REVIEW

The essence of senescence

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Almost half a century after the first reports describing the limited replicative potential of primary cells in culture, there is now overwhelming evidence for the existence of "cellular senescence" in vivo. It is being recognized as a critical feature of mammalian cells to suppress tumorigenesis, acting alongside cell death programs. Here, we review the various features of cellular senescence and discuss their contribution to tumor suppression. Additionally, we highlight the power and limitations of the biomarkers currently used to identify senescent cells in vitro and in vivo.

Replicative cellular senescence

One of the first observations made for primary cells explanted from human tissue was that such cells do not proliferate indefinitely but instead are "mortal." In fact, their proliferative capacity upon explantation consistently displays three phases: phase I, corresponding to a period of little proliferation before the first passage, during which the culture establishes; phase II, characterized by rapid cell proliferation; and phase III, during which proliferation gradually grinds to a complete halt (Hayflick and Moorhead 1961). Commenting on the possible causes of the transition to phase III, Hayflick (1965) hypothesized that "The finite lifetime of diploid cell strains in vitro may be an expression of aging or senescence at the cellular level." The term cellular senescence therefore denotes a stable and long-term loss of proliferative capacity, despite continued viability and metabolic activity.

Consistent with Hayflick's proposal, we now know that, with the propagation of human cells in culture, telomeres (the protective chromosomal termini) are progressively shortened, ultimately causing cells to reach their "Hayflick limit." This barrier has been termed replicative (cellular) senescence, since it is brought about by replication. Telomeres are subject to attrition due to the fact that DNA polymerase fails to completely replicate the lagging strands. In the early 1970s, Olovnikov (1971) and Watson (1972) independently described this so-called "end replication problem", which contributes to telomere shortening. Thus, telomeres act as a molecular clock, reflecting the replicative history of a primary cell (Harley et al. 1990).

When telomeres reach a critical minimal length, their protective structure is disrupted. This triggers a DNA damage response (DDR), which is associated with the appearance of foci that stain positive for γ-H2AX (a phosphorylated form of the histone variant H2AX) and the DDR proteins 53BP1, NBS1, and MDC1. Moreover, the DNA damage kinases ATM and ATR are activated in senescent cells (D'Adda di Fagagna et al. 2003). After amplification of the DDR signal, these kinases activate CHK1 and CHK2 kinases. Communication between DDR-associated factors and the cell cycle machinery is brought about by phosphorylation and activation of several cell cycle proteins, including CDC25 (a family of phosphatases) and p53. In addition, differential expression of p53 isoforms has been linked to replicative senescence (Fujita et al. 2009). Together, these changes can induce a transient proliferation arrest, allowing cells to repair their damage. However, if the DNA damage exceeds a certain threshold, cells are destined to undergo either apoptosis or senescence. The factors bringing about this differential outcome have remained largely elusive, but the cell type and the intensity and duration of the signal, as well as the nature of the damage, are likely to be important determinants (D'Adda di Fagagna 2008).

In addition to p53, replicative senescence is linked to the RB tumor suppressor and its signaling partners, including $p16^{INK4A}$ (a cyclin-dependent kinase inhibitor acting upstream of RB). Indeed, activation of both the p53 and $p16^{INK4A}$–RB pathways is essential for induction of senescence in a variety of human cell strains. The first evidence for this came from experiments employing the viral oncoprotein SV40 large T antigen (LT) and mutants thereof lacking the ability to inhibit p53 or RB. While expression of wild-type LT allowed cellular immortalization to occur, expression of either of the mutants failed to do so (Shay et al. 1991). A variety of experiments subsequently confirmed the critical roles of the p53 and $p16^{INK4A}$–RB pathways (Ben-Porath and Weinberg 2005; Campisi 2005). The relative contribution of these cascades

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to senescence depends on the cell strain: While some are significantly delayed in their onset of senescence upon inactivation of $p16^{INK4A}$ alone, others require a deficiency in p53 or in p53 as well as $p16^{INK4A}$ for the abrogation of senescence. However, such escape from replicative senescence results in only limited further proliferation: Ultimately, cells will undergo telomeric crisis, resulting in chromosomal instability and death (Shay and Wright 2005).

The dependence of replicative senescence on telomere shortening is evident from its bypass by the ectopic expression of the catalytic subunit of the telomerase holoenzyme (hTERT), which elongates telomeres, thereby abrogating the effect of the end replication problem (Bodnar et al. 1998; Vaziri and Benchimol 1998). The limited life span of most primary human cells is explained by the fact that, in contrast to stem cells, telomerase is not expressed in human somatic cells, so they are unable to maintain telomeres at a sufficient length to suppress a DDR (Harley et al. 1990; Wright et al. 1996; Masutomi et al. 2003). Therefore, the ectopic expression of hTERT is a common practice in vitro, allowing for the immortalization of primary human cells. Likewise, tumor cells often express telomerase (Shay and Bacchetti 1997), or elongate their telomeres through a mechanism termed alternative lengthening of telomeres (ALT) (Muntoni and Reddel 2005). As a result, telomeres of human cancer cells are maintained at a length that permits continued proliferation (Shay and Wright 2006).

Premature cellular senescence

Senescence can be induced also in the absence of any detectable telomere loss or dysfunction, by a variety of conditions. This type of senescence has been termed premature, since it arises prior to the stage at which it is induced by telomere shortening. In recent years, evidence for the existence of premature senescence in vivo has been accumulating rapidly and points to a critical role in tumor suppression. We now turn to the various conditions that can induce such premature cellular senescence.

Stress-induced senescence in vitro

In vitro, premature senescence can result from inadequate culturing conditions. When cells are explanted from an organism and placed in culture, they have to adapt to an artificial environment, characterized by abnormal concentrations of nutrients and growth factors and the presence of ambient O_2 levels, as well as the absence of surrounding cell types and extracellular matrix components. One or more of these conditions can induce a culture shock, resulting in stress-induced senescence (Sherr and DePinho 2000). This type of cell cycle arrest is independent of telomere length. It also occurs in mouse cells, which, in contrast to most human cells, express telomerase (Prowse and Greider 1995) and have long telomeres (Kipling and Cooke 1990). Mouse embryonic fibroblasts (MEFs) undergo senescence after a limited number of passages in culture, despite their retaining of long telomeres. Elongation of their life span can be achieved by culturing in serum-free medium supplemented with a number of defined growth factors (Loo et al. 1987) or by culturing under physiological oxygen conditions (Parrinello et al. 2003). Consistent with this, oxidative stress induces cessation of replication in cultured human cells (Packer and Fuehr 1977; Chen et al. 1995; Yuan et al. 1995), while the replicative potential of human melanocytes and epithelial cells depends largely on the composition of the culture medium used, as well as on the use of feeder layers (Ramirez et al. 2001; Bennett and Medrano 2002; Bennett 2003). Senescence of MEFs can be bypassed also by inactivation of *p53* or simultaneous ablation of *RB* family genes (Tanaka et al. 1994; Dannenberg et al. 2000; Sage et al. 2000). Thus, the immortalization 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; for review, see Wright and Shay 2002).

