The role of BRAFE600-induced senescence in tumour suppression

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CHAPTER 5

The essence of cellular senescence and how to recognise it

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The essence of cellular senescence and how to recognise it

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Abstract

More than forty years after the first evidence of the limited lifespan of primary cells in culture, there is increasing evidence for the existence of cellular senescence both *in vitro* and *in vivo*, as well as confirmation of its role in preventing tumourigenesis. In this review we dissect the different categories of cellular senescence and examine their causes as well as their physiological roles in tumour suppression. Moreover, we discuss the power and limitations of the currently available markers that can be used to identify senescence *in vitro* and *in vivo*.

Senescence at the cellular level

One of the first observations made about primary cells explanted from human tissue, was that diploid cultures do not proliferate indefinitely but instead are 'mortal'. In fact, their growth after explantation was characterised by three phases: Phase I of slow growth during which the culture was established (the period before the first passage), phase II characterised by rapid proliferation of the cell strain and phase III during which growth gradually seized (Hayflick & Moorhead, 1961). In search of the reason behind the onset of phase III, Hayflick later hypothesised that 'the finite lifetime of diploid cell strains *in vitro* may be an expression of ageing or senescence at the cellular level' (Hayflick, 1965). The term cellular senescence was therefore introduced to describe phase III as defined by Hayflick: a cellular state in which cells do not proliferate, but remain metabolically active and display a 'spread-out' flattened morphology in comparison to cells growing exponentially. Although it is impossible to say with any certainty what where the causes of the senescence phenotype described by Hayflick, a plethora of experiments have shown that cellular senescence can be caused by a variety of conditions.

Replicative senescence

Primary cells have a limited replicative potential *in vitro*. With every cell division, the cell's telomeres become shorter (Harley et al., 1990), and when they reach a critical minimal length, telomere ends are uncapped, triggering a DNA damage response (d'Adda di Fagagna et al., 2003; Takai et al., 2003), resulting in cell cycle arrest. The term replicative senescence describes cells that are arrested due to telomere attrition or malfunction (Figure 1). The onset of replicative senescence is exclusively dependent on telomere length as it can be bypassed by the ectopic overexpression of the catalytic subunit of the telomerase holoenzyme (hTERT), the enzyme that synthesizes telomeres by the addition of TTAGGG repeats to the chromosomal ends (Bodnar et al., 1998; Vaziri & Benchimol, 1998) (Figure 2). Telomerase is not expressed in human somatic somatic cells with the exception of stem cells (Harley et al., 1990; Wright et al., 1996; Masutomi et al., 2003). Therefore, the ectopic overexpression of hTERT is a common practice *in vitro* allowing for the immortalisation of primary human cells. Tumour cells, on the other hand, very often express telomerase (Shay & Bacchetti, 1997), or more rarely elongate their telomeres through a mechanism

