Review

Regulation of Survival Networks in Senescent Cells: From Mechanisms to Interventions

Abel Soto-Gamez [1,2], Wim J. Quax [1] and Marco Demaria [2]

*1 - **Groningen Research Institute of Pharmacy,** Chemical and Pharmaceutical Biology, University of Groningen, Groningen, the Netherlands*

*2 - **European Institute for the Biology of Aging (ERIBA),** University Medical Center Groningen (UMCG), University of Groningen, Groningen, the Netherlands*

***Correspondence to Marco Demaria:** m.demaria@umcg.nl.*

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Abstract

Cellular senescence is a state of stable cell cycle arrest arising in response to DNA and mitochondrial damages. Senescent cells undergo morphological, structural and functional changes that are influenced by a number of variables, including time, stress, tissue, and cell type. The heterogeneity of the senescent phenotype is exemplified by the many biological properties that senescent cells can cover. The advent of innovative model organisms has demonstrated a functional role of senescent cells during embryogenesis, tissue remodeling, tumorigenesis and aging. Importantly, prolonged and aberrant persistence of senescent cells is often associated with tissue dysfunction and pathology, and is partially the consequence of mechanisms that enhance survival and resistance to cell death. Here, we describe the main molecular players involved in promoting survival of senescent cells, with particular emphasis on the regulation of senescence-associated anti-apoptotic pathways. We discuss the consequences these pathways have in providing resistance to intrinsic and extrinsic pro-apoptotic signals. Finally, we highlight the importance of these pathways in the development of targets for senolytic interventions.

Introduction

Hallmarks of cellular senescence

Cellular senescence is a biological process characterized by a stable proliferative arrest in response to various stimuli (or SAGA, for senescence-associated growth arrest). Unlike quiescent or terminally differentiated cells, the SAGA is accompanied by an altered secretome known as the senescence-associated secretory phenotype (SASP) capable of influencing the microenviroment [1]. Senescent cells also undergo heterogeneous morphological, structural and functional changes that depend on a number of variables, including time, tissue, and cell type. Although few common hallmarks of cellular senescence exist (reviewed in Ref. [2]), the senescent phenotype is highly diverse, with underlying mechanisms not necessarily conserved among the various senescence programs. This diversity is reflected by the lack of specific markers and by the necessity to measure multiple facultative senescence-associated markers for the identification of senescent cells both in cell culture and *in vivo*. These markers include the following: (i) high level of $p16^{INK4a}$ and/or $p21^{CIP1}$, which prevent cell cycle progression by inhibiting the cyclin-dependent kinases CDK4 and CDK6 [3]; (ii) enhanced activity of the lysosomal senescence-associated β-galactosidase (SA-β-gal) [4]; (iii) persistent DNA-damage checkpoint response (DDR) as shown by the presence of p53-binding protein 1 (53BP1) and/or γH2AX foci [5] and (iv) formation of specialized domains of facultative heterochromatin which contribute to the silencing of proliferation-promoting genes (called the senescence-associated heterochromatin foci, SAHF) [6]. Additional predictive markers include a flattened morphology, absence of

