Limits to lifespan

Alison C. Lloyd

It has long-been accepted that normal somatic cells have intrinsic mechanisms that limit their proliferative lifespan. Recent work has now challenged this view by demonstrating that extrinsic factors might be determining proliferative potential.

Many of us are familiar with the image of a senescent cell—large, flat cells that are metabolically active yet are arrested in the cell cycle and stain blue when tested for the expression of senescence-associated β-galactosidase[1] (Fig. 1). Senescent cells arise in cultures of primary cells after serial passaging *in vitro*, a process that has historically been termed replicative senescence. The number of times that a cell divides before entry into senescence is highly variable, depending on both the cell type and the organism from which it was originally derived. However, an intrinsic limitation of proliferative capacity had been thought to apply to all somatic cells, with the possible exception of stem cells. This check on the number of times that an individual cell can divide has been proposed to be an important mechanism for protecting long-lived multicellular organisms from the development of cancer.

However, signals other than extended proliferation have been shown to result in cells developing a phenotype indistinguishable from that of senescent cells at the end of their replicative lifespan. For example, a single double-stranded DNA break is sufficient to trigger normal human fibroblasts to become senescent[2]. Likewise, cellular stress stimuli such as the addition of ceramide[3] or the constitutive activation of the mitogen-activated protein (MAP) kinase signalling cascade by the expression of oncogenic Ras can provoke a senescent phenotype[4]. However, in contrast to replicative senescence, in which an intrinsic mitotic timer acts to regulate proliferative lifespan, senescence in these cases is triggered by extrinsic factors that act independently of replicative age. Because these signals act before the replicative limit of a cell, the process has been termed premature senescence. Irrespective of the trigger, the senescence-associated cell-cycle arrest is the result of the induction of cyclin-dependent kinase inhibitors (CDKIs) that block progress through the cell cycle. Indeed, mimicking the end point of these different signals by artificially inducing high levels of CDKIs in normal cells is sufficient to induce a senescent phenotype[5] (Fig. 1).

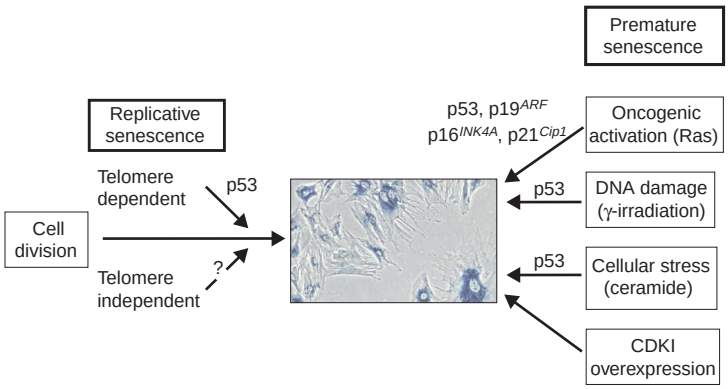

Figure 1 **Mechanisms of inducing senescence. A senescent phenotype can be induced in normal cells by many distinct signals. Replicative senescence results from prolonged proliferation in culture. Premature senescence can be induced by various damage or cellular stress signals and is independent of replicative age. In all cases the cell-cycle arrest is associated with the induction of CDKIs. In telomerase-negative cells, telomere shortening acts as a mitotic clock to define replicative capacity. Evidence for a telomere-independent, cell-intrinsic mechanism of limiting replicative lifespan is currently under debate.**

Replicative potential

Human diploid fibroblasts (HDFs) have been the model of choice for studying replicative potential. In culture, these cells divide a limited number of times (between 50 and 80 population doublings) before entry into senescence[6]. They do not express telomerase and so at each division their telomeres shorten. Shortened telomeres or disrupted telomeres have been shown to trigger entry into senescence in certain cell types[7,8]. That telomere shortening is responsible for entry into senescence in HDFs has been indicated by the finding that the expression of exogenous telomerase in these cells confers immortality[9]. Thus, telomere shortening seems to act as an intrinsic, invariant mechanism for limiting proliferative lifespan. To date, this is the only known mitotic timer shown to regulate replicative lifespan by triggering entry into senescence.