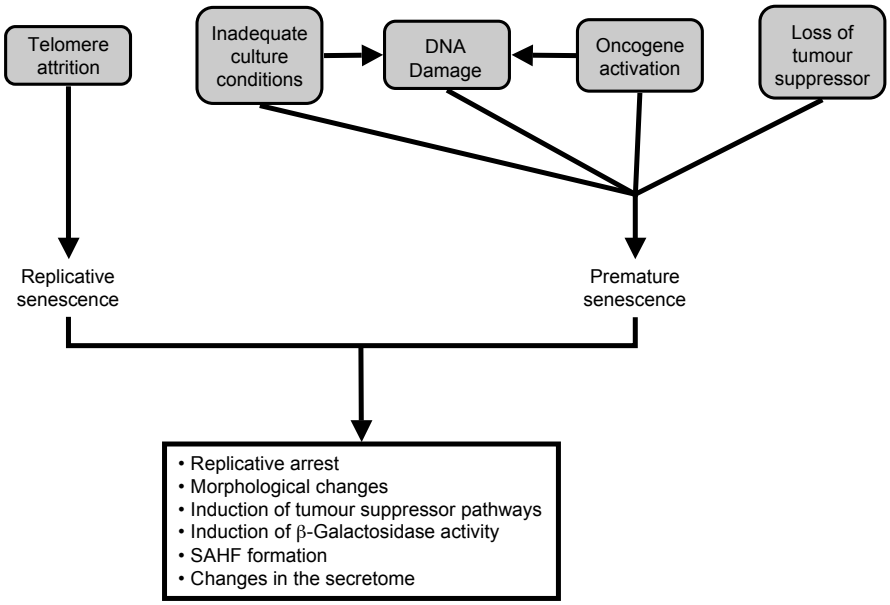

Figure 1. Barriers to indefinite proliferation: achieving senescence induction. Premature senescence can be caused by a variety of conditions such as inadequate culture conditions, DNA damage, oncogene activation or the loss of a tumour suppressor. Moreover, inadequate culture conditions and oncogenic activation can also induce senescence by generating DNA damage. A link between loss of a tumour suppressor and DNA damage has not yet been made. Replicative senescence is caused by the accumulation of cellular divisions, which gradually result in telomere attrition. Cells undergoing replicative or premature senescence are characterised by the same hallmarks.

termed alternative lengthening of telomeres (ALT) (Muntoni & Reddel, 2005). As a result, most human cancer cells have relatively short telomeres, which are maintained at a length compatible with life and proliferation (Shay & Wright, 2006). Existing evidence for replicative senescence *in vivo* is limited to a correlation between aged tissues and the induction of senescence markers (Slagboom et al., 1994; Dimri et al., 1995; Cristofalo et al., 2004; Herbig et al., 2006). The role of replicative senescence as a tumour-suppressive mechanism *in vivo* is indicated by mouse studies: loss of the RNA component of telomerase can prevent tumour formation by inducing senescence in myc-driven tumourigenesis mouse models, in the absence of a functional apoptotic pathway (Cosme-Blanco et al., 2007; Feldser & Greider, 2007).

Premature Senescence

Senescence can be induced also in the absence of any detectable telomere loss, by a variety of conditions (Figure 1). This type of senescence is termed 'premature', to reflect its independence from telomere attrition. Evidence for the existence of premature senescence *in vivo* have been arising lately, indicating that certain types of premature senescence act *in vivo* as genuine tumour suppressing mechanisms. The different conditions that can induce premature senescence are described below.

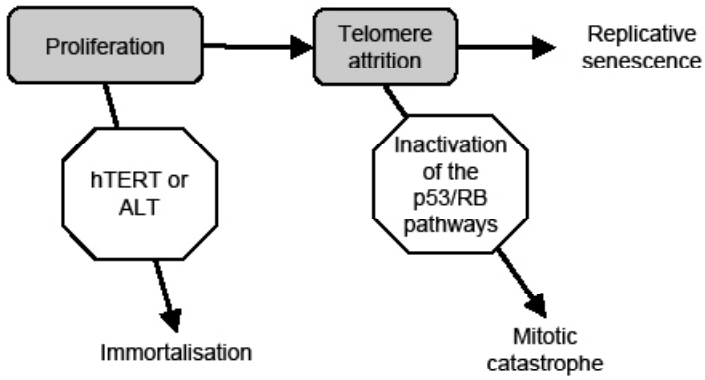

Figure 2. The road to replicative senescence, and how to avoid it. Sustained cellular proliferation leads to telomere attrition and eventually replicative senescence. Blocking the cause of replicative senescence by maintaining telomere length -through expression of hTERT or ALT- results in the immortalisation of human cells. On the contrary, abolishing the consequence of telomere shortening by inactivation of the p53 and RB pathways does not prevent senescence but bypasses it, eventually leading to telomeric crisis and mitotic catastrophe.

Stress-induced senescence

Premature senescence can be induced artificially *in vitro* as a result of inadequate culturing conditions. Following explantation, cells have to adapt to a number of new conditions which can often be suboptimal in supporting proliferation, such as a different abundance of nutrients and growth factors, the absence of other cell types, ambient O_2 levels and the attachment to plastic or lack of extracellular matrix components. One or more of these conditions can induce a 'culture shock', resulting in stress-induced senescence. In contrast to most human cells, mouse cells express telomerase (Prowse & Greider, 1995) and have relatively long telomeres (Sherr & DePinho, 2000). Nonetheless, mouse embryonic fibroblasts (MEF) undergo senescence after just a few passages *in vitro* in the absence of any signs of telomere loss. Elongation of their lifespan can be achieved by culturing in serum free medium supplemented with a number of defined growth factors (Loo et al., 1987) or by culturing in more physiological, low oxygen conditions as opposed to the routinely-used, ambient, 20% O_2 levels (Parrinello et al., 2003). Oxidative stress can also limit the *in vitro* lifespan of human cells as indicated by the longer lifespan of human diploid fibroblasts when cultured under low oxygen (Packer & Fuehr, 1977; Chen et al., 1995; Yuan et al., 1995). Furthermore, the replicative potential of human melanocytes and epithelial cells depends largely on the composition of the culture medium used as well as the use of feeder layers (Ramirez et al., 2001; Bennett & Medrano, 2002; Bennett, 2003). Therefore, the immortalisation of mammalian cells requires not only telomere maintenance, but also optimal culture conditions (Mathon et al., 2001; Ramirez et al., 2001; Tang et al., 2001; Herbert et al., 2002; reviewed in Wright & Shay, 2002).