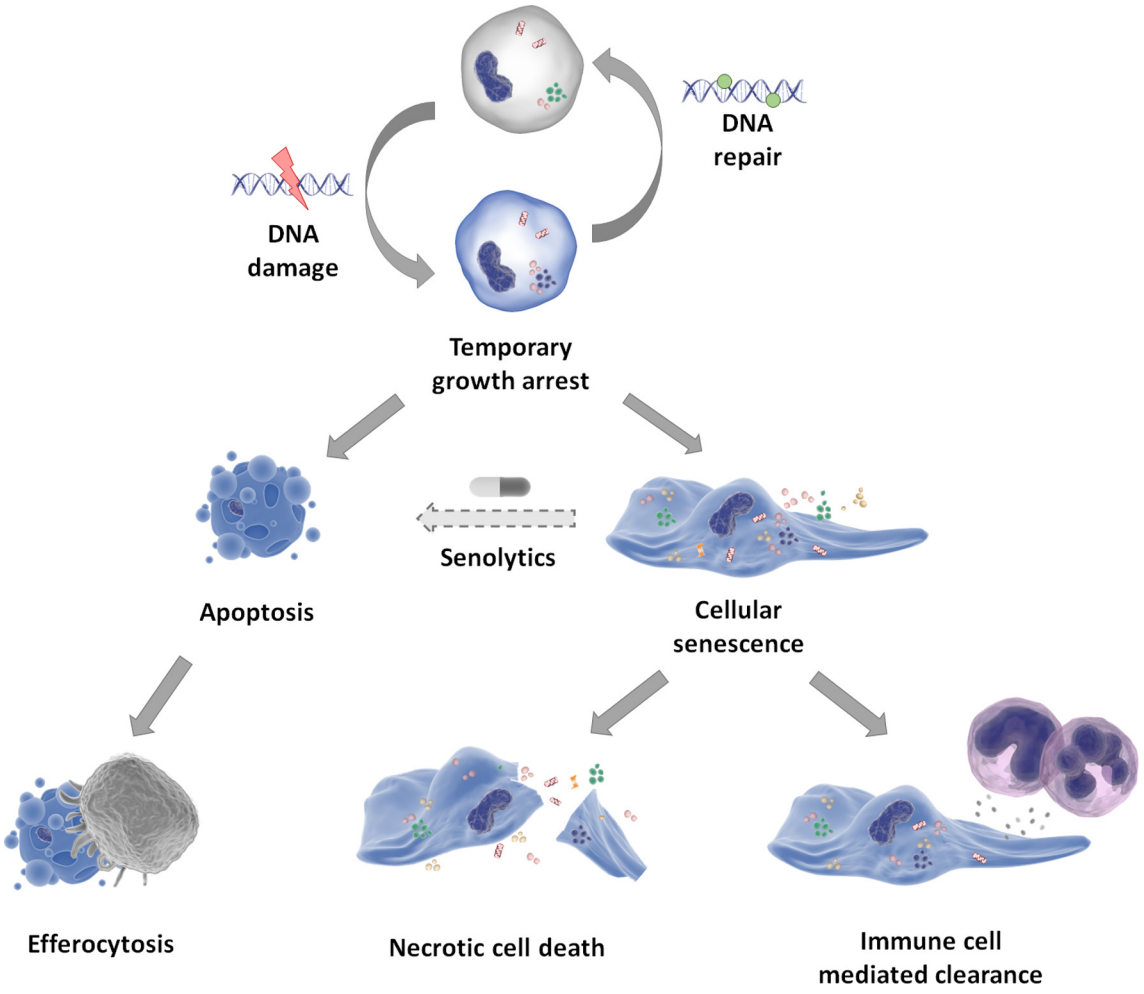

Fig. 1. Cellular fates of damaged cells. Following DNA damage, normal cells engage a temporary growth arrest and attempt to repair their DNA. If unresolved, damaged cells may initiate apoptosis or other forms of regulated cell death and be removed via efferocytosis. Alternatively, damaged cells may become senescent and persist indefinitely within tissues until their removal by the immune system. However, if damage becomes exacerbated, senescent cells may be susceptible to necrotic cell death, further promoting inflammation. Senolytic drugs aim to initiate pro-apoptotic pathways in senescent cells without affecting normal cells.

proliferation markers such as Ki67, lack of EdU/BrdU incorporation, enlarged nuclear size, loss of nuclear high mobility group box 1 (HMGB1) and decreased expression of lamin B1 [7–9]. Cellular senescence was initially described in response to excessive telomere shortening from continued proliferation, and this form is now referred to as replicative senescence or RS [10]. In contrast, premature senescence is triggered by a variety of inducers not associated with short atrophied telomeres. Therapy-induced senescence and oxidative stress-induced senescence arise in response to genotoxic insults. Spontaneous activation of various oncogenes can result in oncogene-induced senescence [11], a form characterized by proliferative bursts and DNA-hyper replication that engage a sustained DDR signaling [12]. Paracrine senescence can originate when normal cells are continuously exposed to senescent cells and the SASP [13]. Lastly, in recent years, a number of programmed senescent states associated with tissue remodeling have been identified both during embryogenesis (developmental senescence [14,15]) and adulthood (tissue repair senescence [16]).

The choice of cellular senescence

Genetic injuries can arise from endogenous (metabolic byproducts of cellular respiration, collapsed replication forks, progressive telomere shortening, etc.) and exogenous sources (UV light, ionizing radiation, harmful environmental agents, or cytotoxic drugs). To face these lesions, the DDR transiently blocks cell cycle progression via $p21^{CIP1}$, while DNA repair is attempted. If the damage is