Oncogene-induced senescence (OIS) in vitro

Early studies on mutant HRAS ($HRAS^{V12}$) led to the discovery that, although it can transform most immortal mammalian cell lines and collaborate with immortalizing genes in oncogenically transforming primary cells, it induces cell cycle arrest when it is introduced alone into primary cells (and at least one immortal rat fibroblast cell line) (Land et al. 1983; Franza et al. 1986; Serrano et al. 1997). Serrano et al. (1997) noted the striking phenotypic resemblance of such nonproliferating cells to those in replicative senescence, and this phenomenon has eventually come to be known as OIS. Unlike replicative senescence, OIS cannot be bypassed by expression of hTERT, confirming its independence from telomere attrition (Wei and Sedivy 1999).

One of the hallmarks shared by cells undergoing replicative senescence and OIS is the critical involvement of the p53 and $p16^{INK4A}$–RB pathways, at least in certain settings. In murine cells, functional inactivation of p53 or its direct upstream regulator, $p19^{ARF}$, is sufficient to bypass RAS^{V12}-induced senescence (Kamijo et al. 1997; Serrano et al. 1997). In human cells, $p16^{INK4A}$ seems to play a more prominent role than p53, as some cells depend solely on $p16^{INK4A}$ for OIS (Ben-Porath and Weinberg 2005). Whereas $p19^{ARF}$ is an exquisite sensor that is activated by oncogenic signals and mediates senescence in cultured murine cells, in human cells it does not seem to play a similarly dominant role (Wei et al. 2001; Michaloglou et al. 2005). Nonetheless, specific mutations affecting human ARF (i.e., $p14^{ARF}$) but not $p16^{INK4A}$ have been identified in some human melanoma (Freedberg et al. 2008). Indeed, OIS mechanisms do not seem to be universal across cell types and genetic contexts. This is also exemplified by the signaling routes relaying OIS by RAS^{V12} versus $BRAF^{E600}$: Whereas RAS^{V12}-induced senescence can be bypassed by abrogation of the $p16^{INK4A}$–RB pathway (Serrano et al. 1997), $BRAF^{E600}$-triggered senescence cannot be bypassed by functional inactivation of $p16^{INK4A}$, be it alone (Michaloglou

et al. 2005) or in combination with silencing of $p14^{ARF}$ (Haferkamp et al. 2009).

Recent evidence suggests the relevance of OIS also in the context of induced pluripotency in vitro. At least two oncoproteins, c-MYC and KLF4, are required for the generation of induced pluripotent stem (iPS) cells. As the INK4A/ARF proteins and p53 limit the efficiency of iPS cell formation, it has been suggested that cellular senescence counteracts the induced conversion of primary cells into pluripotent stem cells (Banito et al. 2009; Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009; Marión et al. 2009; Utikal et al. 2009). Alternatively, increased proliferation rates associated with p53 loss may result in accelerated kinetics of iPS formation (Hanna et al. 2009). To the extent that this can be extrapolated to an in vivo setting, one could imagine that cancer stem cells arise from a similar reprogramming process (Krizhanovsky and Lowe 2009). Thus, cellular senescence might suppress tumor formation not only by inducing a persistent cell cycle arrest, but also by limiting the generation of cancer stem cells.

Tumor suppressor loss-induced senescence in vitro

Similar to oncogene mutation or overexpression, loss of a tumor suppressor can also trigger senescence in mouse and human cells. This was first illustrated for PTEN and NF1. As elaborated further below, for PTEN it was shown that fully deficient MEFs undergo senescence, which is accompanied by induction of p53. Concomitant loss of p53 allows cells to override the cytostatic effects of *Pten* deletions (Chen et al. 2005). Similarly, depletion of NF1 causes senescence in vitro, which is eventually accompanied by decreases in ERK and AKT activities (Courtois-Cox et al. 2006). An elegant model was proposed in which the increase in RAS activity following NF1 loss is dampened by a negative feedback loop. Of note, although loss of NF1 triggers senescence in human diploid fibroblasts (HDFs), it immortalizes MEFs. Another example within this theme is VHL, loss of which triggers senescence in an RB- and p400-dependent manner (Young et al. 2008).

Biomarkers and mechanisms of cellular senescence

While cellular senescence is induced by a wide variety of conditions, senescent cells display a number of characteristics that allow their identification both in vitro and in vivo. Some of these biomarkers reflect the activation of mechanisms (such as the induction of tumor suppressor networks) that contribute to the senescence program. For others that accompany the execution of the senescence program (like the increase in senescence-associated β-galactosidase [SA-β-GAL] activity), it is as yet unclear to what extent they contribute mechanistically.

Cell cycle arrest

Long-term exit from the cell cycle is the central and, in our view, only indispensable marker for the identification of all types of cellular senescence both in vitro and in vivo. However, it is important to realize that, although senescent cells by definition withdraw from the cell cycle, their inability to replicate is far from unique. Terminal differentiation, for example, similarly results in stable replicative arrest. An essential difference is the fact that differentiation is typically triggered by physiological cues and does not commonly involve the activation of tumor suppressor networks. Thus, although cell cycle exit constitutes an indispensable biomarker for senescence, it can by no means be used in isolation to identify senescent cells.

Is cellular senescence strictly irreversible? Early in vitro studies—for example, on $p16^{INK4A}$ and RAF1—suggested that it essentially is (Kato et al. 1998; Zhu et al. 1998), but subsequent studies have argued that, in fact, escape scenarios exist. In spite of the observation that stable RB-dependent heterochromatin structures and several other factors contribute to lock cells in their senescent state (see below), there are multiple ways to reverse the arrest, allowing cells to re-enter the cell cycle. For example, inactivation of the p53 pathway permits senescence reversal, while inactivation of some interleukins also abrogates the arrest (Beauséjour et al. 2003; Dirac and Bernards 2003; Coppé et al. 2008; Kuilman et al. 2008; see below). In some settings, the reversibility depends on the expression of $p16^{INK4A}$ prior to entering senescence (Beauséjour et al. 2003). As discussed in more detail below, there is also the possibility that, in addition to the levels of certain tumor suppressor proteins, the strength of the oncogenic signal contributes to the reversibility of senescence.

It should be noted here that strong selection exists for the small subset of cells that are endowed with the ability to exit the senescent state. While this may be a rare event in cultured cells, it is highly relevant in in vivo settings, where clonal expansion of such reverted cells likely represents a rate-limiting event on the path toward tumor progression. Indeed, activation of senescence-abrogating pathways can be seen in melanomas clonally emerging from melanocytic nevi, which carry the same driver mutation (LCW Vredeveld and DS Peeper, unpubl.). Notably, wound healing can create a microenvironment that induces regrowth in nevi (Schoenfeld and Pinkus 1958; Park et al. 1987). It will be of interest to identify the signaling pathways that apparently relax the cytostatic constraints. A variation on this theme is preventing senescence, rather than reversing it. One could imagine that this can be achieved by either a failure to engage senescence pathways or a lack of fundamental senescence mediators. An example for the first scenario would be the inability of mutant KRAS to induce a senescence response in the small intestine of mice that display evidence of senescence in the colon. In the same study, the rapid development of colon adenocarcinomas in the absence of *INK4a/Arf* is an example of the second scenario (Bennecke et al. 2010; see below).