Oncogene-Induced senescence

Early experiments with activated HRAS ($HRAS^{V12}$) led to the discovery that although $HRAS^{V12}$ can transform most immortal mammalian cell lines, or collaborate with immortalizing oncogenes in transforming primary cells, when introduced alone into primary cells (and at least one immortal rat fibroblast cell line), it induces cell cycle arrest (Land et al., 1983; Franza et al., 1986; Serrano et al., 1997). Serrano and colleagues termed this originally puzzling phenomenon oncogene-induced senescence (OIS), in view of its resemblance with replicative senescence. Unlike replicative senescence, however, OIS cannot be bypassed by expression of hTERT, demonstrating that it exists irrespective of telomere length (Wei et al., 1999). The discovery that primary cells entered senescence when ectopically expressing oncogenes *in vitro* raised the question of its validity as a physiological phenomenon: is OIS a genuine reaction to oncogene expression or is it the artefact of the gross overexpression used in these *in vitro* experiments. Following from that a second question was raised: does

OIS exist *in vivo*? (Peeper & Mooi, 2002; Bennett, 2003; Lowe et al., 2004; Tuveson et al., 2004; Perez-Mancera & Tuveson, 2006). In trying to answer these questions, it became clear that in some cases, expression of low levels of $HRAS^{V12}$ *in vitro* failed to induce OIS but instead triggered increased proliferative activity, whereas OIS was induced with higher levels of oncogene expression (Deng et al., 2004; Sarkisian et al., 2007). Furthermore, MEFs from oncogenic KRAS knock-in ($KRAS^{V12}$ or $KRAS^{D12}$) mice did not senescence in culture, but rather displayed increased proliferation (Guerra et al., 2003; Tuveson et al., 2004). In seeming contrast with these findings, however, $KRAS^{V12}$ knock-in mice develop premalignant lung tumours that show signs of senescence *in vivo* (Collado et al., 2005). Therefore, whatever the conditions *in vitro*, physiological expression of $KRAS^{V12}$ from its endogenous promoter can induce senescence in mouse lungs. Importantly, increased HRAS levels due to *HRAS* amplification can be found often in Spitz naevi, senescent tumours of human melanocytes, indicating that high levels of oncogenic expression can coexist with senescence also *in vivo* (Maldonado et al., 2004). The dependency on the expression levels for induction of senescence may differ among oncogenes. For example, contrary to mutant HRAS, oncogenic BRAF ($BRAF^{E600}$) can induce senescence even when expressed at levels similar to the endogenous protein *in vitro,* or from its endogenous promoter *in vivo* (Michaloglou et al., 2005; Dankort et al., 2007; Dankort et al., 2009; Dhomen et al., 2009). It is therefore clear that although induction of OIS in some instances requires high levels of oncogene expression, it can be a genuine, physiological outcome of oncogene expression, which can also occur *in vivo*.

Tumour-suppressor loss-induced senescence

Similar to oncogene overexpression, loss of a tumour suppressor can also trigger senescence in mouse and human cells both *in vitro* and *in vivo* (Chen et al., 2005; Courtois-Cox et al., 2006). Importantly, loss of NF1 or PTEN correlates with the existence of senescent cells in human neurofibromas or prostate tumours respectively, suggesting that just like OIS, induction of senescence due to the loss of a tumour suppressor can also be involved in limiting tumourigenesis *in vivo*. Interestingly, although loss of NF1 triggers senescence in human fibroblasts, it immortalises MEF, indicating that the onset of senescence depends largely on cellular/genetic context.

