The power behind the throne; senescence and the hallmarks of cancer

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Abstract

The hallmarks of cancer was an attempt to describe the underlying principles of carcinogenesis. In their latest iteration, there is a particular focus on the role that the microenvironment and signalling between cancer cells and their neighbors play in the pathology of tumors. Since the original description of the hallmarks there has been a huge leap forward in our understanding of the biology of cellular senescence promoting it from an autonomous tumor suppressor to a complex, dynamic phenotype that can sometimes be tumor suppressive, but sometimes oncogenic. In particular, our understanding of the diverse non-autonomous effects that senescent cells can have upon both cancer cells and the tumor microenvironment suggests that senescent cells could play a major role in many human cancer types. Here we suggest that cellular senescence could underpin the biology of many of the hallmarks of cancer, making it the true power behind the throne.

1. INTRODUCTION

At the turn of the millennium Hanahan and Weinburg described their hallmarks of cancer to try and distill the essential underlying changes that cells undergo to develop into cancer (Hanahan & Weinberg 2000). This was an attempt to reduce the vast complexity described in various types and stages of cancer into its six underlying principles. One of these characteristics was the insensitivity to anti-growth signals or the loss of tumor suppressor function; their examples centered on the role of the retinoblastoma protein (RB) and TGF-β as archetypes of autonomous and secreted cytostatic factors, respectively. In the report, the description of senescence amounted to a single reference to the recent identification that unrestricted expression of oncogenic RAS could drive senescence (Serrano et al. 1997). At the time cellular senescence was seen solely as an autonomous tumor suppressor mechanism, but little was known of its role throughout the life-cycle and its complex non-autonomous signalling.

Over the intervening time period the hallmarks of cancer have been updated to include two additional hallmarks and two enabling characteristics with particular focus on the tissue microenvironment. Importantly, over the same time period there has been a recognition of the ubiquity of senescence in human diseases and in pre-malignant lesions in particular (Collado & Serrano 2010, Collado et al. 2005). As a tumor suppressor mechanism found in the earliest stages of cancer, senescence is positioned as a master controller of premalignant cells and their ultimate fate to be cleared, remain static, or progress. In addition, senescence has been described in multiple

physiological contexts such as embryological development (Muñoz-Espín et al. 2013, Storer et al. 2013), wound healing (Demaria et al. 2014, Jun & Lau 2010) and normal aging (Burd et al. 2013).

Since the publication of the original hallmarks, it has been the explosion of our understanding of the ability of senescent cells to co-ordinate other players within the tumor microenvironment through secreted signals or cell-contact that suggest that senescence rather than simply being an autonomous tumor suppressor could underpin many of these hallmarks of cancer. Here we take advantage of the hallmarks of cancer (highlighted in **'bold'** in the text) to discuss the role of senescence in the development and progression of cancer, in addition to the therapeutic opportunities that senescence presents to treat both cancer and the side-effects of anti-cancer therapies.

2. SENESCENCE AS AN AUTONOMOUS TUMOR SUPPRESSOR

2.1. Replicative Senescence

As **'replicative immortality and sustained proliferative signalling'** are hallmarks of cancer, the converse, stable proliferative arrest, is the essential hallmark of senescence. Senescence was first defined in cultured human diploid fibroblasts (HDFs), as a state of 'irreversible cell cycle arrest' caused by replicative exhaustion: after a certain number of replications, normal HDFs lose their proliferative capacity, exhibiting morphological (e.g. enlarged cell body and nuclei) and biochemical (e.g. accumulation of senescence-associated β-galactosidase activity, SAβ-GAL) alterations, called replicative senescence (HAYFLICK 1965, Salama et al. 2014, Shay & Wright 2000). The

molecular correlate of 'replicative exhaustion' was found to be critically short telomeres (Harley et al. 1990). Somatic cells in humans lack telomerase activity, the enzyme responsible for telomeric elongation and each round of mitosis causes telomeric attrition; critically short telomeres trigger a DNA damage response (DDR) (Herbig et al. 2004). Thus, the cancer hallmark '**replicative immortality**' is a state of escape from replicative senescence. Indeed, it has been shown that short telomeres in mice can limit tumorigenesis through induction of senescence (Collado et al. 2007, Cosme-Blanco et al. 2007, Feldser & Greider 2007), although it also causes genomic instability, leading to a modest increase in spontaneous tumor formation in highly proliferative cell types (Rudolph et al. 1999). Consistently, most cancers cells aberrantly express telomerase to achieve this state (Kim et al. 1994, Shay & Wright 2011).

2.2. Oncogene-induced senescence (OIS)

It was also shown that various types of cytotoxic stress could induce a similar phenotype, collectively termed premature senescence. Principally, identification of OIS, first in culture (Serrano et al. 1997) and later in vivo (Braig et al. 2005, Chen et al. 2005, Collado et al. 2005, Lazzerini Denchi et al. 2005, Michaloglou et al. 2005), substantiated the role for senescence as a tumor suppressor.

Initially, ectopic expression of constitutively active H-RAS (Serrano et al. 1997) and downstream effectors, MEK (Lin et al. 1998) or RAF1 (Zhu et al. 1998), were shown to paradoxically induce a senescence-like phenotype in

culture. Thus, supra-physiological mitogenic signalling somehow triggers anti-proliferative effectors to avoid transformation. However, this process is liable to bypass in certain situations: when these mitogenic oncogenes are co-expressed with C-MYC or adenoviral E1A, cells fail to undergo senescence and leads to immortalization with increased sensitivity to apoptosis. (Serrano et al. 1997, Zhuang et al. 2008). Indeed, loss of c-MYC expression can lead to a form of senescence (Wu et al. 2007). Considering that senescent cells are typically resistant to apoptosis, these observations support the idea that apoptosis and senescence back up each other for tumor suppression (Narita & Lowe 2005).