Morphological transformation

Cell senescence is generally accompanied by morphological changes, which may be quite striking. Depending

on the senescence trigger, cells can become large, flat, and multinucleated, or rather refractile. A flat cell phenotype is commonly seen in cells undergoing H-RAS^{V12}-induced senescence (Serrano et al. 1997; Denoyelle et al. 2006), stress-induced senescence (Parrinello et al. 2003), or DNA damage-induced senescence (Chen and 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). Melanocytes undergoing RAS^{V12}-induced senescence display extensive vacuolization as a result of endoplasmic reticulum stress caused by the unfolded protein response (Denoyelle et al. 2006).

Activation of tumor suppressor networks

As mentioned above, the p53 and p16^{INK4A}–RB signal transduction cascades commonly mediate the activation of the senescence program (Lowe et al. 2004). Consequently, components thereof have been used as biomarkers to identify senescent cells. 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). The p53 protein can also be phosphorylated on Ser 15 by ATM as part of the senescence response (Calabrese et al. 2009). p53 serves as a node, mediating prosenescence signals emerging from unscheduled oncogene activation, telomere dysfunction, DNA damage, and reactive oxygen species (ROS).

Among the pocket proteins, RB has a unique role in mediating senescence in human cells (Chicas et al. 2010). One of its primary activators, p16^{INK4A}, is commonly induced in senescent cells in many contexts in vitro (Serrano et al. 1997; Campisi 2005). Other proteins in the p16^{INK4A}–RB and p53 pathways, notably p21^{CIP1} and p15^{INK4B}, also often accumulate in senescent cells, and have been used as markers reflecting the activation of these pathways in senescence (Alcorta et al. 1996; Hara et al. 1996; Reznikoff et al. 1996; Serrano et al. 1997; Erickson et al. 1998; Lin et al. 1998; Robles and Adami 1998; Zhu et al. 1998; Hitomi et al. 2007). Induction of p16^{INK4A} is commonly seen in senescent mouse and human lesions in vivo also (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} induction can be evident, too; for example, in senescent mouse prostate tumors (Chen et al. 2005). Disruption of the p16^{INK4A}–RB pathway collaborates with ectopic hTERT expression in the immortalization of both primary human epithelial cells (Kiyono et al. 1998) and melanocytes (Gray-Schopfer et al. 2006). At least in some in vitro settings, this can be bypassed by altering the cell culture conditions (Ramirez et al. 2001), consistent with the view discussed above that inadequate circumstances can launch the senescence program (Sherr and DePinho 2000).

Genetic disruption of the gene encoding p16^{INK4A} predisposes mice and humans to tumorigenesis, including melanoma (Gruis et al. 1995; Krimpenfort et al. 2001; Sharpless et al. 2001). This notwithstanding, at least in melanomagenesis, genetic and immunohistochemical evidence in mice and humans suggests that p16^{INK4A} plays a redundant role in senescence (Gruis et al. 1995; Michaloglou et al. 2005; Dhomen et al. 2009). This may be different in other settings, like human colon adenoma, in which a strict correlation is observed between p16^{INK4A} expression and cell cycle arrest (Dai et al. 2000; Kuilman et al. 2008). In murine cells, its neighbor, p19^{ARF}, appears to take on this dominant role as a common senescence marker. Concordantly, *Arf* acts as a potent tumor suppressor gene in mice (Kamijo et al. 1997) and is induced in response to oncogenic signals in vivo (Zindy et al. 2003).

Induction of SA-β-GAL activity

SA-β-GAL is a commonly used senescence biomarker (Dimri et al. 1995; Debacq-Chainiaux et al. 2009). Its increased activity in senescent cells derives from lysosomal β-D-galactosidase, which is encoded by the *GLB1* gene. Its detection requires tissues to be snap-frozen to preserve enzymatic activity (Debacq-Chainiaux et al. 2009). Also, nonsenescent cells display β-galactosidase activity in the lysosomes that functions most optimally at pH 4 (Lee et al. 2006). Accordingly, the increase in SA-β-GAL activity in senescent cells is likely due to an expansion of the lysosomal compartment, giving rise to an increase in β-galactosidase activity that can be measured also at suboptimal pH 6 (hence, SA-β-GAL) (Kurz et al. 2000; Yang and Hu 2005; Lee et al. 2006). There is, however, as yet no evidence pointing to an actual involvement of this enzyme in the senescence response (Lee et al. 2006).

Senescence-associated heterochromatic foci (SAHF)

Cellular senescence can be associated with an altered chromatin structure, at least in vitro. While DNA dyes display overall homogenous staining patterns in cycling or quiescent human cells, senescent cells often show strikingly different punctate staining patterns. These DNA SAHF (Narita et al. 2003) are specifically enriched in methylated Lys 9 of histone H3 (a modification catalyzed by the histone methyltransferase Suv39h1), while histone H3-Lys 9 acetylation and Lys 4 methylation (both euchromatin markers) are excluded from SAHF. Whereas polycomb group proteins have been shown to repress the *INK4A/ARF* locus (Jacobs et al. 1999; Gil et al. 2004; Bracken et al. 2007), the histone H3-Lys 27 demethylase JMJD3 contributes to its transcriptional activation (Agger et al. 2009; Barradas et al. 2009), thus regulating senescence. Senescent cells display increased binding of heterochromatin-associated proteins in the promoters of several E2F target genes. SAHF formation is circumvented by interference with p16^{INK4A}–RB pathway signaling, correlating with bypass of senescence. Depending on the experimental system, interference with expression of other genes—including those encoding p53, C/EBPβ, or

interleukin-6 (IL-6) (see below)—also reduces SAHF-positivity, which is associated with abrogation of senescence (Chan et al. 2005; Ye et al. 2007; Zhang et al. 2007; Kuilman et al. 2008).

An essential step in the initiation phase of chromosome condensation appears to be the translocation of HIRA (a histone chaperone) to PML (promyelocytic leukemia nuclear) bodies (Zhang et al. 2005; Adams 2007, 2009). These subnuclear organelles are thought to serve as a staging ground for the formation of HIRA/ASF1A-containing complexes, which are subsequently incorporated into chromatin and play an essential role in instigating SAHF formation. RNAi depletion of either ASF1A or HMGA1 (a SAHF component) leads to a partial bypass of senescence (Zhang et al. 2005; Narita et al. 2006).

Secreted factors in senescence

Cells undergoing senescence—whether in response to telomere malfunction, DNA damage, or oncogenic alterations—exhibit profound changes in their transcriptomes. A major consequence of this is the secretion of many dozens of factors, including cytokines and chemokines (Campisi 2005). The first indication of changes in the secretome of human cells accompanying senescence was reported for fibroblasts undergoing replicative senescence. Microarray analysis revealed a strong inflammatory response, as seen in wound healing (Shelton et al. 1999). Subsequent work from various laboratories has revealed that cells undergoing either replicative or premature senescence display profound changes in their secretome, termed the senescence-associated secretory phenotype (SASP) (Coppé et al. 2008; Rodier et al. 2009). A recent study indicates that, for the induction of several of these SASP factors, persistent DNA damage is required. Because DNA damage accompanies some but not all senescence settings (see below), the SASP is not strictly coupled to senescence per se (Rodier et al. 2009).