DNA damage-induced senescence

The involvement of DNA damage in the induction of senescence is best demonstrated

by the ability of telomere attrition to induce replicative senescence. However, DNA damage has been suggested to be involved also in the induction of premature senescence. DNA damaging agents such as ionizing radiation can induce cell cycle arrest in human fibroblasts with characteristics of senescence (Di Leonardo et al., 1994; Robles & Adami, 1998). Furthermore, DNA damage-inducing drugs can cause senescence of tumour cells *in vitro* and *in vivo*, a phenomenon termed drug-induced senescence and exploited for the treatment of cancer (Wang et al., 1998; Chang et al., 1999; Berns, 2002; Schmitt et al., 2002; te Poele et al., 2002; Roninson, 2003). Recently, activation of a DNA damage response (DDR) has been shown to mediate oncogene-induced senescence in some settings *in vitro* (Bartkova et al., 2005; Bartkova et al., 2006; Di Micco et al., 2006; Rodier et al., 2009; Shamma et al., 2009). Furthermore, evidence of a DDR response has also been demonstrated for a variety of premalignant human lesions (Bartkova et al., 2005; Gorgoulis et al., 2005; Bartkova et al., 2006; Di Micco et al., 2006). However, as these lesions contain proliferating cells, and a direct comparison between proliferating and non-proliferating cells in these lesions is not always available, the potential role of DDR in mediating oncogene-induced senescence *in vivo* remains to be addressed in more detail.

Markers of senescence

Although senescence can be induced by a variety of conditions, senescent cells share some characteristics that allow their identification both *in vivo* and *in vitro* (Figure 1). These hallmarks of senescent cells are categorised and discussed below.

Cell cycle arrest

Exit from the cell cycle is probably the only indispensable marker for the identification of all types of cellular senescence both *in vivo* and *in vitro*. However, although all senescent cells without exception undergo cell cycle arrest, the inability to replicate is not unique to senescent cells. Both terminal differentiation, and quiescence -due to lack of growth factor stimulation or contact inhibition-, result also in replicative arrest. Therefore, although cell cycle exit is a crucial marker for senescence, it has little power on its own.

Morphological changes

The cell cycle arrest of senescent cells is often accompanied by a morphological change: cells undergoing replicative arrest become typically large and flat (Bodnar et al., 1998). The same is observed for cells undergoing $HRAS^{V12}$-induced senescence (Serrano et al., 1997; Denoyelle et al., 2006), stress-induced senescence (Parrinello et al., 2003) and DNA damage-induced senescence (Chen & Ames, 1994; Chen et al., 2001). Cells senescing due to $BRAF^{E600}$ expression or the silencing of p400 however, acquire a more spindle shaped morphology (Chan et al., 2005; Michaloglou et al., 2005). Finally, melanocytes undergoing RAS^{V12}-induced senescence display extensive vacuolisation as a result of endoplasmic reticulum stress caused by the unfolded protein response (Denoyelle et al., 2006). The induction of cell cycle arrest and the appearance of morphological changes are usually the first indications of the onset of cellular senescence. But clearly, also this feature is not exclusive to senescent cells. Furthermore, it is questionable whether cell morphology can be used as a senescence marker *in vivo*.

Activation of tumour suppressor pathways

The p53 and RB pathways, the two major tumour suppressor routes in mammalian cells, play a major role in the control of the cell cycle (Figure 3). It is therefore not surprising that both are involved also in the induction of senescence *in vitro* and *in vivo*. In human fibroblasts undergoing replicative or premature senescence RB accumulates in its active, hypophosphorylated form (Stein et al., 1990; Serrano et al., 1997; Lin et al., 1998), and p53 displays increased activity and/or levels (Atadja et al., 1995; Vaziri et al., 1997; Bunz et al., 1998; Wei et al., 2001). Protein levels of members of other factors in the RB and p53 pathways, notably $p16^{INK4A}$ and $p21^{CIP1/WAF1}$ (Figure 3), also accumulate (Alcorta et al., 1996; Hara et al., 1996; Reznikoff et al., 1996; Serrano et al., 1997; Lin et al., 1998; Robles & Adami, 1998; Zhu et al., 1998). Induction of $p16^{INK4A}$ is also seen in senescent mouse and human tumours *in vivo* (te Poele et al., 2002; Collado et al., 2005; Michaloglou et al., 2005; Gray-Schopfer et al., 2006; Dhomen et al., 2009; Goel et al., 2009), while p53 and $p21^{CIP1/WAF1}$ induction is evident in senescent mouse prostate tumours (Chen et al., 2005). Overexpression of $p21^{CIP1/WAF1}$, ARF, or $p16^{INK4A}$ is sufficient to induce cell cycle arrest in HDF as well as to increase some of the senescence markers discussed below (McConnell et al., 1998; Dimri et al., 2000). Furhtermore, also *in vivo*, activation of the p53 pathway can lead to the induction of a senescence response that can act as an efficient tumour suppressive mechanism in certain mouse tissues (Ventura et al., 2007; Xue et al., 2007), demonstrating the role of the p53 pathway in senescence *in vivo* and providing evidence for the potential of senescence as an intrinsic tumour suppressive

mechanism.