resolved, the cell can re-enter the cell cycle; however, not all DNA damage can be readily repaired, and when faced with irreparable damage, normal cells can become senescent, converting their temporary growth arrest into an indefinite one (the SAGA). Alternatively, damaged cells may not become senescent and instead initiate apoptosis or other forms of regulated cell death. Determinants of the molecular cell fate are still under investigation, but the extent of cellular damage has a major influence in the choice between cellular senescence and apoptosis. For instance, treatment with DNA-damaging agents induces cellular senescence at low doses and apoptosis at high doses, as demonstrated after treatment with cisplatin [17,18], etoposide [19], doxorubicin [18,20], temozolomide [18], placlitaxel [18], H_2O_2 [21], UV-B [22,23], and ionizing radiation (reviewed in Ref. [24]). Senescence is similarly observed after treatment with sub-lethal doses of proteotoxic drugs such as thapsigargin or dithiothreitol [25,26]. Remarkably, although transformed cancer cells have partially lost the capacity to signal senescence or apoptosis, various forms of chemotherapy or radiation can inflict severe DNA damage and re-activate these signaling pathways resulting in therapy-induced senescence or apoptotic cell death [27,28]. Indeed, conventional anticancer treatments exploit these outcomes leading to tumor regression and increased tumor-specific immune activity (reviewed in Ref. [29]). However, therapy-induced senescent cells can persist after treatment and contribute to the adverse effects of chemotherapy and to cancer relapse via the SASP [18].

Physiologically, the choice between cellular senescence and apoptosis differs greatly from an immunogenic perspective. Apoptosis is generally recognized as an anti-inflammatory cell-death (reviewed in Ref. [30]) and is normally followed by the rapid engulfment of apoptotic cell by phagocytes in a process called efferocytosis. Efferocytosis prevents the release of the inner contents of apoptotic cells that would otherwise act as damage-associated-molecular patterns and perpetuate an inflammatory response. Likewise, efferocytosis is followed by the release of anti-inflammatory cytokines such as TGF-β and IL-10 [30]. On the other hand, senescent cells are regarded as pro-inflammatory and immunogenic, playing active roles in the recruitment and activation of the immune system (reviewed in Ref. [31]). Furthermore, senescent cells may persist indefinitely within tissues until their removal by the immune system, or after necrotic cell death and other forms of cell death from exacerbated damage (Fig. 1).

Recent evidence suggests that an incomplete or stalled apoptotic phenotype termed “senoptosis” may also occur in human fibroblasts after being subjected to low-dose ionizing radiation [32]. Senoptotic cells are a viable and stable sub-G1 population that has undergone controlled cleavage of nuclear DNA, reminiscent of the chromatin degradation process normally observed in apoptotic cells. Similar to senescent cells, senoptotic cells were unable to proliferate, remained stably viable, and tested positive for SA-β-gal; however, unlike senescent cells, senoptotic cells lacked canonical pro-inflammatory components of the SASP, suggesting that senoptotic cells may not undergo an immunogenic conversion. Importantly, the immunogenic phenotype of senescent cells lies behind its various functions under physiological conditions by facilitating immune infiltration, and activating innate and adaptive immune responses (reviewed in Ref. [31]).

Function of senescent cells

The heterogeneity of the senescent phenotype is exemplified by the intrinsic differences that distinguish each senescent program [33] and by the complex biological roles that senescent cells can cover, appearing at times even paradoxical: acting both as tumor suppressor or tumor promoter, limiting or exacerbating fibrosis, and enhancing tissue repair and regeneration while equally contributing to age-related pathologies via tissue degeneration [34]. Indeed, the phenotype and the impact of senescent cells at the tissue and organismal level greatly depend on the physiological context. Cellular senescence acts beneficially as a barrier to tumorigenesis by limiting the growth of damaged potentially oncogenic cells in a cell-autonomous manner via the SAGA. Meanwhile, components of the SASP such as IL-6 and IL-8 act in autocrine fashion to reinforce growth arrest through a positive feedback loop [13]. Furthermore, the release of SASP growth factors favors tissue repair by promoting the division of new healthy cells, paired with secreted matrix metalloproteinases that enable efficient tissue remodeling and limit fibrosis [1,16].

Importantly however, secretory components may exert beneficial functions in the short term but promote deleterious effects on the surrounding microenvironment if unresolved. For instance, the accumulation of secretory components meant to reinforce cell-cycle arrest may act in a paracrine manner potentially propagating the senescent phenotype to neighboring cells and hampering the regenerative capacity of the surrounding tissue. Similarly, persistent pro-inflammatory signaling meant to promote immune cell infiltration may eventually result in disruptive chronic inflammation and impaired tissue homeostasis via paracrine senescence. The advent of innovative model organisms has demonstrated that prolonged persistence of senescent cells is associated with tissue dysfunction and pathology. Indeed, senescent cells have been implicated in several age related pathologies such as sarcopenia (loss of skeletal muscle mass) [35], lordokyphosis (increased curvature of the

spine) [35], cataracts [35], hair loss [36], osteoporosis [37], aged hematopoietic system [38], vasomotor dysfunction [39], neurodegeneration [40], pulmonary fibrosis [41], and decreased lifespan [42,43]. Accordingly, genetic or pharmacologic clearance of senescent cells is sufficient in delaying these age-related phenotypes and helps restore a pro-regenerative environment [35–44]. The beneficial effects related to senescent cell clearance have sparked great interest in the development of a novel pharmacological strategy, termed senolytics, which aims to selectively eliminate senescent cells by effectively overcoming the survival mechanisms of senescent cells while sparing normal quiescent and proliferating cells.