In a series of studies, oncogene- or loss of tumor suppressor-induced senescence have been identified in various tissues (Braig et al. 2005, Chen et al. 2005, Collado et al. 2005, Lazzerini Denchi et al. 2005, Michaloglou et al. 2005), as exemplified by melanocytic naevi. The majority of human naevi (~80%) carry the oncogenic *BRAF*^{V600E} mutation, as frequently as seen in malignant melanoma; it is rare that naevi subsequently progress to melanoma, suggesting that constitutive activation of BRAF is not sufficient for melanomagenesis (Pollock et al. 2003). Melanocytes from excised human naevi are largely non-proliferative and show multiple markers of senescence including expression of p16 and SAβ-GAL (Gray-Schopfer et al. 2006, Michaloglou et al. 2005).

2.3. Therapy-induced senescence (TIS)

In addition to its tumor suppressive role, senescence is also a potential therapeutic goal in fully developed cancer, termed therapy-induced senescence (TIS). The first genetic evidence for in vivo TIS precedes that of the OIS studies: In murine models of lymphoma, when apoptosis is defective, chemotherapy leads to the accumulation of cells with a p53-dependent senescent phenotype (Braig et al. 2005, Schmitt et al. 2002). Interestingly, in the *Eμ-myc* mouse lymphoma model, tumors in a p53-null background regress after chemotherapy, whereas *Eμ-myc* lymphomas with ectopic *Bcl2* (apoptosis defective) show no tumor regression. Nevertheless, prognosis of the latter is much better than the former (Schmitt et al. 2002). Post-treatment the *Eμ-myc-Bcl2* lymphoma cells exhibit a senescent phenotype, suggesting that a non-apoptotic function of p53 (likely to be senescence) contributes to the efficacy of the therapy (Schmitt et al. 2002). A TIS-like phenotype was also identified in human breast cancer isolated from patients after neo-adjuvant therapy (Poele et al. 2002). Following these initial studies, a number of mouse models have been used to show TIS, some of which are summarized elsewhere (Pérez-Mancera et al. 2014).

2.4. The senescence network

It is well recognized that underpinning the senescence response are two critical tumor suppressor pathways that mediate inhibition of the cell cycle, in addition to numerous other functions: the p53 (Brugarolas et al. 1995, Serrano et al. 1997) and p16/RB pathways (See Fig. 1a & sidebar) (Alcorta et al. 1996, Shay et al. 1991). However, increasing evidence suggest that it might be more appropriate to view senescence as a combinatorial phenotype of diverse

effector programs, which can confer sub-phenotypes, forming a high-order 'pathway network' (Fig. 1b) (Salama et al. 2014). This is in marked contrast to apoptosis, which could be seen as a single entity with clearly defined pathway(s) (Czabotar et al. 2014).

There is significant cross-talk and co-operativity between p16/RB and p53 pathways: both p16 and the canonical p53 target, p21 are endogenous CDK inhibitors, which activate RB to repress cell cycle genes, among others. P16 is in particular has been rather specifically associated with senescence, making it a robust maker of senescence. In contrast, p53 is involved in wider stress responsive contexts, including cell cycle, acute DDR and DNA repair, apoptosis, and metabolism (Levine & Oren 2009). Interestingly, it has recently been shown that chronic activation of p53, such as seen in senescence, drives a distinct transcriptional program to that seen in the acute p53 activation, upon DDR (see sidebar) (Kirschner et al. 2015). In addition, p53 has a range of different functions within the senescence context (Johmura & Nakanishi 2016, Rufini et al. 2013). However, the relationship between p53 and other senescence effectors is not necessary linear: for example, p53 promotes the autonomous aspects of senescence, but as we discuss later, p53 represses the senescence-associated secretory phenotype (SASP) that underpins much of the functionality of the senescent cell (Lujambio et al. 2013, Rodier et al. 2009). In addition, it remains to be elucidated how these two pathways interact within the complex senescence effector network.

2.5. Stable Exit From Cell Cycle

Senescence is distinct from quiescence in terms of the 'irreversible' nature of the proliferative arrest. How is the stability of senescence arrest achieved? This is particularly critical for OIS, which has constitutively '**sustained proliferative signalling'**. A number of autonomous effectors potentially contribute to this arrest (Fig. 1b). It has been shown that mutations causing persistent activation of the RAS pathway provoke a negative feedback response, which inhibits RAS/PI3K pathway in human cells at various levels in the pathway (Courtois-Cox et al. 2006). A similar negative feedback was also observed in the BRAF/MAPK pathway during the BRAF-induced melanocyte senescence (Wajapeyee et al. 2008). This negative feedback was reported to be mediated by secreted protein IGFBP7, although its involvement in OIS might be dependent on cell type or experimental model studied (Scurr et al. 2010, Vizioli et al. 2011, Wajapeyee et al. 2008, 2010). It is also apparent that other SASP components can reinforce the irreversibility of the senescence arrest (Acosta et al. 2008, Kortlever et al. 2006, Kuilman et al. 2008).

Another plausible mechanism is 'persistent' DDR (pDDR) accompanied by chronic activation of the p53 pathway (Fumagalli et al. 2014), as highlighted in the case of telomere dysfunction. This can be caused, not only by replicative stress (d'Adda di Fagagna et al. 2003, Herbig et al. 2004) but also by inhibition of TRF2, a double-stranded telomeric DNA-binding protein essential for telomere protection (Karlseder et al. 2002, Takai et al. 2003, van Steensel et al. 1998). Paradoxically, the protective role for TRF2 at chromosomal ends also appears to contribute to triggering pDDR. It has been suggested that the activity of TRF2 prevents aberrant chromosomal fusions, partly by serving as

an endogenous inhibitor of DNA repair (Bae & Baumann 2007). Thus, DNA damage in telomere regions is essentially irreparable and during senescence induced by genotoxic stress pDDR foci are preferentially located at the telomeres (Fumagalli et al. 2012, Hewitt et al. 2012). Interestingly, pDDR foci have also been identified in non-telomeric regions, collectively called 'DNA segments with chromatin alterations reinforcing senescence' (DNA-SCARS), which also lack DNA repair proteins (Rodier et al. 2011). These DNA-SCARS seem to be persistent double strand breaks that remain unrepaired (Galbiati et al. 2017). Further, the pDDR has also been associated with other forms of senescence including OIS, which typically exhibits an initial mitotic burst, causing an S-phase-associated DDR (Bartkova et al. 2006, d'Adda di Fagagna 2008, Di Micco et al. 2006, Mallette et al. 2007).