In many settings, the induction of senescence depends on the presence of functional RB and/or p53 pathways (Campisi, 2001; Campisi, 2005). Although replicative senescence of human cells cannot be bypassed in the absence of a telomere-elongating mechanism, its induction depends on the p53 and RB pathways as its onset can be delayed by the inactivation of the pathways (Counter et al., 1992; Brown et al., 1997; Brookes et al., 2004). Eventually, though, this leads to telomeric crisis and mitotic catastrophe (Itahana et al., 2001; Shay & Wright, 2005). Also the senescence of MEF *in vitro* can be bypassed by inactivation of the p53 and RB pathways. MEF explanted from $p53^{-/-}$ or $p19^{-/-}$ animals can proliferate indefinitely in culture (Harvey et al., 1993; Tsukada et al., 1993; Kamijo et al., 1997) and the same is true for MEF from animals deficient for all three pocket proteins (RB, p107 and p130; Dannenberg et al., 2000; Sage et al., 2000). Furthermore, p53-silencing in senescent MEF is sufficient to revert senescence (Dirac & Bernards, 2003). Stress-induced senescence in human cells often depends on the presence of $p16^{INK4A}$, as its inactivation can elongate life span and even immortalise cells that express hTERT (Wright & Shay, 2002; Gray-Schopfer et al., 2006). OIS in MEF depends on the presence of intact p53 and RB pathways (Serrano et al., 1997; Lin

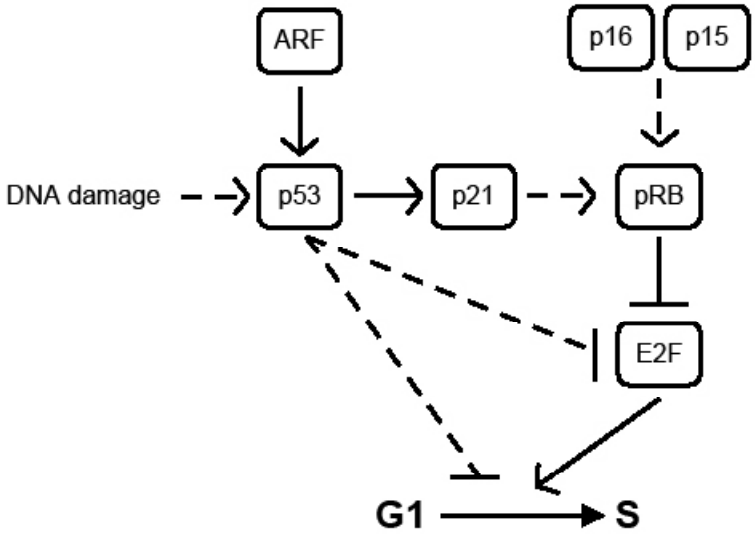

Figure 3: **The p53 and RB tumour suppressor pathways.** p53 is activated by DNA damage as well as ARF, which in turn can be induced by oncogene activation. p53 can block the cell cycle through its transcriptional target, $p21^{CIP1/WAF1}$, which is capable of activating RB and hence connects the two pathways. However, p53 can block the cell cycle also independently of RB. The RB pathway is activated independently of p53, by the cyclin-dependant kinase inhibitors $p16^{INK4A}$ and $p15^{INK4B}$, which are induced during cellular ageing, oncogene activation and tissue culture stress. In its activated form, RB can block the E2F transcription factors, which drive cell cycle progression from G1 to S phase resulting in a G1 arrest. Full and dashed lines indicate direct and indirect interaction respectively.