Proinflammatory cytokines—when secreted by senescent cells—trigger a variety of cellular responses. In certain settings, pro-oncogenic effects result: The proliferative rate, migration, and invasion of premalignant cells are enhanced when they are cocultured with, or grown in medium conditioned by, senescent fibroblasts (Krtolica et al. 2001; Dilley et al. 2003; Parrinello et al. 2005; Yang et al. 2006). For example, a study on ovarian tumorigenesis has revealed that preneoplastic epithelial ovarian cells expressing oncogenic RAS induce expression of GRO1 (a cytokine recognized by CXCR2) (Yang et al. 2006). Once secreted, GRO1 has the potential to induce senescence in ovarian fibroblasts in vitro. In turn, these fibroblasts promote tumor growth when coinjected with preneoplastic epithelial cells into mice. Interestingly, fibroblasts surrounding human ovarian tumors express several senescence markers, suggesting that this two-way communication exists in vivo as well. Similar findings have been reported for several additional weakly oncogenic epithelial cell lines that show increased tumorigenic potential upon exposure to senescent fibroblasts (Krtolica et al. 2001).

Intriguingly, in addition to influencing their microenvironment, some inflammatory cytokines have an indispensable role in the establishment and maintenance of the senescence arrest. For example, signaling through the IL-6 and IL-8 (CXCR2) receptors is essential, in a cell-autonomous fashion, for cells to enter senescence in response to oncogenic BRAF or replicative exhaustion, respectively (Acosta et al. 2008; Kuilman et al. 2008). This is relayed by the C/EBPβ and NF-κB transcription factors and is associated with the activation of an inflammatory transcriptome. Correspondingly, elevated levels of cytokines are found in senescent human neoplasms. Likewise, the ECM regulator PAI-1 has been shown to mediate senescence (Kortlever et al. 2006), although clinical data seem to point to a more complex picture (Kuilman and Peeper 2009).

It thus appears that, while senescent cells can promote cancer by stimulating the proliferation of incipient tumor cells that reside in their microenvironment, senescence also acts as a cell-autonomous tumor suppression mechanism limiting the expansion of early malignant cells. Therefore, the name "senescence-messaging secretome" (SMS) has been proposed in order to emphasize that, next to their requirement for the senescence response (whether or not involving a DDR), many of these factors are also endowed with communicative functions (Kuilman and Peeper 2009). The SMS creates a complex signaling network in which secreted factors affect not only the cells producing them (autocrine effects), but also the microenvironment and hence neighboring cells (paracrine effects). In some cases, SMS factors can stimulate the innate immune system, triggering tumor clearance (Xue et al. 2007; see below). The ultimate effect on target cells may depend largely on their genetic constitution. A classic example is the pro- or anti-proliferative effect of TGFβ on tumor cells as a function of the stage of tumorigenesis (Massagué 2008). Also, the effect of IL-6 on melanomagenesis depends on tumor progression (Lu et al. 1992, 1996). Although in these examples it is as yet unclear to what extent this differential response is linked to the ability of cells to undergo senescence, it is conceivable that the effect of SMS factors on their target cells is influenced by the oncogenic lesions that are carried by the latter.

ROS

As already alluded to, the replicative potential of primary cells is influenced by the oxygen levels in which they are cultured. In fact, lowering the oxygen tension can prevent OIS in HDF (Lee et al. 1999) and tissue culture stress-induced senescence in MEFs (Parrinello et al. 2003). It is widely assumed that not oxygen itself, but rather ROS produced by mitochondria are involved in senescence (Lu and Finkel 2008; Moiseeva et al. 2009). While ROS levels increase in both replicative senescence and OIS (Furumoto et al. 1998; Lee et al. 1999), treatment of HDF with a sublethal concentration of H_2O_2 induces senescence (Chen and Ames 1994; Chen et al. 1998). ROS contribute also to induction of replicative senescence, as evidenced

by its delayed or premature onset upon treatment with antioxidants or inhibitors of cellular oxidant scavengers, respectively (Chen et al. 1995; Yuan et al. 1995). A recent systems biology study has suggested that ROS mediates senescence through induction of DNA damage foci, with a contribution of $p21^{CIP1}$ (Passos et al. 2010).

How do ROS relate to p53 and RB? A study on the maintenance of a senescence-like arrest upon temporary expression of SV40 LT revealed that ROS and PKCδ function in a positive feedback loop to maintain this proliferative halt (Takahashi et al. 2006). Furthermore, ROS have been suggested to impinge either directly or indirectly on the p53 and $p16^{INK4A}$–RB signal transduction cascades (Itahana et al. 2003; Iwasa et al. 2003). For instance, the MINK–p38–PRAK pathway is activated in senescence and controls the activation of $p16^{INK4A}$ and p53, as well as expression of $p21^{CIP1}$, in a p53-independent manner (Wang et al. 2002; Bulavin et al. 2003; Iwasa et al. 2003; Nicke et al. 2005; Sun et al. 2007). These results not withstanding, although ROS undoubtedly plays an important role in senescence, the nature and mechanism of this contribution remains largely unclear (Lu and Finkel 2008). Major questions include how the increased levels of ROS are generated, and what the cellular targets for ROS in senescence are.

DNA damage-induced senescence

The involvement of DNA damage in the induction of replicative senescence by telomere attrition has been established (D'Adda di Fagagna 2008). Also DNA-damaging agents such as ionizing radiation can induce cell cycle arrest with characteristics of senescence in HDFs (Di Leonardo et al. 1994; Robles and Adami 1998). Furthermore, DNA damage-inducing drugs can cause senescence of tumor cells in vitro and in vivo, a phenomenon termed drug-induced senescence, which may be 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).

Activation of a DDR has also been shown to contribute to OIS in several settings in vitro; among others, in the context of oncogene-induced DNA hyperreplication (D'Adda di Fagagna 2008). The DDR and p53 often function in a common signal transduction cascade in which interference with specific DDR components can substitute for the loss of p53 function (Bartkova et al. 2006; Di Micco et al. 2006; Moiseeva et al. 2006; Mallette et al. 2007; Kim et al. 2009; Santoriello et al. 2009). For example, a shRNA targeting ATM can serve as a proxy for HPV E6 (which inactivates p53), contributing to bypass of senescence by STAT5 (Mallette et al. 2007).

However, DDR activation is not a universal feature of OIS. For example, in mice, in the context of several types of senescent adenomas, no DDR is observed, nor is there a requirement for ATM in (oncogene-induced) senescence (Efeyan et al. 2009). Furthermore, senescence triggered by RAF1 or $BRAF^{E600}$ and, in some cases, RAS^{V12} does not require p53 (Olsen et al. 2002; Nicke et al. 2005; Kuilman et al. 2008) and fails to mount an appreciable DDR (C Lenain and DS Peeper, unpubl.). In line with this, senescence induced upon genetic loss of *Skp2* in the context of *Pten* heterozygosity is not associated with the emergence of DDR markers and does not depend on p53 signaling (Lin et al. 2010). Shedding some light on a possible link between the DDR and senescence, an elegant study showed that, in the context of *Rb* loss-driven murine adenomas, the DDR preceded cellular senescence (Shamma et al. 2009; see also below). While early and late adenomas similarly expressed DDR markers, senescence was apparent only in late (arrested) lesions, suggesting that, at least in this experimental system, it is primarily senescence and not DNA damage signaling that acts cytostatically (Peeper 2009). It thus seems that DDR activation is involved in certain, but not all, OIS settings.