Although pDDR appears to underline many types of senescence, senescence can be induced without a DDR. For example, ectopic expression of p16 or p21 is able to drive a senescent phenotype without a DDR (Coppé et al. 2011). More recently, developmental senescence has also been found to be a critical mediator of embryological development and is not associated with a DDR (Muñoz-Espín et al. 2013, Storer et al. 2013).

It has also been suggested that the multiple nuclear and chromatin alterations seen in senescence might be involved in underpinning the stable cell cycle arrest (Salama et al. 2014). This was also highlighted in mouse genetic models of OIS/TIS, where *Suv39h1* is essential for senescence: Suv39h1 is a histone H3 lysine 9 methyl-transferase, which marks heterochromatin (Braig

et al. 2005, Dörr et al. 2013). Although not described as a cancer hallmark, epigenetics might be considered to be an 'enabling characteristic' for other hallmarks. Another 'autonomous' effector, potentially associated with senescence arrest is a metabolic switch: Senescence has a distinct metabolic state compared to cancer, which is accompanied by '**deregulating cellular energetics'**. We recently reviewed these features of senescence in detail: epigenetics (Parry & Narita 2016); and metabolism (Pérez-Mancera et al. 2014). Interestingly, increasing evidence suggest interplay between them. For example, senescence-specific downregulation of the nuclear lamina protein Lamin B1 has been implicated in gene regulation and senescence-associated heterochromatic foci (SAHF) formation during OIS (Sadaie et al. 2013). Lamin B1 has recently been shown to be a substrate of autophagy (Dou et al. 2015), a nutrient-sensing bulk-protein degradation process, demonstrated to be activated during senescence (Young et al. 2009). In addition, histone-modifying enzymes are often controlled by metabolic intermediates of glycolysis and the tricarboxylic acid (TCA) cycle (Lu & Thompson 2012), both of which appear to be hyper-activated during senescence (Dörr et al. 2013, Kaplon et al. 2013). Although any causal link between senescence-associated metabolic changes and histone modifications is unclear, such a link has been highlighted in cancer by mutations of isocitrate dehydrogenases (IDH), which normally metabolizes intermediates of the TCA cycle. Mutant IDH converts α-ketoglutarate (α-KG) to 2-hydroxyglutarate (2-HG), which inhibits α-KG-dependent enzyme activities. Such enzymes include the JmjC-domain family of demethylases and TET proteins; thus, 2-HG inhibits the demethylation of histones and DNA, respectively (Lu et al. 2012, Turcan et al. 2012).

Oncogenic mutations in another TCA cycle enyzme fumarate hydratase (FH) have been shown to induce senescence (Zheng et al. 2015). The mechanism for induction of this form of senescence appears to be oxidative stress, but it would be interesting to know how such perturbation of the TCA cycle affects the epigenetic state and chromatin structure of senescent cells.

4. NON-AUTONOMOUS ACTIVITIES OF SENESCENCE

Senescence was once viewed as a functional end point of stress response, but senescent cells are able to express and secrete factors that can exert profound effects upon surrounding players in the microenvironment, such as parenchymal, stromal and immune cells, in addition to the extracellular matrix (Fig. 1c). This secretome consists of a wide variety of cytokines, chemokines, matrix-modifying enzymes and growth factors that have been linked with diverse, sometimes contrasting, downstream functional outcomes in different contexts. The SASP has been linked with outcomes in 6 different domains: (1) tumor suppression through autocrine reinforcement of senescence; (2) tumor suppression through paracrine transmission of senescence; (3) tumor suppression through promotion of immune-mediated surveillance of senescent cells; (4) oncogenesis through paracrine promotion of tumorigenesis, through '**tumor-promoting inflammation**'; (5) paracrine enhancement of 'stemness' and plasticity; (6) promotion of angiogenesis.

4.1. Senescence and Immunity

It has long been recognized that many cancers, such as viral hepatitis-related hepatocellular carcinoma (HCC) or inflammatory bowel disease-related

colorectal carcinoma, can develop in the context of chronic tissue inflammation (Coussens & Werb 2002). Further, inflammation and subsequent immune cell infiltration portends an improved prognosis in several human cancers (Pagès et al. 2010, Stanton et al. 2016). Inflammation not only provides mitogenic stimulation, but also leads to modification of the extracellular matrix and promotes angiogenesis; therefore, '**tumor-promoting inflammation'** is proposed to be an enabling characteristic of other hallmarks of carcinogenesis, and it is perhaps coupled with the hallmark, **'avoiding immune destruction'** (Hanahan & Weinberg 2011). Increased evidence suggests that senescence, either in tumor or stromal cells, is involved in both of these processes.

Within the diverse factors of the SASP, inflammatory cytokines and chemokines, including IL6, IL8, and IL1, have been used as a representative subset. These factors are pleiotropic and, not surprisingly, have been reported to contribute to diverse processes. One feature of activation of the inflammatory SASP in vivo is immune cell recruitment and subsequent immune-mediated clearance of senescent cells: senescent cells signal to different components of the immune system to drive this process (Yevsa et al. 2012). The first evidence for this was shown in a 'TIS model' (in a broader sense), where endogenous p53 is reactivated in fully established cancer (Xue et al. 2007). In this study, embryonic mouse liver progenitor cells expressing oncogenic H-RAS and tetracycline-responsive sh-p53 develop cancer when they are seeded to the livers of mice lacking adaptive immunity; reactivation of endogenous p53, by switching off the RNAi, results in tumor regression.

