note that there is strong evidence from cell fusion data for a third M1 checkpoint in many cell types (not shown).

Control by both p53- and pRb-dependent pathways

We have described [76] a second, minor subpopulation of normal thyroid epithelial cells which lack thyroid-specific morphology and most differentiated characteristics, but retain expression of cytokeratin 18 and the thyroid-associated transcription factor PAX-8, supporting their relationship to classical thyrocytes, either as a less-differentiated precursor or as a metaplastic derivative. Although a tiny minority *in vivo*, in cell culture they rapidly assume greater proportions due to their much greater proliferative rate and lifespan [76]. Clearly this is highly reminiscent of the contrast between "stem-type" and early passage epithelial cells in the breast described above.

These "variant" poorly-differentiated epithelial cells exhibit a proliferative lifespan of at least 20 PD in culture (in contrast to < 3 PD in their well-differentiated counterparts), after which they enter a viable state of growth arrest accompanied by morphological changes closely resembling M1 senescence in human fibroblasts. Unlike the irreversible M1 arrest described above in well-differentiated thyrocytes, this is readily overcome by expression of SV40 T [72], which permits further proliferation until, after a further 30–50 PD, a classical crisis supervenes. Variant cells therefore appear to follow a conventional M1, M2 model of proliferative ageing. Recent gene transfer experiments (J.A. Bond, University of Wales College of Medicine, Cardiff, U.K.) reveal a very similar "dual control" to that of human fibroblasts, with abrogation of both *RB* and *TP53* being needed to escape M1.

In some epithelia, such as keratinocytes, the picture is more confused. *RB* is clearly a key player, since immortalisation can be achieved with E7 alone [63] but not with E6. However, the overall yield is increased by abrogation of *TP53* function [77]. This indicates a dual control in some cells; it is also possible that there is heterogeneity in the target cell population (K. Parkinson, Beatson Institute for Cancer Research, Glasgow, U.K.) and that some cells are solely regulated by *RB*.

Overview of lifespan checkpoints

The diversity in number, timing and control of lifespan checkpoints is illustrated in Figure 2, which is a compendium of the small number of cell types for which such data are available. Some trends can be tentatively suggested.

First, there appear to be two groups with respect to the initial rate limiting step. In well-differentiated epithelia, this occurs relatively early (M_0-type) and is solely *RB*-dependent; in poorly-differentiated epithelia (whether de-differentiated derivatives or stem-type precursors), as in mesenchymal cells, there is a later M1-type arrest which is at least partly dependent on *TP53*.

Second, although all may share a single ultimate crisis (M2), there are differences in the number of checkpoints prior to M2. As might be predicted, most have at least one back-up to the initial checkpoint, although breast (both well- and poorly-differentiated subtypes) appears to have only one. Such differences may be more apparent than real since one feature not evident from Figure 2 is the probability that a nascent tumour clone will effectively stop its checkpoint timer e.g. by re-activation of telomerase. Indeed, it has already been noted that this event may be unusually frequent in breast epithelium; it is conceivable therefore that such cells may fail to exhibit later checkpoints because they have already stopped the clock before reaching them!

Finally, there is the question of the identity of the underlying "clock". The M1 arrests all appear to share common features of classic senescence with respect to phenotype and timing and in these there is every reason to expect a telomere-based mechanism. The earlier M_0 arrests are more disparate. Although there is a common dependence on the *RB* pathway and indifference to *TP53*, they differ in phenotype and timing, and, as noted above, the behaviour of thyroid epithelium in particular is suggestive of a different mechanism.

CLINICAL SIGNIFICANCE OF CELL-SPECIFIC LIFESPAN CHECKPOINTS

Tumour aggressiveness and selection for loss of tumour suppressor genes

If lifespan checkpoints are a significant hurdle to tumour development, it should follow that the diversity of the normal control pathways maintaining senescence in different cell types should have a profound influence on the subsequent behaviour of the corresponding tumours.