et al., 1998; Peeper et al., 2001). In human cells however, the involvement of the p53 and RB pathways in the onset of oncogene-induced senescence is not as clear: whereas IMR90 and LF1 human diploid fibroblasts (HDF) do not depend on p53 for $HRAS^{V12}$-induced senescence (Serrano et al., 1997; Wei et al., 2001), BJ HDF do (Voorhoeve & Agami, 2003). WI-38 HDF undergo E2F-induced senescence that is mediated by p53 (Dimri et al., 2000). Furthermore, IMR90 undergo also RAF1-induced senescence independently of a functional p53 pathway (Zhu et al., 1998), indicating that p53-dependency is cell-line-specific. $HRAS^{V12}$ induces senescence in human epithelial cells that can be bypassed by $p21^{CIP1/WAF1}$ silencing (Nicke et al., 2005), whereas in human melanocytes it is independent of p53 inactivation (Denoyelle et al., 2006). Similarly, although human fibroblasts homozygous for a deletion that renders $p16^{INK4A}$ inactive (Leiden fibroblats) do not undergo $HRAS^{V12}$-induced senescence (Brookes et al., 2002), BJ and primary melanocytes do so in the presence of a $p16^{INK4A}$-silencing hairpin (Voorhoeve & Agami, 2003; Denoyelle et al., 2006). Furthermore, $p16^{INK4A}$ is dispensable also for the induction of $BRAF^{E600}$- or $NRAS^{K61}$-induced arrest in HDF and human primary melanocytes (Michaloglou et al., 2005; Haferkamp et al., 2009). It is therefore evident that although the p53 and RB pathways are involved in the induction of OIS in human cells, the individual contributions of each pathway are cell type-, cell line- and context-dependent. The existing evidence on tumour suppressor loss-induced senescence (e.g., in the context of PTEN loss) indicates that it, too, depends on the p53 and RB pathways. However, this type of senescence has been identified more recently and has not yet been studied as extensively as OIS, making it premature to generalise this. Finally, human cells deficient for the p53 pathway fail to undergo senescence upon DNA damage *in vitro* (Di Leonardo et al., 1994; Chang et al., 1999; te Poele et al., 2002), and mouse lymphomas undergo DNA damage-induced senescence only in the presence of p53 and $p16^{INK4A}$ (Schmitt et al., 2002). These results point at an important role for the p53 and RB pathways also in this type of premature senescence.

In conclusion, activation of at least one of the RB and p53 pathways has been identified in almost all cases of senescence induction, both *in vitro* and *in vivo*. In many cases, a causal link has been established between the activation of the tumour suppressor and the induction of senescence. As such, activation of one or more components in two tumour suppressor pathways serves not only as an important marker for the presence of cellular senescence but can also in some instances explain its onset.

Induction of senescence-associated β-Galactosidase activity (SA β-Gal)

In 1995, the group of Judith Campisi identified the presence of β-Galactosidase (β-Gal) enzymatic activity measured at pH 6.0 as a marker of replicative senescence both *in vitro* in human fibroblasts, melanocytes and epithelial cells, as well as in human skin (Dimri et al., 1995). They showed that, although all cells tested where

positive for β-Gal activity measured at the optimal pH 4.0, only senescent, and not proliferating, quiescent or terminally differentiated cells, displayed enzymatic activity at pH 6.0. They named this enzymatic activity at pH 6.0 senescence-associated β-Gal activity (SA β-Gal) and demonstrated that it is also increased in human skin of aged donors. Later experiments revealed that β-Gal activity at pH 4.0 and SA β-Gal activity at pH 6.0 corresponded to the same enzyme (β-Gal, with optimal pH 4.0), whose levels increase during senescence, making its activity measurable at the suboptimal pH 6.0 (Kurz et al., 2000; Lee et al., 2006). Other studies showed that β-Gal activity can increase also as a function of quiescence, induced by cell confluency, or even in some cases, serum deprivation (Severino et al., 2000; Yang & Hu, 2005). Importantly, the fact that SA β-Gal corresponds to the β-Gal enzyme, whose activity can be induced by a variety of factors, implies that, contrary to common practice, β-Gal activity cannot be used as a single marker for the identification of senescence, whether *in vitro* or *in vivo*. Nevertheless, when used in combination with other senescence markers, it is a credible senescence marker. Indeed, increased β-Gal activity is seen in cells undergoing premature senescence *in vitro* such as OIS and DNA damage-induced senescence (Serrano et al., 1997; Robles & Adami, 1998; Narita et al., 2003; Michaloglou et al., 2005). Increased β-Gal activity has also been demonstrated in several senescent mouse and human pre-malignant lesions *in vivo* (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005; Courtois-Cox et al., 2006; Gray-Schopfer et al., 2006; Dhomen et al., 2009; Goel et al., 2009).