Interestingly, the primary response to the p53 restoration is not cell death but senescence and a subsequent innate immune response that in turn targets the senescent tumor cells. This is in contrast to the aforementioned *Eμ-myc-Bcl2* lymphoma model, where the primary response to genotoxic chemotherapy is p53-dependent senescence with no tumor regression (Schmitt et al. 2002).

Pre-malignant OIS cells also have significant interactions with the immune system (Kang et al. 2011). Hydrodynamic tail-vein delivery of $NRAS^{G12V}$-containing transposons in the mouse results in mosaic integration and induction of hepatocyte OIS, which is accompanied by the SASP-mediated recruitment of immunocytes and subsequent clearance of senescent cells, termed senescence surveillance (Kang et al. 2011). In the absence of functional adaptive, and more specifically CD4+ T-lymphocyte, immunity persistent RAS-senescent hepatocytes develop into HCC with reduced expression of $p19^{Arf}$, a strong senescence effector in mice (Kang et al. 2011), placing senescence surveillance as an additional tumor suppressive layer. A more recent study showed that OIS hepatocyte-induced CCL2-CCR2 signaling promotes recruitment of immature myeloid cells (iMC) to the liver and their differentiation to macrophages, likely to be the major effector cells downstream of CD4+ T-helper cells, for elimination of OIS hepatocytes (Eggert et al. 2016).

This model offers a unique opportunity to study the dynamic mechanisms underpinning immune destruction and its avoidance, but as mentioned above,

earlier in vivo OIS studies do not describe immune elimination of senescent cells (Narita & Lowe 2005). Thus, in both TIS and OIS, the efficiency of senescence surveillance appears to be model dependent. The reason for the variation of the long-term fate of senescent cells in vivo, either in TIS or OIS, is unclear. In the liver OIS model, a CD4+ T-cell-dependent, mutant NRAS-specific reaction critical for senescence surveillance develops in adult mice after delivery of a genuinely novel tumor antigen. In other genetically modified mouse models oncogenes (such as K-Ras and BRaf), mostly driven by endogenous promoters, are expressed during embryonic development and therefore may be ignored as self-antigens.

It is also possible that the SASP composition varies with different senescence drivers. Indeed, in the context of senescence induced by the loss of *Pten* in murine prostatic epithelial cells, the SASP is immunosuppressive when compared to the immunostimulatory SASP of Ras-induced senescence in the same tissue (Di Mitri et al. 2014, Toso et al. 2014). This Pten-loss SASP drives the recruitment and function of myeloid-derived suppressor cells, impairing CD8+ T-lymphocyte-mediated immune surveillance. Utilising pharmacological inhibition of the JAK/STAT signalling pathway, it is possible to reprogram the functional output of the SASP in order to repress this SASP-dependent immunosuppressive microenvironment, promoting immune-mediated clearance and improving cancer-related outcomes (Toso et al. 2014).

Furthermore, it was recently shown that functionally distinct secretomes are dynamically regulated during the transition to OIS (Hoare et al. 2016). NOTCH1, a highly conserved cell surface receptor, previously identified as both oncogenic and tumor suppressive in different human cancers (Aster et al. 2017), is transiently activated during the transition to senescence, temporally associated with a phase of the SASP rich in TGFβ ligands, collagens and fibronectin. By full senescence, when the SASP includes pro-inflammatory cytokines and fibrolytic matrix-modifying enzymes, NOTCH activity had returned to baseline. Mechanistically NOTCH achieves this, in part, through inhibition of C/EBPβ, a key transcription factor controlling the inflammatory SASP (see sidebar). Importantly, in the liver OIS surveillance model (Kang et al. 2011), inhibiting Notch signaling in OIS hepatocytes facilitates their immune clearance, possibly due to a shift towards a more inflammatory SASP (Hoare et al. 2016), highlighting a therapeutic opportunity to modulate SASP activity to control tumorigenesis.

These immunosuppressive aspects of the SASP, represented in the *Pten* loss-induced prostate pre-neoplastic tumor (Toso et al. 2014) or NOTCH-type SASP (Hoare et al. 2016) might explain, at least in part, the varying extent of senescence surveillance depending on the model. This view might be extended to the context of advanced cancer, where an immunosuppressive SASP, derived from either senescent tumor or stromal cells could also be involved in the cancer hallmark, **'avoiding immune destruction'** through diverse mechanisms (Eggert et al. 2016, Ruhland et al. 2016). In recent years, the recognition that anti-tumoral immunity is actively suppressed

through inhibitory immune check-points (such as PD-1 and CTLA4) has led to an explosion in the interest in blocking these mechanisms to release the endogenous anti-cancer function of the patients own immune system. It would be interesting to know whether the SASP is also involved in this process.

In addition to the tumor suppressive consequence of OIS/TIS, it is important to explore the opposite scenario, where senescent cells are the source of the cancer hallmark, **'tumor promoting inflammation'**. The same SASP factors have been shown to promote tumorigenesis or tumorigenic inflammation, particularly for cells that have escaped senescence arrest in some contexts (Jackson et al. 2012, Pribluda et al. 2013). In the liver OIS model mentioned above (Kang et al. 2011), the same tumor suppressive environment that eliminates OIS hepatocytes can also promote the growth of established HCC cells. This is mediated through a combination of the SASP (CCL2) and HCC-derived secretory factors: the former recruits iMC to the liver and the latter inhibits subsequent differentiation of the immunosuppressive iMC into tumor suppressive macrophages (Eggert et al. 2016), highlighting a complex interaction between simultaneous secretomes derived from senescent and tumor cells. This study also reinforces the varying impacts of the same SASP on tumorigenesis depending on the cell receiving the signal.