But other cell types seem to have mechanisms distinct from telomere shortening that regulate their replicative lifespan. Human cell types such as keratinocytes and mammary epithelial cells have a lower proliferative capacity (20–30 population doublings) *in vitro* than HDFs and this is apparently telomere-independent because cells artificially expressing telomerase senesce at the same population doubling[10]. Instead, in response to an unknown signal, levels of the CDKI $p16^{INK4A}$ increase, as these cells proliferate *in vitro*. Both the loss of $p16^{INK4A}$ and the expression of ectopic telomerase are required to immortalize these cells. Many rodent cell types have also been shown to have a limited proliferative capacity *in vitro*. Because rodent cells express telomerase and maintain long telomeres in culture, and cells from telomerase-knockout mice undergo senescence as wild-type cells[11], it seems that a telomere-independent mechanism regulates the proliferative lifespan of rodent cells.

The findings that DNA damage, cellular stress and oncogenic activation can trigger the premature senescence of normal cells led many to question the nature of the apparent replicative limit of cell types in which telomere shortening does not seem to have a role[12,13]. Is there a telomere-independent intrinsic mechanism limiting the proliferative capacity of these cells? Or has our failure to expand these cells indefinitely in culture been the result of imperfect culture conditions leading to the activation of cellular checkpoints resulting in a form of premature senescence? Recent work, discussed here, provides strong evidence that extrinsic factors are responsible for both limiting the lifespan of rodent cells and acting in concert with telomere shortening in some types of human cells. This evidence against

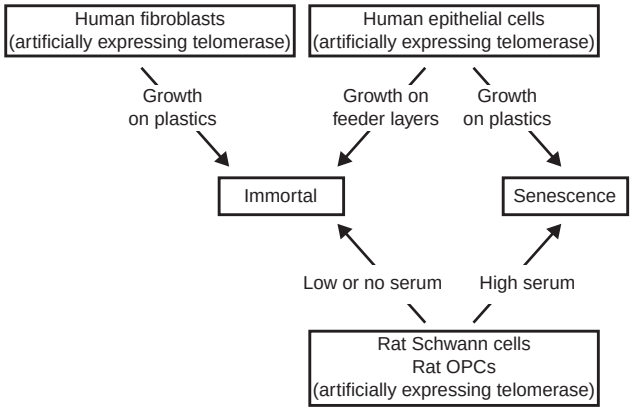

Figure 2 **Culture conditions can activate a checkpoint response that triggers senescence. Proliferative capacity in telomerase-expressing cells can be changed by altering culture conditions. Transfected human epithelial cells expressing telomerase are immortal when grown on feeder layers but senesce when grown on plastic. Rat Schwann cells and OPCs normally retain expression of telomerase. In medium containing a low concentration of serum they proliferate indefinitely; switching to a high serum concentration triggers senescence.**

the existence of an additional cell-division clock suggests that any cell expressing telomerase could proliferate indefinitely if optimal culture conditions could be found.

Unlimited lifespan

The first new findings describe conditions in which two rodent cell types, Schwann cells and oligodendrocyte precursor cells (OPCs), can proliferate indefinitely in culture, providing evidence against the dogma that all somatic cells have an intrinsic limitation to their proliferative lifespan[14,15]. Schwann cells are the glial cells that myelinate axons in the peripheral nervous system. If a nerve is cut, either by removal from the animal or by injury, the associated Schwann cells dedifferentiate and can proliferate in the presence of mitogen. These cells are able to remyelinate regenerated axons *in vivo* or can be expanded *in vitro*. Rat Schwann cells cultured in a medium containing a low concentration of serum in the presence of a specific mitogen, glial growth factor, proliferate indefinitely at a constant rate. This is in contrast to perineural fibroblasts isolated from the same nerves that senesce after fewer than 10 population doublings in culture. Similarly, rat OPCs that produce the glial cells that myelinate axons in the central nervous system are able to proliferate indefinitely when cultured under serum-free conditions.

In long-term culture both Schwann cells and OPCs maintain the checkpoint mechanisms that arrest the cell cycle in response to DNA damage and oncogenic Ras activation. The tumour suppressor proteins p53, $p16^{INK4A}$ and $p19^{ARF}$ that mediate these checkpoint responses are the same proteins that enforce the cell-cycle arrest seen in senescent cells. As Schwann cells and OPCs have an unlimited proliferative life-span, at least in culture, but are not particularly cancer-prone, it is tempting to speculate that the role of these tumour suppressors is to act as checkpoints to inappropriate oncogenic activation rather than limiting proliferative lifespan.