Activation of Survival Mechanisms

Senescent cells seem to be more resistant to apoptosis compared to normal proliferating cells. An increased apoptosis resistance is reported in response to several stimuli, including serum withdrawal [45], UV damage [23,36,46], oxidative stress [47,48], extrinsic apoptosis inducers [36,49], and treatment with cytotoxic drugs such as staurosporine or thapsigargin [23,50]. Given the underlying stress present in senescent cells prior to exposure to apoptotic stimuli, an increased susceptibility to cell death would perhaps be expected, and may well be the case in some cell types, such as endothelial cells [51]. However, the observations of increased survival, at least in senescent fibroblasts and keratinocytes, attest to the role of cellular senescence as an adaptive response. In recent years, a number of studies have shown that the enhanced survival of senescent cells depends on the activation of various mechanisms referred to as senescent cell anti-apoptotic pathways (SCAPs). SCAPS are directly related to many features of the senescent phenotype such as the SAGA, the SASP, the unfolded protein response (UPR), and the PI3K-Akt signaling. Importantly, SCAPs are now under investigation as targets for senolytic drugs.

The SAGA

Since proliferation is an essential feature for the effectiveness of many DNA-damaging agents [52], the SAGA may provide a survival advantage to senescent cells. $p21^{Cip1}$ is an early effector of cell cycle arrest following DNA damage and p53 signaling. However, $p21^{Cip1}$ is also induced in p53- and DNA damage-independent situations such as tissue development, serum stimulation, and cellular differentiation [53]. Recently, $p21^{Cip1}$ was revealed to play a role in maintaining senescent cell viability under persistent DDR signaling [54]. Knockdown of $p21^{Cip1}$ in DNA damaged-induced senescent cells, but not oncogene-induced senescence cells, resulted in the accumulation of DNA lesions that activated ATM and NF-κB, TNF-α secretion, JNK signaling, and increased cell death. Importantly, these effects were not limited to normal fibroblasts but were also observed in a p53 mutated non-small cell lung cancer cell line (H1299) [54]. Importantly, cell death was only partially rescued with the pan-caspase inhibitor Q-VD-OPh, but fully rescued in combination with JNK inhibition. Importantly, JNK activation can shift the balance of TNF-stimulated cell death from apoptosis to necrosis by increasing the production of cytotoxic reactive oxygen species (ROS) [55]. These findings suggest alternative modes of cell death additional to apoptosis may also be involved. $p21^{Cip1}$ may therefore prevent the exacerbation of DNA damage from the continued proliferation of DNA-damaged cells, eventually tipping cell fate balance from cellular senescence into apoptosis or necrosis [54]. However, two important notions challenge that the pro-survival effects of $p21^{Cip1}$ are exclusively related to cell cycle progression. First, knockdown of the other main senescence-associated CDKi, $p16^{INK4a}$, is normally not lethal for senescent cells [56]. Second, p21-proficient, p53-null cancer and near-normal cell models showed that after an initial senescence-like phase, a subpopulation of p21-expressing proliferating cells emerged, featuring increased replication stress, genomic instability, aggressiveness, and chemoresistance [57].

The SASP

In contrast to quiescent cells, the SAGA is accompanied by a secretory phenotype (the SASP) composed of major inflammatory factors. SASP IL-6, IL-8, IL-1, and TNFα help reinforce growth arrest via autocrine NF-κB signaling and ROS generation. However, activation of NF-κB may also promote survival responses by transcriptional induction of anti-apoptotic proteins of the Bcl-2 family [58–61] often overexpressed in senescent cells [36,45,62].

The SASP also includes several growth factors like IGF-1, PDGF, and VEGF proposed to act in a paracrine manner to fuel the proliferation of nearby healthy cells, thereby promoting tissue repair and regeneration [16]. In tumor contexts, these growth factors may be taken up by surrounding cancer cells and be responsible for some of the pro-tumorigenic effects of senescent cells [63]. However, growth factors may also promote survival in established senescent cells. IGF-1 binds to the tyrosine kinase receptor (TKR) IGF1R and leads to the upregulation of the PI3K/AKT pathway, leading to cell growth and proliferation during acute signaling but inducing premature senescence after prolonged signaling in a p53-dependent manner [64]. In normal cells, p53 negatively regulates basal-levels of IGF-1 but upregulates IGF-1 in response to chemotherapy,

ionizing radiation, and genomic instability providing a survival advantage in a p53-ATM-DDR dependent manner [65]. Accordingly, IGF-1 inhibition delays senescence in proliferating cells, while inducing apoptosis in senescent fibroblasts [65].