4.2. Senescence and Stroma

In addition to senescence of parenchymal cells critically altering the microenvironment, there is increasing evidence that senescence of stromal cells within the tumor-microenvironment can modulate the fate of neighboring

cancer cells and cancer-related outcomes. In several culture and xenograft models, senescent fibroblasts have been demonstrated to promote the proliferation and invasiveness of neighboring epithelial tumor cell lines through the SASP and stimulation of the epithelial-mesenchymal transition (Bavik et al. 2006, Coppé et al. 2008, Krtolica et al. 2001, Parrinello et al. 2005), suggesting tumorigenic effects of the SASP on epithelial cells that are already primed for transformation. In addition, senescence-reporter mice have highlighted stromal senescence in some tumor models (Burd et al. 2013, Yoshimoto et al. 2013).

4.2.1. Stromal SASP and tumorigenesis. The similar effects of stromal senescence on established cancer have been shown in vivo. For example, murine melanoma cell lines become more invasive and metastasized more frequently when implanted into the skin of aged mice, with increased numbers of senescent dermal fibroblasts (Kaur et al. 2016). Wnt signaling has been implicated in melanoma 'phenotype switching' (i.e. switch between proliferative and invasive states) (Webster et al. 2015), and this study showed that sFRP2, a Wnt antagonist, secreted from these fibroblasts is critical for melanoma invasion and metastasis in this model (Kaur et al. 2016). Stromal senescence has also been shown to facilitate murine breast cancer recurrence and metastasis after chemotherapy or surgical removal (Demaria et al. 2017), suggesting that the stromal SASP contributes to the cancer hallmark, **'activating invasion and metastasis'**. In these studies, the interaction between stromal senescence and immunophenotypes was not addressed. However, stromal SASP was reported to promote tumorigenesis

through immune modulation in a different model: stromal-specific induction of senescence or skin-injection of senescent fibroblasts is sufficient to establish a immunosuppressive microenvironment characterized by increasing myeloid-derived suppressor cells that are capable of inhibiting CD8+ T-cell function (Ruhland et al. 2016). This environment appears to promote growth of injected murine tumor cell lines, supporting the potential role for senescence in the **'avoiding immune destruction'** hallmark. Stromal senescence in tumor-microenvironments was highlighted in a senescence-reporter mouse model, and curiously, those senescent cells include bone-marrow derived cells: the significance of a senescence-like state in these cells remains to be elucidated (Burd et al. 2013).

While the stromal SASP is mostly tumorigenic, its impact on tumor 'initiation' appears context-dependent. Senescence has been shown to play a key role in tissue homeostasis and damage repair through the SASP (Demaria et al. 2014, Jun & Lau 2010, Krizhanovsky et al. 2008) and the same process could also affect tumorigenesis. For example, in a mouse model of liver fibrosis induced by the liver-damaging reagent, CCL4, quiescent hepatic stellate cells (HSCs) differentiate into proliferative and fibrogenic myofibroblasts (activated HSCs), which subsequently develop senescence with an inflammatory and fibrolytic SASP. Thus, the SASP limits fibrosis and, and simultaneously, recruits immune cells to eliminate the senescent HSCs (Krizhanovsky et al. 2008). In this context, chemically induced HCC development is limited through tumor suppressive M1 macrophages. However, when HSCs escape senescence, due to loss of p53, they secrete factors promoting polarization of

macrophages into a tumorigenic M2 state (Lujambio et al. 2013). HSCs can also be induced to senescence in the context of genetic and dietary obesity, where the gut microbiota is altered (Yoshimoto et al. 2013). These bacteria induced increased concentrations of the toxic bile acid deoxy-cholic acid (DCA), absorbed through the enterohepatic circulation and provoking the SASP in HSCs. In contrast to the CCL4-liver damage model, the SASP in this case promotes chemically-induced HCC.

The opposing effects of the HSC-SASP on HCC development in these two models might be, in part, due to the different metabolic state. The extent of liver damage and fibrosis, the latter appearing absent in the obesity model, might also need to be considered. These factors might affect the nature of the immune microenvironment, particularly at the initial stage of tumorigenesis, potentially an important determinant for the difference in long-term tumorigenic effects of SASP. Interestingly, the shift from fibrogenic to fibrolytic secretome in HSCs during senescence development in the CCL4 model is reminiscent of the NOTCH-regulated SASP switch described above (Hoare et al. 2016) and NOTCH-mediated stromal senescence was noted in a skin cancer model (Procopio et al. 2015). Detailed analysis of the dynamic and context-dependent stromal SASP-immune axis might help to reconcile this question.

4.2.3. Senescence and cellular re-programing. One potential mechanism for the non-autonomous tumor-promoting activity of senescence is the recently recognized role that senescence and the SASP plays in controlling

differentiation and the ability to re-program into different cellular fates (Chiche et al. 2017, Mosteiro et al. 2016, Ocampo et al. 2016, Ritschka et al. 2017).

Somatic cells can be re-programed into induced pluripotent stem cells in response to expression of the four Yamanaka factors: Oct3/4; Sox2; Klf4; c-Myc (OSKM). It was previously shown that senescence is a barrier to re-programing in culture (reviewed in (Salama et al. 2014)). Paradoxically, a recent series of studies indicate that OSKM-mediated reprograming does occur or even is facilitated in the context of aging or senescence in vivo (Chiche et al. 2017, Mosteiro et al. 2016, Ocampo et al. 2016, Ritschka et al. 2017). Mosteiro et al. show that the OSKM induction in vivo is associated, not only with reprograming, but also extensive tissue damage and the development of senescent cells within the same microenvironment, where reprograming tends to occur in close proximity to more abundant senescent cells (Chiche et al. 2017, Mosteiro et al. 2016). Loss of the $p16^{INK4A}/p19^{ARF}$ locus, which abrogates the senescence barrier, thus enhancing reprograming in vitro, suppresses in vivo reprograming (Mosteiro et al. 2016). These observations suggest that reprograming of 'some cells' is promoted indirectly by neighboring senescent cells in vivo. Indeed, selective killing of senescent cells also suppresses in vivo reprograming (Chiche et al. 2017, Mosteiro et al. 2016). Consistently, the researchers identified that the SASP (IL6 in particular) facilitates in vivo reprograming. Thus, senescence is both an autonomous barrier and non-autonomous promoter of reprograming (Chiche et al. 2017, Mosteiro et al. 2016).