Autophagy

Increasing evidence points to a link between cellular senescence and autophagy. This catabolic process is mediated by the formation of double-membraned vesicles (autophagosomes) engulfing cytoplasmic content that fuse with lysosomes. Ultimately, this leads to the breakdown of the vesicles and their constituents (Levine and Kroemer 2008). Early studies have demonstrated that chaperone-mediated autophagy is decreased in senescent human cells (Cuervo and Dice 2000). Treatment of human umbilical vein endothelial cells (HUVECs) with glycated collagen I (GC) results in an increase of SA-β-GAL activity (Patschan et al. 2008). This seems to be correlated with a transient induction of the formation of autophagosomes, as evidenced by the up-regulation of the LC3-II protein. The fusion of autophagosomes with lysosomes, as measured by colocalization of LC3 and LAMP1 (a lysosomal marker), correlates with expression of LC3-II. Thus, both autophagosome formation and its fusion with lysosomes are eventually decreased compared with nonsenescent cells. Inhibition of autophagosome formation by treatment with the pharmacological inhibitor 3-methyladenine decreases the number of SA-β-GAL-positive cells upon treatment with GC (Patschan et al. 2008). This might indicate a decrease in the amount of senescent cells, although one cannot exclude that it reflects a drop in the number of lysosomes due to a decreased demand for lysosome–autophagosome fusion. However, senescence and autophagy have recently been connected more tightly. It was shown that autophagic activity increases in OIS, as monitored by the activation of LC3-II, through a delayed activation of the mTORC1 pathway. Furthermore, autophagy contributes to cell cycle arrest and production of senescence-associated interleukins (Young et al. 2009).

Identifying senescent cells

As described above, the execution of senescence programs is associated with the appearance of several markers, which are commonly used in order to identify senescent cells in vitro and in vivo. They include the loss of proliferation, morphological changes, increased SA-β-GAL activity, the production of SAHF, and a rise in

the levels of DDR markers as well as the cell cycle inhibitors $p16^{INK4A}$, $p15^{INK4B}$, $p21^{CIP1}$, and p53. DEC1, DCR2 (Collado et al. 2005), and PAI1 (Goldstein et al. 1994) have also been used as senescence biomarkers (Collado and Serrano 2010). Furthermore, a number of inflammation-linked factors have been associated with senescence. However, it should be noted that different senescent lesions may be characterized by different biomarkers.

It should be emphasized that, in isolation, none of these markers can indisputably identify senescent cells, whether in vitro or in vivo. Indeed, several of the markers used in vitro await in vivo confirmation. For example, due to the lack of appropriate antibodies, it has remained unclear whether the senescence-associated cell cycle protein $p15^{INK4B}$ can be used to specifically identify senescent human cells in vivo. Moreover, although recently identified markers encoded by the inflammatory transcriptome—particularly IL-6, IL-8, and CXCR2 (Acosta et al. 2008; Coppé et al. 2008; Kuilman et al. 2008)—have been demonstrated to identify senescent cells in some early murine and human tumors, it remains to be determined to what extent they can be used across different tissues and genetic contexts.

In spite of its popularity, SA-β-GAL should not be regarded as a unique identifier for senescent cells. In addition to senescent cells, contact-inhibited or serum-starved cells in tissue culture, as well as proliferating dysplastic epithelium in the gastrointestinal tract, can be positive for SA-β-GAL (Severino et al. 2000; Going et al. 2002; Yang and Hu 2005). Furthermore, one would expect that increases in the number or activity of lysosomes unrelated to senescence would also result in elevated lysosomal β-galactosidase protein levels, and thus in increased SA-β-GAL activity. Thus, employing SA-β-GAL in the absence of additional senescence markers may inappropriately classify cells as being senescent. For these reasons, SA-β-GAL can serve as senescence marker only in conjunction with additional markers.

A major challenge to the field, therefore, is to increase the number as well as the reliability of markers to identify senescent cells, especially in vivo. Ideally, one needs to uncover biomarkers that are causally involved in this unique type of proliferative arrest. For the time being, in the absence of unambiguous single markers, we would recommend that, for the identification of senescent cells, one uses, in addition to a proliferation marker such as the Ki-67 antigen (by immunostaining) or the thymidine analog bromodeoxyuridine (BrdU) (by in vivo labeling), at least two additional senescence markers, the choice of which may vary for different settings. For example, $p16^{INK4A}$ often correlates with senescence both in vitro and in vivo, and as such it is a common (but not universal) senescence marker. Also, tissue sections for immunostaining should be analyzed with multiple senescence markers. Ideally, this is done on consecutive sections or, even better, on single sections by double staining, if feasible. Only careful and multiparameter analyses will allow the appropriate detection of presumed senescent lesions.

Cellular senescence as a pathophysiological phenomenon

Ever since the discovery of cellular senescence in vitro, researchers have wondered whether there are in vivo correlates of this program. Hayflick (1965) described possible implications of cellular senescence for carcinogenesis and aging: "Thus the phenomenon of the alteration of a cell strain to a cell line is important because, in its simplest terms, it can be regarded as oncogenesis in vitro." Indeed, the conversion from a mortal to an immortal cell reflects a rate-limiting step in tumorigenesis. The identification of OIS in cultured cells opened a debate on the possible role of this type of proliferation arrest in vivo. Many were quick to realize that, in principle, OIS could serve as a powerful line of defense against tumorigenesis. It would allow an incipient neoplastic cell harboring an oncogenic lesion to withdraw itself from the proliferative pool, so that tumor formation is prevented. This is consistent with the fact that $p16^{INK4A}$ and p53, two factors often associated with senescence, are among the most commonly mutated proteins in cancer (The Cancer Genome Atlas Gene Ranker GBM 6000, http://cbio.mskcc.org/tcga-generanker/index.jsp).

Early in vitro experiments on senescence commonly used supraphysiological expression levels of RAS^{V12}. Subsequently, several groups reported that RAS^{V12} and mutant RAF proteins act mitogenically or, conversely, drive cells into senescence as a function of increasing expression levels (Woods et al. 1997; Guerra et al. 2003; Deng et al. 2004; Tuveson et al. 2004; Sarkisian et al. 2007; Chen et al. 2009). Some of these observations, and the fact that commonly used culturing conditions differ markedly from those in intact tissue and presumably act as a further stressor, initially caused some skepticism about a physiological role for senescence. In the last 5 years, however, a plethora of mouse models as well as human lesions have provided convincing and independent arguments in favor of OIS serving as a bona fide and effective tumor suppressor mechanism.

Although some in vitro senescence models may have been artificial, they did not at all rule out a possible role for senescence in vivo. For instance, increased expression of activated RAS genes is a common feature of human tumors (Elenbaas et al. 2001; Johnson et al. 2005; Okamura et al. 2006; Chen et al. 2009) that can be associated with the conversion from benign to malignant tumors (Quintanilla et al. 1986; Finney and Bishop 1993; Rodriguez-Puebla et al. 1999). Amplification of $HRAS^{V12}$ is common in human Spitz nevi, which rarely progress to malignancy (Maldonado et al. 2004), probably representing the best available evidence of high levels of a mutant RAS gene in a growth-arrested benign neoplastic lesion.