Additional SASP components and their TKRs may act in parallel or play autonomous roles in a cell type-dependent manner. For instance, multiple TKRs act as dependence receptors: specialized surface receptors that activate classic signaling pathways implicated in cell survival in the presence of their cognate ligand, while eliciting caspase-dependent apoptotic signals in the absence of a ligand (reviewed in Ref. [66]). Senolytic screens have identified the upregulation of ephrin dependence signaling in senescent cells via the ephrin ligands (EFN) B1, and EFNB3 [62]. Targeting EFN signaling with the antioxidant and promiscuous drug quercetin increased cell death of human senescent preadipocytes, but was much less effective on senescent endothelial cells. Only the combination of quercetin and dasatinib (a PI3K and serpine inhibitor) resulted in cell death of both senescent cell types, highlighting the reliance of senescent cells on multiple pro-survival pathways.

Importantly, mTOR signaling is a major regulator of the SASP by promoting translation of IL-1A [67] and of the mitogen-activated protein kinase activated kinase 2 (MAPKAPK2), responsive to multiple stress stimuli (e.g., cytokines, ultraviolet and ionizing irradiation, ROS) [68,69]. Recently, a senescence-associated secretory switch mediated by mTOR and PI3K/Akt signaling has been described in endothelial cells, exerting various chemoprotective effects [70]. Inhibition of mTOR with the drug rapamycin strongly represses the SASP of senescent cells [69], while preventing senescence-associated morphological changes and delaying senescence in treated cells [67]. Put together, these findings highlight the importance of mTOR in incorporating inputs from multiple stresses, as well as the role of the SASP in establishing the senescent phenotype.

The UPR

The SASP exerts proteotoxic stress through the accumulation of unfolded proteins in the endoplasmic reticulum (ER) and triggers an UPR. The UPR is an adaptive mechanism intended to restore protein homeostasis by activating a cascade of transcription factors that regulate genes encoding chaperones, and components of the ER-associated protein degradation system and of the autophagy machinery (reviewed in [71]). Importantly, the UPR is a key player in the regulation of multiple morphological aspects of the senescent phenotype such as cytoskeleton-mediated shape changes, size enlargement, and organelle remodeling [25]. Treatment of normal fibroblasts with sub-lethal doses of the drug thapsigargin, an ER stress inducer, culminates in premature senescence [25]. Interestingly, if ER stress precedes additional DNA damage, $p21^{Cip1}$ is suppressed, and the apoptotic threshold to genotoxic treatments is lowered [72]. However, senescent fibroblasts showed an increased resistance to ER stress compared to young non-senescent fibroblasts [73], presumably due to pre-established UPR signaling serving as a coping mechanism.

Oxidative stress is a common feature of senescent cells, which can act either as inducer or sustainer of the senescent phenotype (reviewed in Ref. [74]). However, ROS inflict damage to various cellular components including proteins, lipids, and nucleic acids and contributes to ER stress and UPR signaling. When ER stress is unresolved, cells heavily rely on autophagy to avoid cell death [75]. How ER stress activates autophagy remains unclear, but the UPR may play an active role by enabling the transcription of autophagy components [71]. Autophagy allows the degradation of cytosolic proteins and contributes to the clearance of all irreversibly oxidized biomolecules, thereby preventing oxidative stress from culminating in cell death [76]. Indeed, lack of autophagy leads to lysosomal and mitochondrial impairment in senescence and oxidative stress-induced senescence [77], while increased autophagy is a feature of senescent fibroblasts and endothelial cells [71,78,79]. In contrast, high levels of dysregulated autophagy may result in the degradation of important cellular structures leading to autophagy-dependent cell death and not apoptosis, as described for senescent keratinocytes [80,81]. These findings suggest that senescent cells may depend on tightly regulated levels of autophagy: on one hand, enhanced autophagy attenuates existing proteotoxic stress and promotes cell survival [82]; on the other hand, limiting autophagy is a necessary step to prevent autophagy-dependent cell death. A balanced level of autophagy in senescent cells may be regulated via Bcl-2 and homolog proteins. Although anti-apoptotic Bcl-2 proteins are primarily known for their inhibitory role in the intrinsic pathway of apoptosis, Bcl-2 can directly interact with the evolutionarily conserved autophagy protein Beclin1 and inhibit autophagosome formation [83]. As some senescent cells overexpress Bcl-2, Bcl-2 may serve an anti-autophagic function and protect from autophagy-dependent cell death, in addition to its anti-apoptotic roles.