Then which cells are preferentially reprogramed? Using the same inducible OSKM mice, Chiche et al. show that, in skeletal muscle, reprograming occurs during muscle regeneration after damage, which triggers senescence. Reprograming mostly occurs in satellite cells, suggesting that progenitor/stem cells are the preferential origin for reprograming at least in skeletal muscle (Chiche et al. 2017).

While these studies used 'reprogramable mice', a similar observation was made in OIS models. Ritschka et al. showed that transient exposure to senescent-conditioned media induces a stem cell-like gene signature within differentiated keratinocytes (Ritschka et al. 2017). Thus, senescence induced after tissue damage is crucial for co-ordinating the response of immune cells to limit damage, but also for promoting regeneration through induction of stemness and tissue proliferation, a feature that might be utilized by cancer in some contexts.

4.2.2. Senescence and the vasculature. 'Angiogenesis' is a critical hallmark of most cancers, where increased vascularization provides the increased oxygen and nutrient delivery to permit tumor development and progression. Senescence has been demonstrated to modulate angiogenesis in both benign degenerative and malignant diseases through the SASP. Elimination of senescent cells from the murine skin wound healing model leads to a reduction in angiogenesis within the healing wound (Demaria et al. 2014). Senescent fibroblasts produce VEGF, a potent trophic factor for vascular growth and angiogenesis (Coppé et al. 2006); when co-injected with

tumor cells, senescent fibroblasts promote increased tumor vascularization compared to non-senescent fibroblasts (Coppé et al. 2006). Senescence-induced angiogenesis has been demonstrated in other models. Retinal ischaemia, frequently induced by diabetes mellitus and a major cause of sight loss, is associated with the development of both retinal cell senescence and secretion of VEGF, in a mouse model (Oubaha et al. 2016). Therapeutically, antibody-mediated depletion of VEGF reduced progression of the retinopathy in this model. In the field of cancer, multiple myeloma is able to induce stromal cell senescence and subsequent secretion of FGF2 which underpins increased growth and angiogenesis of xenografted myeloma tumors (Kanehira et al. 2016). Senescent dermal fibroblasts, through secretion of sFRP2, are also able to provoke increased vascularization of implanted melanoma tumors (Kaur et al. 2016).

5. SENESCENCE AS THERAPEUTIC TARGET IN CANCER

As discussed earlier, TIS represents the therapeutic induction of senescence in fully transformed cells. TIS typically implies genotoxic chemotherapy-induced senescence, but this has been extended. For example, p16 acts to inhibit CDK4/6, which are activated or amplified in several human cancer types and therefore the development of specific inhibitors, such as palbociclib, hold promise as negative regulators of cellular proliferation and therapeutic inducers of senescence (O'Leary et al. 2016). In the context of breast cancer the addition of palbociclib to conventional treatment led to a doubling of progression-free survival (Finn et al. 2015, Turner et al. 2015). In melanoma, palbociclib is able to induce cellular senescence, even in the context of

tumors that are resistant to the $BRAF^{V600E}$ inhibitor vemurafenib (Yoshida et al. 2016).

However, TIS often involves complex non-autonomous activities, of which net impact on the cancer microenvironment is difficult to predict. One approach could be to exploit the autonomous arrest of senescence with minimal SASP induction. In addition to TIS, effectors of senescence surveillance could be a potential target to enhance this process, as exemplified by NOTCH inhibition in OIS hepatocytes (discussed above) (Hoare et al. 2016). But, here we focus on an alternative avenue that has gained increasing attention: targeted killing of senescent cells (or 'senolytics') of either tumoral or stromal origin. The pioneering work in this area was made in aging contexts: elimination of senescent cells in genetically-modified mice, which harbor *INK-ATTAC*, the 'killing cassette' for inducible apoptosis in p16-expressing senescent cells, extends healthy life span in both premature and naturally aging mice (Baker et al. 2011, 2016).

Following this initial genetic approach, several small molecules have been identified that 'selectively' kill senescent cells (Baar et al. 2017, Chang et al. 2016, Yosef et al. 2016, Zhu et al. 2015). Senescent cells are known to be relatively resistant to apoptosis and transcriptional analyses have revealed upregulation of several anti-apoptotic factors in some (Yosef et al. 2016, Zhu et al. 2015), but not all contexts (Baar et al. 2017). Targeting these anti-apoptotic survival pathways with dasatinib and quercertin (Zhu et al. 2015) or ABT-263/ABT-737 (pan-Bcl inhibitors) (Chang et al. 2016, Yosef et al. 2016)

leads to reduced viability of senescent, but not proliferating cells. Similar to the *INK-ATTAC* model, treatment of aged or irradiated mice with these compounds results in fewer senescent cells, as well as some improvements in organ functions.

More recently, a novel senolytic agent has been developed: a modified FOXO4 peptide, FOXO4-D-Retro-Inverso (DRI), which interferes with FOXO4-p53 binding (Baar et al. 2017). Treatment with FOXO4-DRI releases active p53, which is excluded from the nucleus, triggering apoptosis in senescent cells, presumably through recruitment of active p53 to mitochondria. Additionally, treatment with FOXO4-DRI in vivo reduces the burden of senescent cells and restores physical fitness, hair density and renal function in both premature and natural aged mice.