Does the ability of Schwann cells and OPCs to proliferate indefinitely reflect something special about these cell types and perhaps their role in the nervous system? Schwann cells and OPCs are both glial cells, although they derive from separate lineages—Schwann cells from the neural crest and OPCs from ventral neuroepithelium. What these cell types have in common, apart from the ability to myelinate axons, is that they can produce new cells throughout the life of the animal. However, this is a property of many other cell types: liver cells and fibroblasts, for example, maintain the ability to proliferate in response to injury, yet *in vitro* these cell types senesce. It is therefore difficult to correlate regenerative potential *in vivo* with replicative potential *in vitro*.

"Culture shock"

A key finding was that the lifespan of these cells could be altered by changing the culture conditions. Transferring Schwann cells or OPCs into higher concentrations of serum resulted in the onset of senescence (Fig. 2). This effect did not seem to be due to a rapid activation of damage checkpoints, because the Schwann cells in higher serum proliferated at a faster rate for the initial few passages before exiting from the cell cycle and developing a senescent phenotype. These experiments demonstrate that apparent limits to a cell's proliferative lifespan can be the result of culturing cells in particular conditions. This raises the question of whether senescence induced in other cells is also the result of the culture environment. This issue has been discussed previously after the incongruous finding that rodent embryonic fibroblasts deficient in certain DNA damage responses senesce rapidly in culture, whereas if they remained in the animal they should continue to divide[12,13]. Common sense thus seemed to indicate that culture conditions were responsible for triggering senescence and were, in effect, masking the intrinsic proliferative potential of these cells. However, this idea will remain conjecture until culture conditions are discovered that permit the extended proliferation of other cell types. These have yet to be found, despite a previous report that mouse embryo fibroblasts can proliferate indefinitely when grown under defined conditions[16]. Subsequent experiments demonstrated that the conditions used were leading to the selective outgrowth of neural precursor or stem cells and that it was these cells that had unlimited proliferative potential[17].

A further recent report[18] has strengthened the argument that the culture environment is responsible for the telomere-independent limits to proliferative lifespan seen *in vitro* (Fig. 2). Two human epithelial cell types were studied that have previously been shown to senesce in a telomere-independent, $p16^{INK4A}$-dependent manner after a relatively short period in culture; growing one of these cell types, keratinocytes, on a feeder layer rather than on plastic extended their proliferative lifespan. Moreover, expressing telomerase under these conditions allowed the cells to proliferate indefinitely. Similar experiments with mammary epithelial cells demonstrated that growing these cells on feeder layers also increased their proliferative lifespan. Returning the keratinocytes to plastic resulted in the onset of senescence within a few passages. In each case, entry into senescence was correlated with the induction of $p16^{INK4A}$.

The results in the human epithelial cells and rodent glia demonstrate that the onset of telomere-independent replicative senescence in these cells is not the result of an invariant cell-intrinsic mechanism that counts cell divisions. Instead, proliferation in distinct environments differentially determines whether a cell will senesce. What these results do not tell us are the nature of the signals that trigger senescence in these cells after an extended period in culture and whether these checkpoint mechanisms have any role *in vivo*.

In Schwann cells and OPCs, senescence is triggered by culturing the cells in high serum. One possible explanation for this is that certain culture conditions can result in 'overstimulation' of mitogenic pathways. This idea is based on the observation that strong activation of MAP kinase pathways in primary cells, by oncogenic Ras expression for example, triggers entry into senescence, acting possibly as a tumour suppressor mechanism.