Importantly, mTOR is a master regulator of autophagy. When nutrients like glucose and aminoacids are freely available, mTOR is found in complexes where it blocks autophagy and promotes protein synthesis (reviewed in Ref. [84]). Specifically, mTOR complex 1 (mTORC1) inactivates autophagy by inhibiting unc-51 like autophagy activating

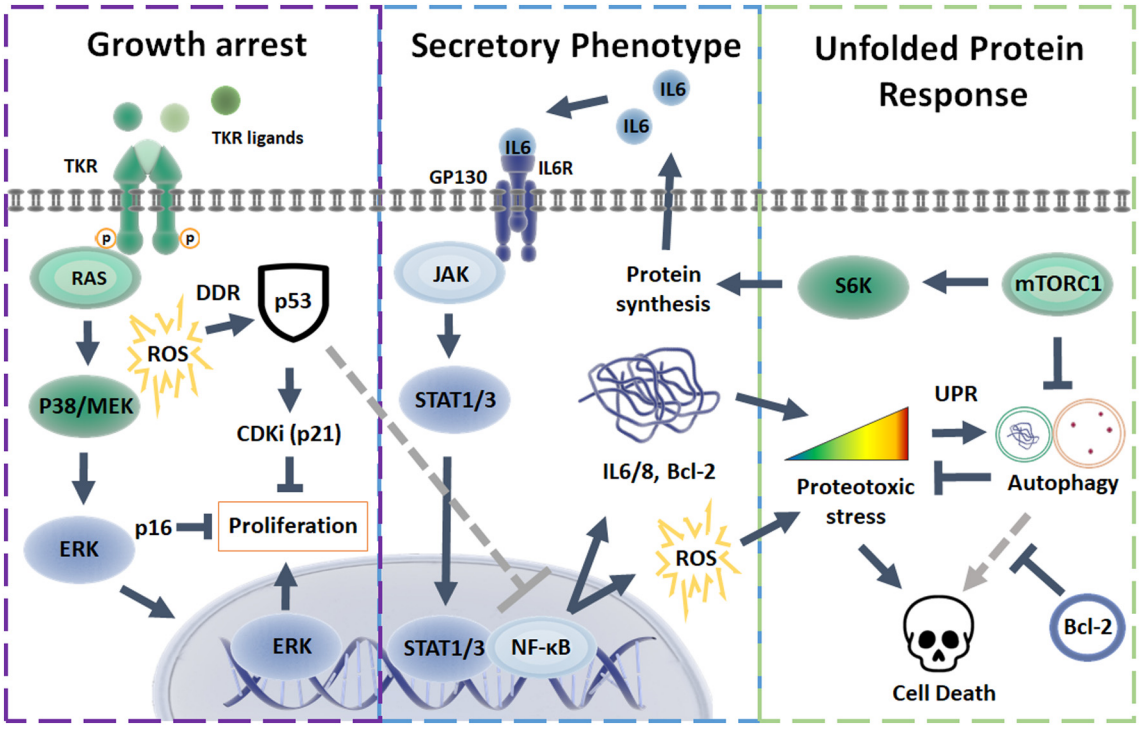

Fig. 2. Crosstalk between SAGA, secretory phenotype and UPR. Senescent cells are insensitive to extracellular mitogenic stimuli signaling from TKRs due to the overexpression of cyclin dependent kinase inhibitors (e.g., p21, p16) resulting in a senescent-associated growth arrest (the SAGA). Growth arrest is reinforced by the secretory phenotype of senescent cells (the SASP) through the amplification of autocrine loops. An overactive SASP results in ROS generation and proteotoxic stress, which engage the UPR responsible for many adaptive morphological changes associated with senescent cells. However, unresolved proteotoxic stress results in autophagy, which helps attenuates proteotoxic stress and limit cell death. In turn, autotophagy is limited by mTORC1 signaling and Bcl-2, which may serve protective effects against autophagic cell death in senescent cells.

kinase 1/2 (ULK1/2) and promoting protein synthesis via S6 kinase (S6K). In contrast, when nutrients are low, mTOR complexes are inactivated and the cell can engage in autophagy, allowing the degradation and recycling of cytosolic proteins to satisfy a shortage of aminoacids. mTORC1 therefore plays an important role in promoting the SASP while limiting autophagy and potentially autophagic-programmed cell death (Fig. 2).