Such ‘rejuvenation’ effects of senolytics in mice are highly promising. In the natural aging context, clearance of senescent cells in the *INK-ATTAC* mouse does not reduce the overall lifetime incidence of cancer, but it significantly increases both cancer- and non-cancer-related survival (Baker et al. 2016). However, the efficacy of selectively killing senescent tumor cells (e.g. tumor TIS plus senolytics) remains to be tested. Nevertheless, mice without cancer, treated with doxorubicin, a standard anti-cancer agent, developed systemic senescence and impaired physical function, that could be rescued by elimination of the senescent cells either by FOXO4-DRI or in a second genetic mouse model, *p16-3MR*, containing a thymidine kinase, which kills p16-expressing cells in the presence of ganciclovir (GCV). Thus, senescence

elimination can neutralize the side-effects of chemotherapy (Demaria et al. 2017). Furthermore, when the *p16-3MR* mice, implanted with a murine breast cancer cell line (with no *p16-3MR*) are treated with the chemotherapy with or without GCV, mice with GCV show better survival with a lower frequency of metastasis (Demaria et al. 2017). Thus, clearance of senescent cells either for preventing the development of cancer or reducing the toxicity of chemotherapy regimes is also a potential therapeutic target.

SUMMARY POINTS:

1. The hallmarks of cancer describe the mechanisms underlying the transformation of cells from normal to cancer. Senescence may underpin a number of these hallmarks.
2. Senescence is an autonomous tumor suppressor, leading to long-term cell cycle arrest. Senescence can develop after oncogene activation, loss of tumor suppressors or anti-cancer therapies.
3. Through the senescence-associated secretory phenotype (SASP), senescent cells have myriad effects upon the microenvironment that can be pro- or anti-tumorigenic.
4. Through the SASP, senescent cells trigger their own immune-mediated destruction; if this process is interrupted, persistent senescent cells are tumorigenic.
5. Senescence is a potential therapeutic target, through modulation of the composition of the SASP or targeted killing of senescent cells to prevent cancer or the side effects of anti-cancer therapies.

FUTURE ISSUES:

Some of the most pressing research needs in the field of senescence and

cancer are:

1. The development of ‘pure’ autonomous senescence inducers;
2. Understanding the context-dependent nature of the SASP in different stages of cancer development; what are the mechanisms of antagonist secretomes in the same microenvironment?
3. The development of diverse methods for ‘mechanism-guided’ senescence elimination;
4. Understanding the effects of senolytics on (pre)neoplastic senescence in vivo.

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Sidebars:

CHRONIC P53 DATABASE

P53 is constantly produced and degraded through the MDM2-mediated proteasomal system (Kruse & Gu 2009). MDM2 is a canonical transcriptional target of p53, forming a strong negative feedback loop. Thus, p53 is rapidly but transiently stabilized upon DNA damage (herein we call this 'acute p53'). However, it was recently shown that comparable levels of p53 accumulate on chromatin in senescent cells in culture ('chronic p53'). Importantly, it has been suggested from in vivo genetic models that it is the persistent activation of p53, rather than the acute p53 response that is more important for the tumor suppressive activity of p53 (Brady et al. 2011, Christophorou et al. 2006). Yet genome-wide mapping of p53 binding sites had been conducted almost exclusively in acute p53 conditions. However, chronic p53-specifc targets were recently evaluated through integrating p53 ChIP-seq and transcriptomic datasets, revealing more abundant and distinct p53 targets in the context of OIS compared to acute DDR (Kirschner et al. 2015). Examples of knowledge-based pathway models of chromatin p53 can be found at http://australian-systemsbiology.org/tp53 (Kirschner et al. 2015). This resource may represent many tumor-associated p53-targets, which have previously been poorly recognized.

P16 REGULATION IN SENESCENCE

p16 is an inhibitor of cyclin dependent kinase (CDK4/6)-dependent phosphorylation of RB, thus blocking G1/S cell cycle transition, and the p16-

CDK-RB axis is often inactivated in cancer (Gil & Peters 2006). Senescence is typically defined by combination of multiple senescence features in addition to long-term cell cycle exit. This is because of the lack of single definitive markers for senescence (Salama et al. 2014). However, p16 upregulation is probably one of the most specific features of senescence. p16/RB is also functionally important for senescence: for example, ectopic expression of p16 is sufficient to induce senescence and loss of p16/RB leads to bypass of senescence. Thus, p16 is a very useful senescence reporter both in culture and tissues. *p16* transcription is, at least in part, regulated by ETS transcription factors (TFs), but the entire regulatory TF network remains unclear. *p16* expression is also under complex regulation, involving polycomb, non-coding RNAs, and high-order chromatin structure (LaPak & Burd 2014). Nevertheless, the *p16* promoter or its relatively small parts have been successfully used to not only faithfully report *p16* expression and senescence induction, but also induce cell death specifically in *p16*-expressing senescent cells in vivo (Baker et al. 2011, Burd et al. 2013, Demaria et al. 2017, Yamakoshi et al. 2009).

SASP REGULATION

SASP regulation is best-studied for its inflammatory components. Critical to SASP expression is a pDDR, dependent on ATM and CHK2, but not on p53 (Coppé et al. 2008, Rodier et al. 2009). Indeed, p53 plays an inhibitory role in the SASP (Rodier et al. 2009). Transcriptionally, two transcription factors (TFs), RelA and C/EBPβ, cooperatively induce ‘proximal’ inflammatory SASP

components, such as IL1 and IL6, which in turn activate these TFs, forming an amplifying loop (Acosta et al. 2008). Thus, blocking these cytokines collapses the SASP network (Acosta et al. 2013, Kuilman et al. 2008, Orjalo et al. 2009). SASP factors, including these cytokines are also under epigenetic regulation, where the chromatin 'reader' BRD4 (Tasdemir et al. 2016) and chromatin architectural protein HMGB2 positively regulate the SASP (Aird et al. 2016). Several other factors, including post-transcriptional mechanisms, are involved in the cytokine-TFs loop: p38MAPK activation induces the SASP by activating NFkB in a DDR-independent manner (Freund et al. 2011); DDR can activate NFkB by inhibiting 'selective' autophagy-degradation of GATA4, which positively regulates IL1a and TRAF3IP2, both NFkB activators (Kang et al. 2015); NOTCH1 inhibits the inflammatory SASP through blocking the C/EBPβ activity (Hoare et al. 2016); and mTOR facilitates IL1a translation (Laberge et al. 2015). mTOR also promotes the SASP through stabilization of SASP transcripts (Herranz et al. 2015) or through forming mTOR-('global') autophagy spatial coupling compartment (TASCC) (Narita et al. 2011, Young et al. 2009), although the relationship between the TASCC and the other mTOR-mediated mechanisms is unclear.