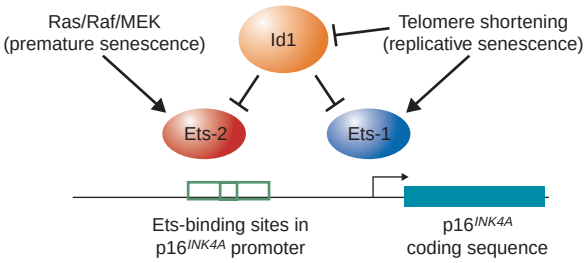

Figure 3 **Signals that induce senescence induce p16^{INK4A} expression by distinct mechanisms. p16^{INK4A} induction associated with telomere shortening (replicative senescence) and Ras expression (premature senescence) in human diploid fibroblasts is associated with increased activity of Ets transcription factors. An increase in Ets activity is both necessary and sufficient to induce p16^{INK4A} and trigger senescence. However, different signalling pathways are responsible for the increase in Ets activity in the two responses. Cells approaching replicative senescence have high levels of Ets-1 and lower levels of Id1 (a negative regulator of Ets activity), whereas activation of the Ras/Raf/MAP kinase pathway results in a strong increase in Ets-2 levels and activity.**

Perhaps the mitogen concentrations found in culture, in particular the rich mix of mitogens in serum, mimic this situation. This suggestion is consistent with the genetic analysis of senescence in mouse embryo fibroblasts. The p19^{ARF}/p53 signalling pathway that mediates culture-induced senescence is also required for Ras to induce senescence, whereas DNA damage signals to p53 via alternative pathways[19]. How this signal would gradually accumulate over several generations is, however, difficult to envisage. Another possibility is that high levels of serum result in higher rates of metabolism, resulting in increased oxidative damage that eventually signals to the senescence pathways. With human epithelial cells, growing cells on feeder layers rather than on plastic allows extended proliferation. Again, an explanation for this is not obvious; perhaps the feeder layers provide anchorage signals which modulate signalling pathways that would otherwise trigger senescence. An alternative explanation is that the feeder layers permit the survival of a population of cells that would be lost on plastic; it is possible that these are able to continuously replenish the proliferating population.

Signals for senescence

Understanding the signalling pathways involved in the onset of senescence is likely to be important for the development of culture conditions that will permit the unlimited proliferation of many normal cell types. It might also lead to a greater understanding of checkpoint controls that could have a role in both the development of cancer and the ageing process of organisms. p16^{INK4A} induction is a common feature of both replicative and premature senescence in human cells and is thought to be important in maintaining the associated cell-cycle arrest[20,21]. In rodent cells, although genetic analysis has revealed, at least in mouse embryo fibroblasts, that p16^{INK4A} is not essential[22,23], the levels of p16^{INK4A} do increase sharply in culture and in Ras-induced senescent cells[4]. As a marker of senescence in response to different stimuli, studying p16^{INK4A} regulation might help us to understand the pathways limiting proliferative capacity both *in vitro* and *in vivo*. Earlier studies have indicated that different stimuli use different signalling pathways. Now, recent experiments exploring the regulation of the p16^{INK4A} promoter have confirmed that distinct mechanisms are involved and have identified some of the relevant transcription factors.

Disrupted telomeres mimic DNA damage and activate the p53 damage-response pathway[24]. Interestingly, it has been reported that inducing senescence by DNA damage in HDFs results in a prolonged p53 response, followed by a strong induction of p16^{INK4A} that maintains the cell-cycle arrest[25]. It would therefore follow that the induction of p16^{INK4A} seen when HDFs become senescent is the result of a damage signal from uncapped telomeres. It remains unclear whether p16^{INK4A} in this situation is induced directly by a damage signal or results from a stable cell-cycle arrest. In contrast, activation of the Ras/Raf/Map kinase pathway leads to a rapid increase in p16^{INK4A} levels in HDFs that is independent of p53 and is coincident with the onset of the cell-cycle arrest[26,27].

Experiments on primary HDFs have shown that binding sites for the transcription factor Ets located in the p16^{INK4A} promoter are required for the upregulation of p16^{INK4A} levels in response to senescence signals[28]. Constitutive expression of Ets is sufficient both to induce p16^{INK4A} and to provoke entry into senescence, whereas the expression of a dominant-negative form of Ets is able to block these events. The activity of Ets is modulated by the levels of Id proteins that act as negative regulators of the Ets family. The balance of Ets and Id proteins therefore determines the overall level of Ets activity. Interestingly, the increase in Ets activity responsible for p16^{INK4A} upregulation seems to be achieved by different mechanisms in Ras-induced senescence and in replicative senescence. Activation of Ras leads to a rapid increase in the levels and activity of Ets-2, an effect mediated by the MAP kinase pathway. In contrast, the increase in Ets activity as cells approach the end of their replicative lifespan seems to be due to both an increase in Ets-1 levels and a marked decrease in Id levels (Fig. 3). We and others have shown that the induction of p16^{INK4A} in rodent glial cells and human epithelial cells, as a result of proliferation in culture, is due to signals arising from a particular culture environment. Analysis of the transcription factors known to regulate the p16^{INK4A} promoter should help to identify the signalling pathways involved.