Metabolic reprogramming

In addition to autophagy, senescent cells display other metabolic changes such as increased glycolysis and increased mitochondrial metabolism [82,85]. Metabolic reprogramming may serve as a cellular adaptation to an overactive secretory phenotype resulting in increased nutrient requirements in the form of energy and amino acids. Indeed, SASP production has been shown to rely on increased glycolysis and enhanced ATP production in mitochondria [82], while a shortage of amino acids may be overcome by coupling autophagy to protein synthesis [86]. In addition to restore protein homeostasis, the UPR controls other pathways of lipid and energy metabolisms, suggesting metabolic reprogramming [71]. Importantly, the increased energetic demands and metabolic reprogramming of the senescent response can sensitize senescent cells to blockade of glucose usage [82], inhibition of autophagy [82], and mitochondrial targeting [87]. Metabolic pathways might thus represent a therapeutically exploitable target.

Mitochondrial metabolism in cellular senescence remains controversial. If senescent cells would depend on increased mitochondrial activity to meet energy requirements, mitochondrial depletion would perhaps result in cell death. Instead, mitochondrial depletion resulting in senescence bypass and ATP production is compensated by increased glycolysis [88]. Indeed, mitochondria appear to be required for the establishment of the senescent phenotype through a DDR-dependent feedback loop involving ROS-ATM-Akt-mTORC1 and leading to PGC-1β-dependent mitochondrial biogenesis [88]. Inhibition of mTORC1 via rapamycin or PGC-1β deletion prior to DDR activation reduced the expression of various mitochondrial proteins pertaining to oxidative