Senescence-associated heterochromatic foci (SAHF)

Cells undergoing senescence display a distinct nuclear DNA pattern, which is evident as foci when cells are stained with a DNA dye (Narita et al., 2003). Heterochromatic markers such as histone H3 methylated on lysine 9, the histone H2A variant macroH2A and HP1 proteins (α, β and γ) co-localise with these DNA foci, whereas euchromatic markers such as histone H3 methylated on lysine 4, or acetylated on lysine 9, are excluded (Narita et al., 2003; Zhang et al., 2005). Whole chromosomes are found condensed within individual foci, and the levels of linker histone H1 diminish (Funayama et al., 2006; Zhang et al., 2007). RNA polymerase II and active sites of transcription localise outside SAHF, whereas promoters of some E2F target genes are heterochromatinized in senescent cells (Narita et al., 2003; Funayama et al., 2006). Because of these features, these areas are named senescence-associated heterochromatic foci (SAHF) (Narita et al., 2003; reviewed in Adams, 2007). SAHF form during replicative and premature senescence but not quiescence, and as such they are not markers of cell cycle arrest in general but are specific markers of senescence (Narita et al., 2003). It is hypothesized that as the formation of SAHF signifies the extensive heterochromatinization of DNA and specifically the silencing of E2F target

genes, it plays a functional role in the onset and maintenance of the senescence response. Indeed, overexpression of SAHF components can induce senescence *in vitro* and silencing of SAHF components can, in some cases and to a certain extent, rescue cells from senescence (Zhang et al., 2005; Narita et al., 2006). However, although senescence bypass *in vitro* often correlates with the inability to form SAHF (Narita et al., 2003; Chan et al., 2005; Ye et al., 2007; Kuilman et al., 2008), there are also cases where cells remain arrested in the absence of SAHF formation (Narita et al., 2006). As such, the inability to detect SAHF is not necessarily an indication of the lack of a senescence response. Nevertheless, in combination with the induction of cell cycle arrest, SAHF formation remains a strong senescence marker.

Senescence-associated secretome

Cells undergoing senescence, whether replicative or in response to (oncogenic) stress, exhibit changes in their transcriptome. Part of this change is reflected by the number and abundance of factors secreted by the cells (secretome) (Campisi, 2005; Kuilman & Peeper, 2009). The first indication of senescence-induced changes in the secretome of human cells was reported for fibroblasts undergoing replicative senescence. Microarray analysis of these cells indicated a strong inflammatory response, reminiscent of wound healing (Shelton et al., 1999). Although Shelton and colleagues did not analyse protein levels, their results are in agreement with more recent work indicating that cells undergoing senescence display changes in their secretome, collectively termed the Senescence-Associated Secretory Phenotype (SASP; Coppe et al., 2008; Rodier et al., 2009). As these secreted factors turn out to be prominently involved in the communication between senescent cells and their microenvironment, it is also referred to as the Senescence-Messaging Secretome (SMS, after the mobile phone short messaging service; Coppe et al., 2008; Kuilman & Peeper, 2009; Rodier et al., 2009). Induction of the SMS is evident during both replicative and premature senescence (induced by oncogene expression or DNA damage) and is observed not only in fibroblasts but also in epithelial cells. Furthermore, this phenomenon is not limited to primary cells: prostate cancer cell lines activate an SMS when treated with the chemotherapeutic agent mitoxantrone (MIT) *in vitro*, and evidence for the induction of an SMS is found also *in vivo*, in prostate tumour samples of patients that have undergone MIT chemotherapy. Of note, these tumours display also induction of other senescence markers. A number of other studies have reported the secretion of factors by senescent cells, for example, IGFBP3, PAI1 and IFI16, during replicative senescence of human diploid fibroblasts (Goldstein et al., 1991; Goldstein et al., 1994; Xin et al., 2004) and TGFβ during $HRAS^{V12}$-induced senescence of mouse keratinocytes (Tremain et al., 2000) (reviewed in Kuilman & Peeper, 2009).