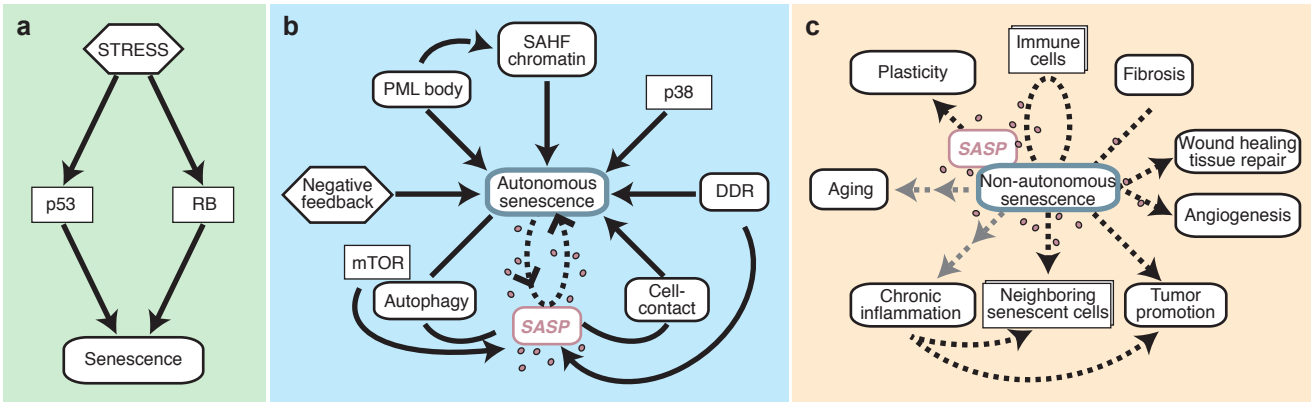
FIGURE LEGENDS

Figure 1. Features of cellular senescence. **a)** The classical view of the senescence pathway. Premature senescence develops as a consequence of a diverse range of stressors, such as ionising radiation, unrestricted oncogene activation or anti-cancer therapies. The cell-cycle arrest of senescence is underpinned by the p53 / p21 pathway and p16 / RB pathway, which co-

operatively contribute to senescence establishment and maintenance. **b-c)** A current view of the senescence network involving both autonomous (**b**) and non-autonomous (**c**) features of senescence. Multiple effector mechanisms contribute to the autonomous senescence phenotype including the DNA-damage response (DDR) and autocrine reinforcement through the senescence-associated secretory phenotype (SASP). Oncogene-induced senescence is often accompanied by senescence-associated heterochromatic foci (SAHF) and negative feedback loops repressing activity of the RAS / MAPK / PI3K pathways. **c)** Senescence is associated with profound non-autonomous effects upon multiple players within the microenvironment. Either through SASP-paracrine signaling or cell-contact-dependent juxtacrine signaling, senescent cells are able to promote plasticity, modulate immunity (typically inflammatory, but can also be immunosuppressive), fibrosis (typically fibrotic, but can also be fibrolytic), orchestrate tissue repair, drive angiogenesis and, if persistent, drive chronic inflammation. Dependent on the context of the signal-receiving cell, these non-autonomous effects can be tumour suppressive or pro-oncogenic. Dotted lines represent remote activities through secretory factors. 'No arrow head' lines indicate the effect can be either promoting or suppressing.

Figure 2. Senescence and the Hallmarks of Cancer. Recent work has highlighted the diverse range of autonomous and non-autonomous features of senescence in various models. Some of these features are tumour suppressive, consistent with the previous view of senescence as a tumour

suppressor mechanism, but other features serve to promote development and progression of cancer. We summarise these features here in this pseudo-heatmap and link them to the hallmarks of cancer in a simplified manner.



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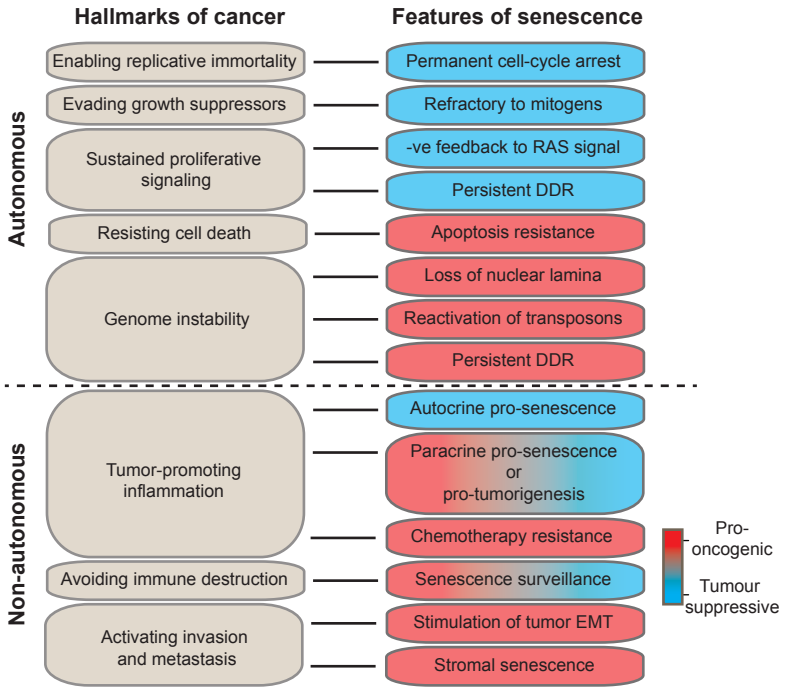

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