In the case of fibroblasts, for example, escape from M1 should require loss of function of both the *TP53* and *RB* pathways, either directly, or indirectly (through expression of inhibitors such as mdm2 [74] or loss of mediators such as p16 [14]), and this accords broadly with molecular analysis of human sarcomas [78, 79]. The same dual-knockout applies to epithelial populations in which both pathways are involved, a good example being carcinomas of the head and neck derived from squamous epithelium in which again *TP53* mutation often coincides with loss of p16 [61].

On the above reasoning, it would be predicted that the minority of human cancer types which do *not* show high frequencies of *TP53* mutation might be those derived from cells which do not have a *TP53*-dependent M1 lifespan checkpoint. The limited studies of this point to date are indeed tantalisingly suggestive of this, as seen in the analysis of breast and thyroid cells [64–66, 69, 71–73, 76] discussed at length above, and especially for the breast are perhaps of even greater significance than has been appreciated.

It is widely accepted that a minority (around one-third) of invasive ductal breast cancers fall into a more aggressive subgroup which can be defined on the basis of a range of clinico-pathological parameters including poor differentiation (high grade), high proliferative rate, ER-negativity (with hormone-independence), and high expression of EGF-R (epidermal growth factor-receptor) [80]. These features, together with their intermediate filament profile (expression of basal markers CK14 and vimentin), has led to the suggestion [70] that this subgroup in contrast to the majority, arises not from the luminal cell but from a less-differentiated breast epithelial cell with features intermediate between classical luminal and basal (myo-epithelial), perhaps representing a stem cell.

Clearly, this distinction *in vivo* could correspond to the different populations of normal breast epithelium identified *in vitro* in mammoplasty cultures by Band and associates [66] and Galloway and Foster [71], the highly proliferative "stem" type regulated by a p53-dependent lifespan checkpoint only, contrasting with the more restricted proliferative capacity of the differentiated (early passage) cell, regulated by a p53-independent pathway.

Analysis of p53 status of breast cancers has consistently indicated a very strong correlation between the occurrence of *TP53* mutation and the ER-negative, EGFR-positive, poorly differentiated phenotype [81, 82], although of course such correlations can never be expected to be absolute, since, for example, p53 function may be lost in indirect ways, and some ER-negative tumours may be derived from luminal cells which have lost ER expression (p53 status has not, as far as we are aware, been directly correlated with cytokeratin profile). If these clinical data are put together with the data from cell biology, then an obvious explanation emerges for the two different phenotypes, namely that the predominant well-differentiated "luminal" tumour type retains wt p53 because it arises from a cell which uses only a p53-independent lifespan control in which there is therefore no selection for *TP53* mutation, whereas the converse accounts for the need for *TP53* mutation in the "stem type" tumours.

Viewed in this way the significance of the association of *TP53* mutation with the more aggressive phenotype is quite different from the conventional view, i.e. it is not that the tumour is aggressive and less differentiated *because* it has *TP53* mutation, but that the *TP53* mutation is merely an inevitable reflection of the controls operating in the cell of origin. In other words, the differences in tumour phenotype may be determined more by the respective properties of their cell of origin than by the nature of the mutational events which supervene.

Tumour progression

The above argument is particularly important for studies attempting to define the molecular basis of progression to hormone insensitivity in breast cancer. The assumption in such work is nearly always that tumours become unresponsive through some further mutational event. However, the line of reasoning above suggests that this may not be entirely appropriate, in that the switch in behaviour may instead be a reflection of a partial or complete trans-differentiation of the tumour cell from luminal to stem type, selected for by therapy-induced hormone deprivation. It will