Importantly, the induction of secreted factors in senescent cells is often causally related to the onset of senescence, as their RNAi-mediated silencing allows for bypass of senescence, both in human and mouse cells (Tremain et al., 2000; Xin et al., 2004; Kortlever et al., 2006; Kim et al., 2007; Acosta et al., 2008; Kuilman et al., 2008; Wajapeyee et al., 2008; reviewed in Kuilman & Peeper, 2009). Thus, secreted factors affect not only the cells that produce them (autocrine effects) but also the microenvironment and hence neighbouring cells (paracrine effects; whether this works also at a distance (endocrine effects) is unknown). The proliferative rate, migration and invasion of premalignant cells are enhanced when they are co-cultured with, or grown in medium conditioned by, senescent fibroblasts (Krtolica et al., 2001; Dilley et al., 2003; Parrinello et al., 2005). Presenescent fibroblasts have no effect on either phenotype. Moreover, senescent fibroblasts can induce the proliferative activity and tumourigenic potential of malignant cells (Krtolica et al., 2001). The paracrine effects of senescent fibroblasts raise the possibility of a novel, oncogenic role for senescence (also called pleiotropic antagonism; Campisi, 2005). Indeed, although it has so far been considered a tumour suppressive mechanism limiting the indefinite expansion of cells, senescence could also promote cancer by stimulating the expansion of (pre) malignant neighbouring cells.

Current status and future perspectives

Since the discovery of the Hayflick limit, senescence has been the subject of numerous studies. As a result, a variety of stressful conditions that can cause senescence have been identified. A clear distinction has been made between replicative senescence, caused by the inevitable cellular ageing of most cell types, and premature senescence, manifesting itself only as a result of cellular stress. Furthermore, a number of new senescence markers have been identified, allowing for the study of a broader range of cellular responses to senescence-inducing mechanisms, probably most notably changes in the DNA structure and the secretome. Nonetheless, as a single marker none of the currently available senescence biomarkers universally and unequivocally suffices to identify senescent cells, whether *in vitro* or *in vivo*. While replicative arrest is to be considered the most important marker, it too cannot be used in the absence of other markers as it does not mark senescent cells exclusively, but also cells that have undergone other types of cell cycle arrest. The absence of exclusive and global markers could reflect the fact that senescence can be triggered by a variety of conditions and in various contexts, and therefore must engage a number of different cellular pathways.

Indeed, in spite of our rapidly increasing understanding of senescence, a number of key questions have remained unsolved. For example, it is unclear what determines the response of different cells to oncogenic stimulation. Whereas $KRAS^{V12}$ expression

from its endogenous promoter induces senescence in mouse lung cells (Collado et al., 2005), prostate epithelium reportedly fails to show any signs of senescence when an endogenous $KRAS^{D12}$ allele is expressed (Tuveson et al., 2004). Is this due only to the different KRAS mutants studied or is it caused by differences in the inherent ability of various cell types to senesce? Furthermore, why do some HDF strains undergo $p16^{INK4A}$-dependent OIS *in vitro*, whereas others depend more on $p21^{CIP1/WAF1}$ and p53? And why are HDF that lack $p16^{INK4A}$ resistant to $HRAS^{V12}$-induced senescence (Brookes et al., 2002), whereas knockdown of $p16^{INK4A}$ does not rescue other HDF strains from $HRAS^{V12}$-induced senescence (Voorhoeve & Agami, 2003; Denoyelle et al., 2006)? Moreover, why does $BRAF^{E600}$ induce SA β-Gal in senescent melanocytic tumours (Dhomen et al., 2009) but fails to do so (efficiently) in senescent lung adenomas (Dankort et al., 2007)? How much does the induction of a specific set of senescence markers depend on the particular oncogene, the cell type or even the inherent differences between individual organisms of the same species? Understanding why and how senescence occurs in certain settings and not in others will provide us with valuable information on the interconnection of the various molecular pathways involved in the onset and maintenance of senescence.

In conclusion, the original concern by some that senescence is merely the result of artificial culturing or experimental conditions *in vitro* and, therefore, bears little physiologic relevance, has now been refuted by the discovery that senescence biomarkers can be identified also *in vivo*. Clearly, although inadequate culturing conditions can also serve as a trigger, cellular senescence is now widely recognised as a physiological phenomenon limiting the indefinite expansion of cells. It is seen *in vivo* in the context of telomere shortening or malfunction, oncogenic stress and DNA damage resulting from various insults including genotoxic drugs. Intriguingly, the possibility of exploiting the reactivation of senescence for cancer treatment is being explored. In fact, senescence induction is likely to reflect at least one effect of the cytostatic actions of chemotherapeutic drugs. And, although achieving cell death should remain the top goal of cancer treatment, activation of senescence programs may be an option for specific tumour types, for example those that are notoriously chemo- and radioresistant, such as melanomas.

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