As already alluded to above, the propensity to launch a senescence program in response to an oncogenic alteration differs among tissue and cell types. In murine pulmonary tumors, for example, $p19^{ARF}$ is induced to relatively low levels upon expression of $KRAS^{G12D}$, while it is highly elevated in sarcomas (Young and Jacks 2010). Along these lines, KRAS-driven senescence is observed in

several mouse tissues, including lung adenomas, pancreatic intraductal neoplasia, and colonic epithelium, but not small intestinal epithelial cells (Collado et al. 2005; Bennecke et al. 2010). Similarly, reactivation of p53 in established murine tumors causes senescence in sarcomas but apoptosis in lymphomas (Ventura et al. 2007; see below). Thus, while senescence can be considered as a pathophysiological phenomenon, several physiological factors influence its onset.

Replicative senescence in vivo

A number of studies have suggested that senescent cells accumulate in aging individuals. An increase in SA-β-GAL activity can be detected in the skin of elderly people (Dimri et al. 1995). In addition, a small but significant decrease in telomere length has been detected in aged tissue, although this is limited to certain cell types (Cristofalo et al. 2004). These studies, which relied on single senescence markers, were later supported by the finding that several DNA damage and heterochromatinization markers—including 53BP1, γ-H2AX, phospho-Ser 1981-ATM, HP-1β, and HIRA—are increased in dermal fibroblasts from aging baboons compared with young ones. DNA damage markers were shown to colocalize with telomeres, and this correlated with increased $p16^{INK4A}$ expression (Herbig et al. 2006), suggesting that dysfunctional telomeres can create DNA damage signals leading to the onset of senescence also in vivo. In line with the idea that replicative senescence results from telomeric attrition upon prolonged proliferation, senescence is detected mainly in tissues experiencing substantial cellular turnover (Jeyapalan et al. 2007).

Telomere dysfunction has been suggested to be connected to age-related pathology, and this warrants further study. For example, atherosclerosis is accompanied by an induction of SA-β-GAL activity (Minamino et al. 2002) and telomere shortening (Foreman and Tang 2003). Senescent cells have also been observed in osteoarthritis (Price et al. 2002). Since these studies used single senescence markers, they await confirmation. This notwithstanding, the observations are in agreement with the proposed deleterious effects of senescent cells on their microenvironment, possibly contributing to age-related disease (Campisi 2005).

Conversely, beneficial aspects of replicative senescence have also been uncovered, including some that are unrelated to cancer. In a mouse model of liver fibrosis, senescent cells were detected, as evidenced by positivity for SA-β-GAL, $p16^{INK4A}$, p53, $p21^{CIP1}$, and HMGA1. These senescent cells derive from activated hepatocellular stem cells and limit the progression of fibrosis (Krizhanovsky et al. 2008). In addition, two reports (Cosme-Blanco et al. 2007; Feldser and Greider 2007) have provided evidence for the proposal by Hayflick (1965) that replicative senescence limits tumorigenesis. Both studies report that mice lacking the RNA component of telomerase (mTR) display decreased tumor formation in the context of either one or two copies of mutant $p53^{R172P}$ replacing endogenous p53 (Cosme-Blanco et al. 2007) or Eμ-MYC/BCL2-driven lymphomagenesis (Feldser and Greider 2007). The lack of telomerase activity had been shown previously to result in an induction of apoptosis, but, in these two models, this response was abrogated owing to expression of the p53 mutant or BCL2, respectively. These studies show that cells display extensive induction of senescence in intestine, kidney, and spleen (Cosme-Blanco et al. 2007), or in microlymphomas (Feldser and Greider 2007). This has provided the first direct evidence of replicative senescence preventing tumorigenesis, at least in the context of an apoptosis block.

Consequently, a colorful picture is emerging for the role of telomeres in senescence and cancer biology. Telomere shortening or dysfunction initially triggers a robust stress response that, through the induction of cell cycle inhibitory proteins, limits the replicative potential of cells. As such, this aspect of telomere biology is likely to act as a potent anti-cancer mechanism. This is consistent with a significant drop in the tumor take of telomerase-deficient ($mTR^{-/-}$) mice in the context of *INK4a/Arf* loss (Greenberg et al. 1999). In the absence of p53, however, telomere dysfunction instead promotes tumorigenesis (Chin et al. 1999). This could occur through the inability of cells to engage a full DDR, and through the perturbation of the apoptosis and senescence responses (Artandi and DePinho 2010).

OIS in vivo

The past 5 years have witnessed a boost in the number of studies demonstrating senescence in vivo in both murine and human lesions. Senescence markers have been demonstrated in several contexts in which oncogenes or tumor suppressor genes were perturbed (Fig. 1). Four studies that were published in concert provided the first evidence for OIS in vivo. Schmitt and colleagues (Braig et al. 2005) reported on the tumor-suppressive role of the chromatin remodeling enzyme *Suv39h1* in Eμ-NRAS-driven lymphomagenesis, which correlates with bypass of OIS in vitro in $Suv39h1^{-/-}$ splenocytes. Serrano and coworkers (Collado et al. 2005) showed that conditional expression of $KRAS^{V12}$ from its endogenous locus in mice results in the emergence of lung adenomas, as well as premalignant pancreatic intraductal neoplasia, most of which fail to progress to malignancy. The adenomatous stage is specifically characterized by a low proliferative index and the appearance of several senescence markers, including SA-β-GAL, $p15^{INK4B}$, $p16^{INK4A}$, and several new markers. Peeper and coworkers (Michaloglou et al. 2005) showed that congenital human melanocytic nevi, which frequently harbor activating BRAF or NRAS mutations, display several characteristics of senescence (see below). The first demonstration of tumor suppressor (PTEN) loss-induced senescence in vivo (Chen et al. 2005) is discussed below.

As a sequel to this first series of observations, several other groups have further supported the view that OIS inhibits tumor formation. For example, both established and newly identified chemokine and cytokine markers suggest the presence of senescent cells in human colon adenoma and prostatic intraepithelial neoplasia (PIN)