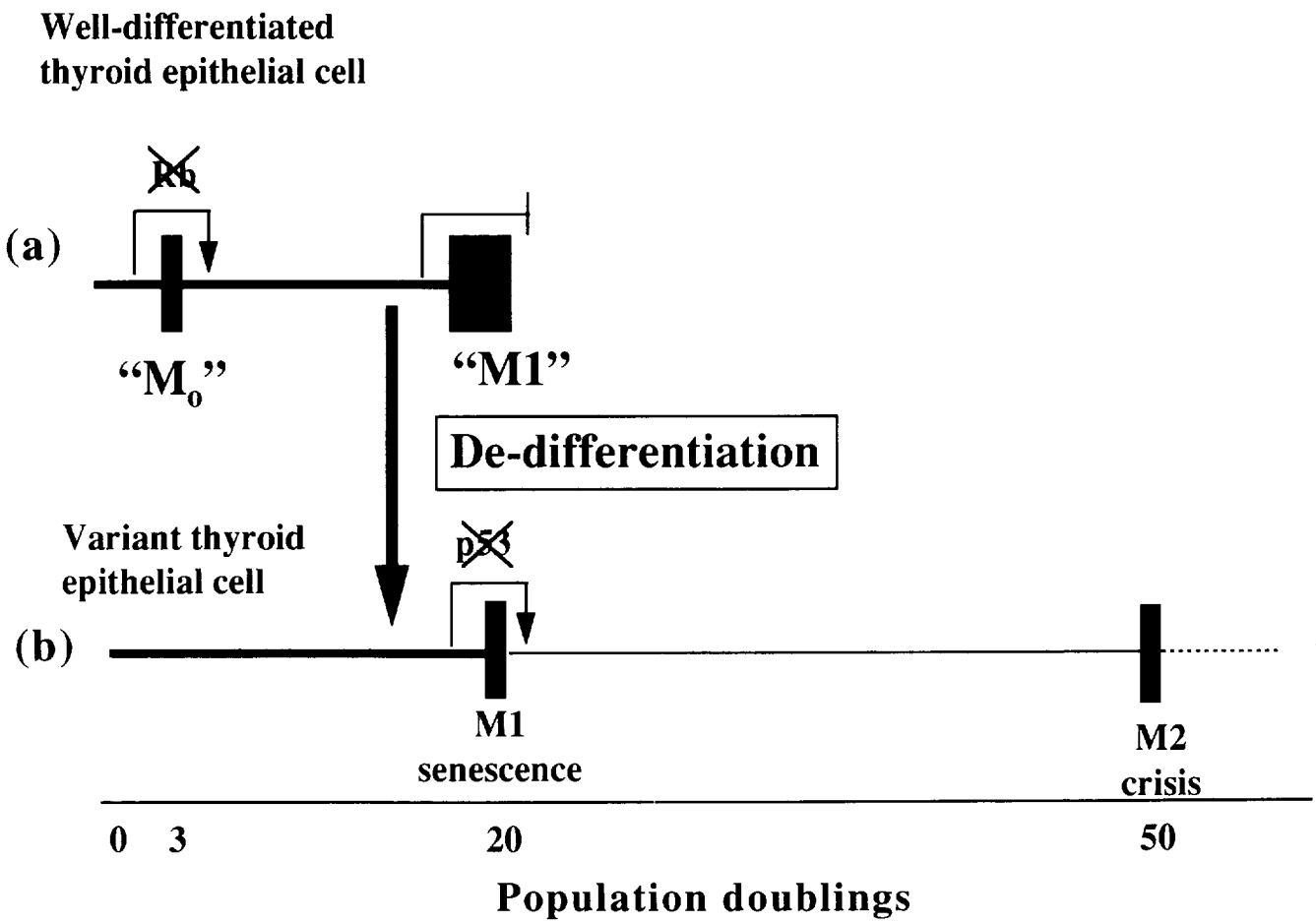

Figure 3. Trans-differentiation and lifespan checkpoint switching: a novel mechanism of tumour progression. The predominant, well-differentiated, thyroid epithelial cells (a) undergo early growth arrest (M_0) which can be overcome by abrogation of pRb to confer 5–15 extra PD. After this, however, a second irreversible growth arrest supervenes (M1) which cannot be overcome even by combined loss of p53 and pRb function. (b) In contrast, the poorly-differentiated "variant" thyroid epithelial cell has a much longer normal proliferative lifespan ending in a typical M1 senescent state which can be overcome by loss of *RB* and *TP53*, leading to a further 30–40 PD of lifespan, after which the cells enter a conventional M2 crisis. Our data show that well-differentiated cells in which *TP53* and *RB* have been abrogated can escape irreversible M1 arrest by undergoing a spontaneous de-differentiation event, which effectively switches their ageing programme to that of the variant cell. We speculate that a comparable event is involved in the transition of well-differentiated to undifferentiated (anaplastic) thyroid cancer *in vivo* and that the resulting additional proliferative lifespan plays a crucial part in the dramatic increase in malignant potential which ensues.

be of interest to test the prediction that such tumours should have acquired the need to inactivate p53.