The ability to culture large numbers of normal cells in culture has huge therapeutic potential. Understanding the mechanisms underlying the limitations of proliferative lifespan will be crucial in making this possible. □

The author is a Cancer Research Campaign Senior Research Fellow in the MRC Laboratory for Molecular Cell Biology and Dept. of Biochemistry, University College London, Gower Street, London WC1E 6BT, UK.
e-mail: alison.lloyd@ucl.ac.uk

1. Dimri, G. P. *et al. Proc. Natl Acad. Sci. USA* **92,** 9363–9367 (1995).
2. Di Leonardo, A., Linke, S. P., Clarkin, K. & Wahl, G. M. *Genes Dev.* **8,** 2540–2551 (1994).
3. Venable, M. E., Lee, J. Y., Smyth, M. J., Bielawska, A. & Obeid, L. M. *J. Biol. Chem.* **270,** 30701–30708 (1995).
4. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. *Cell* **88,** 593–602 (1997).
5. McConnell, B. B., Starborg, M., Brookes, S. & Peters, G. *Curr. Biol.* **8,** 351–354 (1998).
6. L. Hayflick. *Exp. Cell Res.* **37,** 614–636 (1965).
7. McEachern, M. J. & Blackburn, E. H. *Genes Dev.* **10,** 1822–1834 (1996).
8. van Steensel, B. Smogorzewska, A. & de Lange, T. *Cell* **92,** 401–413 (1998).
9. Bodnar, A. G. *et al. Science* **279,** 349–352 (1998).
10. Kiyono, T. *et al. Nature* **396,** 84–88 (1998).
11. Blasco, M. A. *et al. Cell* **91,** 25–34 (1997).
12. Sherr, C. J. & DePinho, R. A. *Cell* **102,** 407–410 (2000).
13. Wright, W. E. & Shay, J. W. *Nature Med.* **6,** 849–851 (2000).
14. Mathon, N. F., Malcolm, D. S., Harrisingh, M. C., Cheng, L. & Lloyd, A. C. *Science* **291,** 872–875 (2001).
15. Tang, D. G., Tokumoto, Y. M., Apperly, J. A., Lloyd, A. C. & Raff, M. C. *Science* **291,** 868–871 (2001).
16. Loo, D. T., Fuquay, J. I., Rawson, C. L. & Barnes, D. W. *Science* **236,** 200–202 (1987).
17. Loo, D. T., Althoen, M. C. & Cotman, C. W. *J. Neurosci. Res.* **42,** 184–191 (1995).
18. Ramirez, R. D. *et al. Genes Dev.* **15,** 398–403 (2001).
19. Palmero, I., Pantoja, C. & Serrano, M. *Nature* **395,** 125–126 (1998).
20. Alcorta, D. A. *et al. Proc. Natl Acad. Sci. USA* **93,** 13742–13747 (1996).
21. Kiyono, T. *et al. Nature* **396,** 84–88 (1998).
22. Krimpenfort, P., Quon, K. C., Mooi, W. J., Loonstra, A. & Berns, A. *Nature* **413,** 83–86 (2001).
23. Sharpless, N. E. *et al. Nature* **413,** 86–91 (2001).
24. Blackburn, E. H. *Nature* **408,** 53–56 (2000).
25. Robles, S. J. & Adami, G. R. *Oncogene* **16,** 1113–1123 (1998).
26. Lin, A. W. *et al. Genes Dev.* **12,** 3008–3019 (1998).
27. Zhu, J., Woods, D., McMahon, M. & Bishop, J. M. *Genes Dev.* **12,** 2997–3007 (1998).
28. Ohtani, N. *et al. Nature* **409,** 1067–1070 (2001).