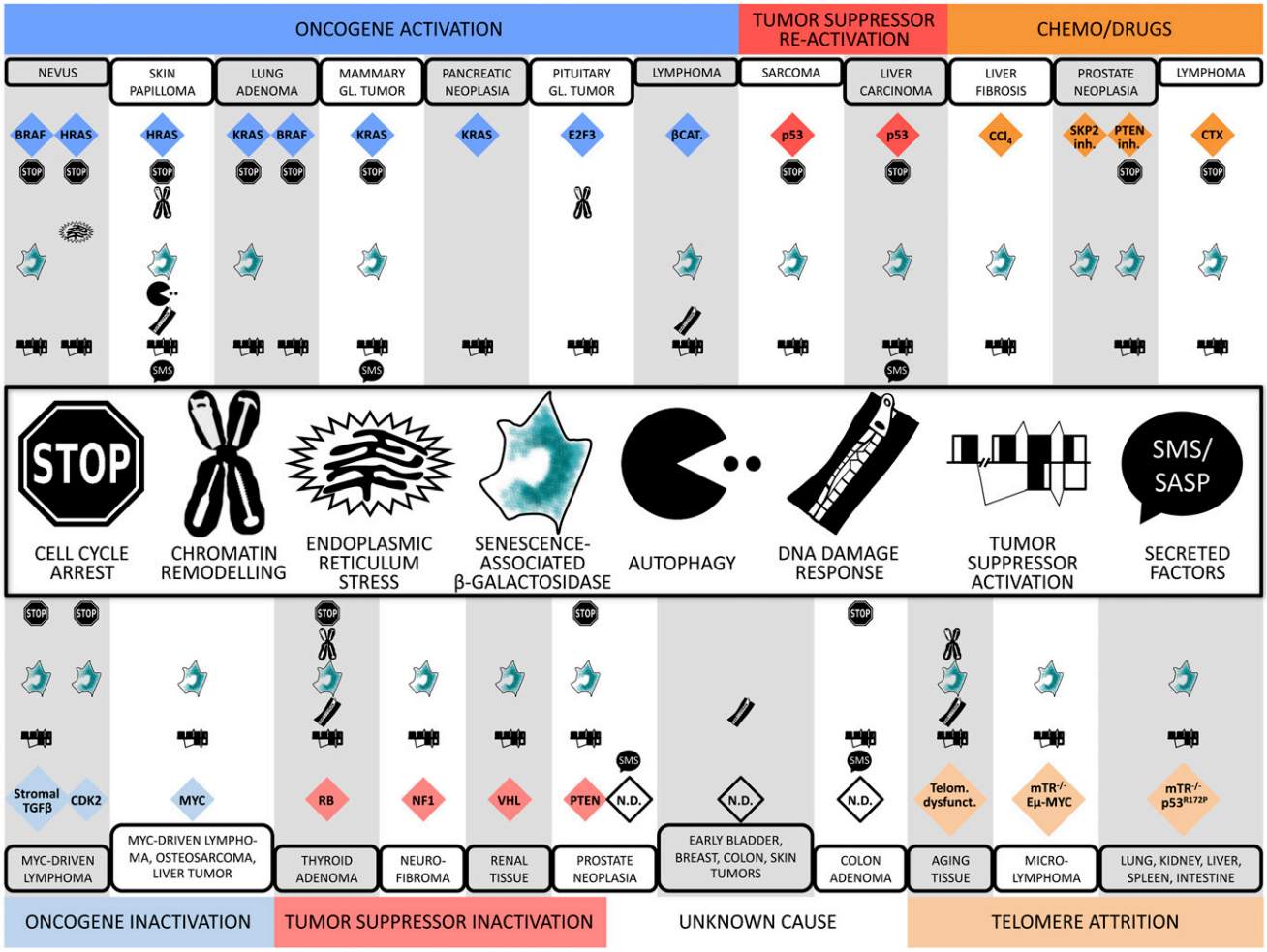

Figure 1. The essence of senescence: biomarkers in vivo. Several markers of cellular senescence have been identified in early neoplastic lesions in both mice and humans. Representative examples are shown in the various columns, with the type of genetic lesions or other types of stress (stress categories, color-coded) that instigate senescence at the very *top* and very *bottom*, and from thereon inward: the tissue types, specific stress signals, biomarkers, and symbols legends, respectively. For instance, in BRAF mutant nevi, the stress category is oncogene activation (blue), the tissue type is nevus, the specific stress signal is BRAF, and the biomarkers reported are cell cycle arrest, increase in SA-β-GAL activity, and tumor suppressor activation. (CTX) Cyclophosphamide; (βCAT) β-catenin; (GL) gland; (N.D.) not determined.

lesions (Acosta et al. 2008; Kuilman et al. 2008). In addition, murine papillomas driven by $HRAS^{V12}$ expression or induced upon DMBA/TPA treatment express several senescence markers (Collado et al. 2005; Acosta et al. 2008; Chen et al. 2009; Yamakoshi et al. 2009). Recent evidence indicates that Myc, known mainly for its proapoptotic function, drives a subset of murine lymphomas into senescence through stromal secretion of TGFβ (Reimann et al. 2010), providing support for the premise that senescence is not solely implemented in a cell-autonomous manner. In line with the idea that OIS represents a barrier for cancer development, a senescence index of tumor cells has been associated with better treatment outcome in high-grade colorectal cancer (Haugstetter et al. 2010). Moreover, expression of several senescence-associated genes is reduced when tumors progress beyond the senescent stage (Chen et al. 2005; Collado et al. 2005; Lazzerini Denchi et al. 2005; Acosta et al. 2008). However, particularly for cell cycle-regulating genes like $p16^{INK4A}$, abundant expression may, in fact, be maintained during malignancy, which is explained by the occurrence of other (epi)genetic aberrations (including events occurring in parallel and downstream) that neutralize their functions. As such cases are expected to coincide with immunopositivity for proliferation markers, this reinstates the need for careful, multiparameter analysis of senescence in vivo.

OIS in models of nevus and melanoma formation

Melanocytic nevi, benign tumors that have a low propensity to progress toward melanoma, are a well-studied system for OIS in vivo in humans, mice, and fish. In 2002, $BRAF^{E600}$ was identified as a frequent mutation in human

cancer, predominantly melanoma (Davies et al. 2002). At least as remarkable was the finding that the same mutation is present in the large majority of nevi (Pollock et al. 2003). In spite of the presence of an oncogenic BRAF (or, in some cases, NRAS) allele, an important and common feature of nevi is their exceedingly low proliferative activity. This characteristic is typically maintained for decades until the lesion gradually disappears. Nevi express elevated levels of $p16^{INK4A}$ and display increased SA-β-GAL activity (Michaloglou et al. 2005). Arguing against a role for replicative senescence, it was found that telomere length in nevi is indistinguishable from that in normal skin (and longer than in melanoma cells). This strongly suggests that nevi undergo OIS in vivo. These findings have been confirmed by Bennett and coworkers (Gray-Schopfer et al. 2006). Secreted IGFBP7 has been reported to play a central role in the initiation and maintenance of the senescent state of nevus cells (Wajapeyee et al. 2008), but these findings were challenged recently (Schrama et al. 2010; Scurr et al. 2010; Wajapeyee et al. 2010).

The studies on senescent nevi are consistent with earlier observations that a mutant *BRAF* transgene in the melanocytic compartment induces the formation of nevus-like lesions in zebrafish (Patton et al. 2005). Although senescence markers were not assessed in this study, Patton et al. (2005) describe that, in combination with loss of p53, the fish develop invasive melanomas. In a similar transgenic mouse model, expression of $BRAF^{E600}$ in the melanocytic lineage drives the formation of nevi with several signs of senescence (Goel et al. 2009). These animals develop melanoma with a long latency, depending on the expression of the transgene. Melanoma formation was strongly accelerated in *Cdkn2a* or *p53*-deficient backgrounds.

Recently, two advanced mouse models have been created that more closely resemble spontaneous mutation of the oncogene by conditionally expressing $BRAF^{E600}$ from its endogenous promoter (Dankort et al. 2009; Dhomen et al. 2009). In these models, specific expression of mutant BRAF in the melanocytic compartment results in the formation of nevus-like benign lesions, which, depending on the model used, remain stable for several months to more than a year. Importantly, these melanocytic cell groups express several senescence markers, corroborating earlier observations on RAS^{V12} that physiological expression levels of an activated oncogene can also give rise to senescence in neoplasms. In line with the idea that $p16^{INK4A}$ is not always strictly required for OIS in vivo (Michaloglou et al. 2008), nevus formation was unimpaired in a $p16^{INK4A}$-deficient background, although increased melanoma penetrance and reduced latency were observed (Dhomen et al. 2009). In contrast, in combination with a mutant *Braf* knock-in allele, loss of *Pten* produced aggressive tumors much resembling human metastatic melanoma (Dankort et al. 2009). These elegant models have convincingly shown that mutation of BRAF can drive nevus formation, and that a specific secondary lesion (e.g., *Pten* loss) collaborates to drive melanomagenesis. Whether this classical genetic oncogenic cooperation denotes an essential role of PTEN in $BRAF^{E600}$-induced senescence remains to be established.