Our laboratory has recently obtained strong evidence [72] that it is exactly this cooperation between an epigenetic and a genetic mechanism which is responsible for another major switch in human cancer behaviour, the transition from differentiated to anaplastic carcinoma of the thyroid.

We have observed that well-differentiated (majority phenotype) thyrocytes which have escaped the initial lifespan checkpoint (M_0 in Figure 2) by expression of SV40 T undergo two mutually-exclusive fates. They either (i) remain well-differentiated, in which case they undergo irreversible growth arrest after 5–15 PDs at M1 in Figure 2; or (ii) spontaneously develop poorly-differentiated subclones which exhibit greatly extended proliferative lifespan (up to 75 PDs). The correlation between de-differentiation and extension of lifespan is absolute, suggesting that it is not just a passive epiphenomenon but is causally linked to the change in growth control. The frequency of this event—estimated at greater than 1 per 3000 cell divisions—is much higher than that expected from somatic mutation (although we cannot of course exclude the possibility that mutation in any one of several alternative genes might generate the same phenotypic change). Furthermore, the loss of differentiated features is identical to that which characterises the spontaneously arising variant cells in normal thyroid cultures [76] and for which there is no reason to suspect a mutational basis. Taken together, this suggests an epigenetic model, in which the thyrocyte is liable, with a given probability, to undergo a spontaneous switch in the differentiation programme the result of which is (i) to extinguish most of its thyroid-specific characteristics, and (ii) to convert effectively its proliferative behaviour to that of a pseudomesenchymal cell (Figure 3). Such epithelial–mesenchymal transitions have been well documented in other tumour progression models, and indeed in normal development [83, 84].

This leads to a model (Figure 3) in which escape from the otherwise insurmountable M1 checkpoint (Figure 2) is achieved not by directly overcoming it, but by a side-step in which it is substituted by a new checkpoint (effectively that of a fibroblast) which *can* now be overcome provided that the functions of the *TP53* as well as the *RB* pathway are abrogated. This is consistent with the failure of cells expressing E7 (*RB* only inactivated) to undergo this escape process.

Our data go further than the breast data of Wazer [66], in that they not only describe two distinct "tracks" for proliferative ageing in different subpopulations of epithelial cell, but also suggest that it is possible for a cell to switch from one to the other.

Loss of differentiation is a widespread feature of tumour progression, and frequently accompanies more aggressive behaviour. The thyroid provides a particularly clear-cut example in the abrupt phenotypic switch which occasionally occurs from the common well-differentiated papillary cancer with limited proliferative potential and excellent prognosis, to the exceptionally aggressive undifferentiated (anaplastic) form [85]. 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 our *in vitro* model and the conversion of well- to undifferentiated thyroid cancer *in vivo* suggest that, at least in this example of progression, increased proliferative potential may be dependent on epigenetic as well as mutational mechanisms. We speculate that progression *in vivo* results from a synergism between the differentiation switch discussed here and the occurrence of *TP53* mutation, which is a hallmark of undifferentiated thyroid cancers [86]. The rarity with which the transition to undifferentiated cancer is seen clinically in the thyroid can be explained by the need for the differentiation switch and the *TP53* mutation to arise independently in the same cell before any selective advantage is obtained.

CONCLUSION

Novel therapies

One final implication of cell-type diversity of lifespan checkpoints deserves mention. This relates to the current excitement [87] surrounding the therapeutic potential of mutant adenoviruses which lack the ability to inactivate host-cell p53 and should therefore selectively replicate in and kill cancer cells (most of which lack wt p53) but not normal cells. The observations on breast and thyroid cells discussed above point to the possibility that some *normal* cell types are intrinsically resistant to activation of the wt p53. Clearly this would have major implications for the toxicity of any therapy which relies on the assumption that only tumour cells lack functional p53!

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