Tumor suppressor loss-induced senescence in vivo

Pandolfi and colleagues (Chen et al. 2005) were the first to show that senescence in vivo can also be triggered by the loss of a tumor suppressor gene. They found that conditional *Pten* deletion in murine prostate cells results in the formation of high-grade PIN, which display characteristics of senescence. In conjunction with *p53* loss, these lesions progress to malignancy. Senescence induced by *Pten* loss can be enhanced by inactivation of the E3 ubiquitin ligase *Skp2* (Lin et al. 2010). Similarly, Cichowski and coworkers (Courtois-Cox et al. 2006) showed that, in benign dermal neurofibromas carrying NF1 mutations, several senescence markers are up-regulated, while phospho-ERK and phospho-AKT are present at low levels. Finally, kidneys from *Vhl* knockout mice display an increase in SA-β-GAL activity as well as induced immunostaining for $p27^{Kip1}$ and the senescence marker DcR2 (Young et al. 2008).

The Takahashi laboratory (Shamma et al. 2009) used a model of senescence in vivo in which genetic loss of one allele of *Rb* drives thyroid adenoma formation. These lesions display enhanced isoprenylation and activation of N-Ras and express several senescence markers, as discussed above. Homozygous loss of *NRas* in the context of *Rb* heterozygosity allowed for progression to carcinomas that was accompanied by the loss of senescence markers. Together, these examples provide the first pieces of evidence that, similar to activated oncogenes, loss of expression of tumor suppressors can also cause incipient cancer lesions to activate features of senescence, serving to limit tumorigenesis in vivo.

Restoration of cellular senescence in vivo

In light of the steadily increasing evidence that senescence protects humans and other organisms against cancer, ideas are being launched to restore this process in tumor cells in which it has become deficient. Induction of apoptosis in tumor cells, whether by chemotherapeutics or signaling molecules, is successfully used therapeutically. Would reactivation of senescence be similarly rewarding clinically? In particular, for those tumors that are highly resistant to drug- or radiotherapy-induced cell death, the concept of activating senescence in (early) tumors may sound appealing. But is it feasible? To explore the viability of such an approach, the Jacks (Ventura et al. 2007) and Lowe (Xue et al. 2007) laboratories have employed conditional mouse models. In the system used by Ventura et al. (2007) a transcription–translation stop site flanked by *loxP* sites within the p53 gene could be excised upon expression of Cre recombinase. Upon activation of p53 in already established lymphomas and sarcomas, dramatic tumor regression was observed. While this was associated with induction of apoptosis in lymphomas, sarcomas failed to undergo apoptosis, but instead displayed several characteristics of senescence. In contrast to tumor cells, restoration of p53 function in normal tissue did not

result in detectable expression of the tumor suppressor. This suggests that restoration of p53-dependent senescence occurs only in the context of stress signals (Ventura et al. 2007). In the model by Xue et al. (2007), liver progenitor cells were infected ex vivo with retrovirus encoding $HRAS^{V12}$ and an inducible shRNA targeting p53. Upon transplantation into the spleen of recipient mice, the hepatoblasts seeded in the liver and produced invasive hepatocarcinomas. Reactivation of p53 caused these tumors to undergo senescence. Remarkably, an innate immune response was subsequently elicited, eventually resulting in the clearance of the tumors (Xue et al. 2007). Thus, these models have established that, at least under conditions in which widespread and acute activation of senescence can be achieved, this can culminate in tumor regression. Conversely, as has been shown for Myc, oncogene inactivation can also set off a senescence program in several tumors (Wu et al. 2007) in a TGFβ-dependent manner (van Riggelen et al. 2010). These studies may therefore serve as proof of principle that reactivation of senescence is a realistic means to intervene with cancer.

In several examples above, a tumor suppressor (p53) that contributed to the tumor initiation and maintenance was restored, resulting in senescence. Since inactivation, rather than restoration, of a tumor suppressor can also induce senescence in vivo (as discussed above for PTEN and NF1), it thus appears that tumor suppressors can differentially influence senescence. These effects can be seen even for a single tumor suppressor. For example, as ectopic expression of PTEN or suppression of PI3K also causes senescence (Courtois-Cox et al. 2006), together these observations suggest that major pathways like PI3K/PTEN require fine-tuning to maintain cell proliferation: Too little or too much activity may act cytostatically. Recently, the window in which reduction in PTEN levels induces senescence (PICS) has been proposed as a means to establish a prosenescence therapy (Alimonti et al. 2010). It was shown that pharmacologic inhibition of PTEN caused increased SA-β-GAL activity along with a reduction in cell proliferation both in vitro and in tumor xenografts. Along these lines, Lin et al. (2010) reported that a Skp2–SCF inhibitor triggers senescence and inhibition of tumor growth of *PTEN*-deficient cancer cells. In further support of this emerging theme, Amati and colleagues (Campaner et al. 2010) observed that oncogenic stress caused by the *Myc* oncoprotein sensitizes *Cdk2*-deficient cells to undergo senescence in vitro and in vivo. Similarly, in advanced non-small-cell lung carcinoma driven by activation of oncogenic *KRas*, tumor progression is halted and senescence is induced upon genetic ablation of *Cdk4* (Puyol et al. 2010). The rising number of examples of enforced senescence in vivo justifies further exploration of this possibility by addressing several more clinically oriented questions.

Concluding remarks

The discovery of cellular senescence in cultured cells came with exciting hypotheses about the potential roles it may have in tumorigenesis and aging. Despite intensive research on this topic, four decades have passed before solid evidence was first presented that senescence occurs not only under artificial conditions in vitro, but also under pathophysiological conditions and in several genetic settings in vivo. Attempts to increase our understanding of this process were boosted by the discovery of OIS, which strengthened the link with cancer research. In spite of this important progress, however, many challenges remain. Probably most importantly, there is an urgent need for better senescence biomarkers. It is clear that, although a plethora of markers has been proposed and in fact are commonly used, no single one can reliably identify senescent cells in vitro or in vivo. Results from genome-wide expression analyses of senescent cells could serve as a convenient starting point for the identification of new and better senescence markers. Integrating these data with systematic RNAi approaches is likely to yield biomarkers with causal relationships to senescence. The contribution to senescence in vitro and in vivo of several cellular processes—including activation of DDR, SAHF formation, and induction of ROS and autophagy—also requires further elucidation. In addition, the question remains of which factors determine whether tissues primarily undergo senescence or other stress-induced programs like apoptosis. This may be determined by the abundance of cell-intrinsic survival proteins, but other factors, perhaps of stromal origin, are likely to add to the complexity of this choice. Several of these issues can be effectively investigated in mouse models; for instance, in animals conditionally expressing activated oncogenes from their endogenous promoters, or conditional tumor suppressor genes. It is conceivable that these as well as newly generated models will prove instrumental in validating results obtained in vitro. As therapeutic exploitation of enforced cellular senescence may become a realistic goal, answers to these questions may eventually provide us with new tools to effectively treat cancer patients.

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The essence of senescence

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