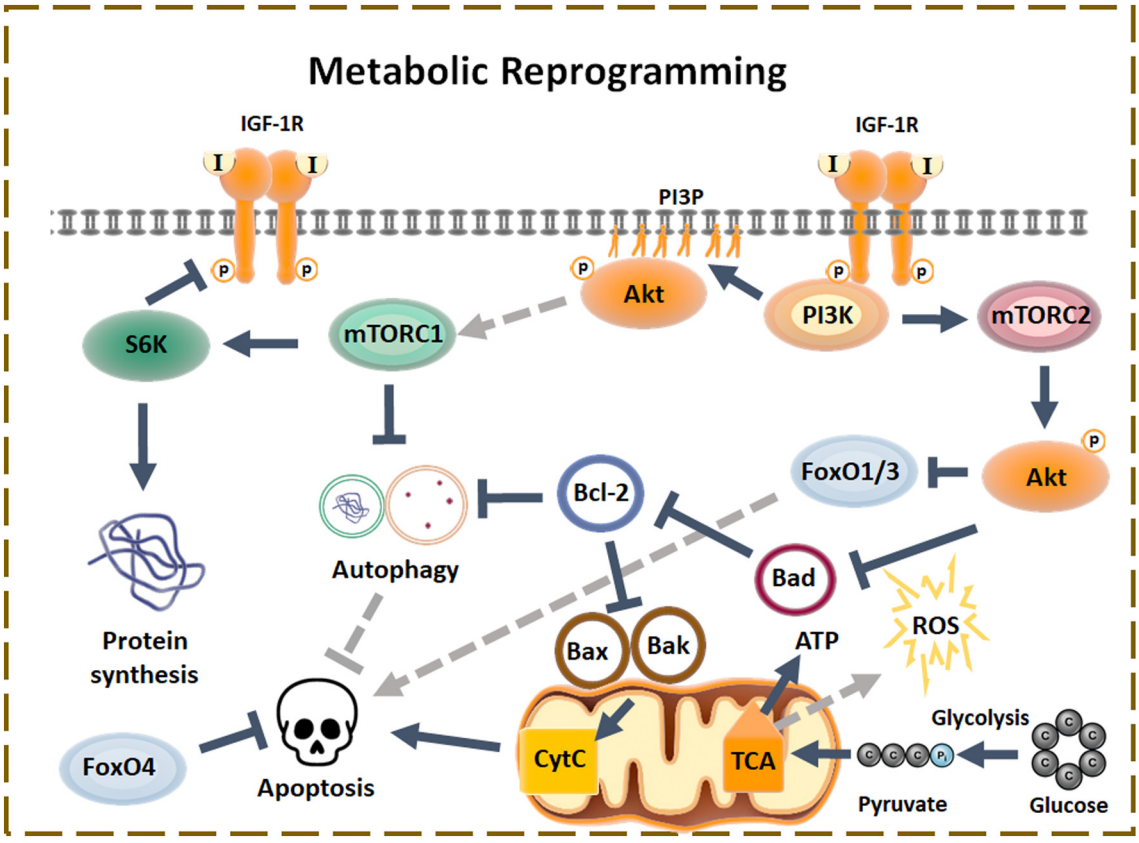

Fig. 3. Metabolic reprograming of senescent cells. IGF-1R signaling activates PI3K leading to membrane PIP3 enrichment serving as an anchor for 3-phosphatidylinositol-dependent kinase 1 (PDK1) and Akt activation at position T308. Among its multiple functions, Akt indirectly enables the formation of mTORC1. mTORC1 positively regulates protein translation through S6K, which exerts negative feedback inhibition at the level of IGF-1R. PI3K also activates mTORC2, which further activates Akt, increasing its kinase activity. Fully activated Akt can then efficiently inhibit pro-apoptotic Bad. Akt may also inhibit pro-apoptotic FoxO transcription factors FoxO1/3, thereby preventing apoptosis. In contrast, anti-apoptotic FoxO4 present in senescent cells also supports senescent cell viability [93].

phosphorylation complexes. Accordingly, increased respiration and oxidative stress are crucial mediators of oncogene-induced senescence, and rely on the mitochondrial gatekeeper pyruvate dehydrogenase for enabling the use of pyruvate in the tricarboxylic acid cycle [85]. Intriguingly, in the absence of DNA damage, mitochondrial impairment leads to modified form of senescence with a p53-mediated SAGA and a modified SASP lacking the IL-1-dependent inflammatory arm, presumably due to p53 inhibition of NF-κB [89]. This form of senescence was termed mitochondrial dysfunction-associated senescence (MiDAS) and appears to be independent of ROS. Antioxidant treatment inhibiting mitochondrial ROS failed to prevent MiDAS, whereas treatment with pyruvate prevented MiDAS growth arrest but restored NF-κB activity, supporting the notion that the SAGA and the SASP may be uncoupled. Resistance to apoptosis was not explored in MiDAS cells, and the levels of mitochondrial pro-apoptotic and anti-apoptotic components were not characterized. As NF-κB activity signaling was absent in MiDAS cells, it is tempting to speculate that Bcl-2 homologs may be reduced compared to “conventional” senescent cells.

It is unclear whether increased energy requirements are truly a limitation in senescent cells, as nutrients can be made freely available in cell cultures, well-fed animal models, and aging humans, without necessarily delaying senescence. In fact, the opposite may be true as caloric restriction delays senescence [90] and is widely recognized as a lifespan extension intervention [91]. Nutrient availability may therefore play a role in the survival senescent cells. For instance, high glucose uptake leads to insulin release, a hormone structurally similar to IGF-1. Although both hormones have different affinities to the IGF-1 receptor, both are capable of binding and initiating downstream signaling. Activated IGF-1R recruits phosphatidylinositol-3 kinase (PI3K) to the cell membrane, which then phosphorylates phosphatidylinositol-biphosphate

Table 1. Apoptotic ligands, receptors and decoy receptors

Apoptotic ligand	Death receptor	Decoy receptor
TRAIL	TRAIL-R1/DR4, TRAIL-R2/DR5	DcR1, DcR2, OPG
FasL	CD95/Fas	DcR3
TNF-alpha	TNFR1	TNFR2

(PIP2) into phosphatidylinositol-triphosphate (PIP3). The enrichment of PIP3 phospholipids at the cell membrane serves as an anchor for 3-phosphatidylinositol-dependent kinase 1 (PDK1), which next activates the pro-survival kinase Akt at position T308. Among its multiple functions, Akt indirectly enables the formation of mTORC1. In turn, mTORC1 positively regulates protein translation through S6K, which exerts negative feedback inhibition at the level of IGF-1R. However, in contrast to mTORC1, mTORC2 has a direct role in cell survival, and IGF-1R signaling can also activate mTORC2 in a PI3K-dependent manner. Once activated, mTORC2 further phosphorylates Akt in position S473, increasing its kinase activity over 10-fold. Activated Akt can then efficiently inhibit the pro-apoptotic protein Bad, which initiates Bax-Bak dependent mithochondrial pathway of apoptosis [92]. Alternatively, Akt can also inhibit forkhead box O (FoxO) transcription factors FoxO1/3, thereby preventing apoptosis (reviewed in Ref. [84]). Moreover, anti-apoptotic FoxO proteins such as FoxO4 may be present in senescent cells [93]. FoxO4 inhibition prior to senescence induction resulted in Bax/Bak-dependent release of mitochondrial cytochrome C and apoptosis, suggesting that FoxO4 favors senescence over apoptosis. Accordingly, inhibiting the interaction of FoxO4 and p53 with a therapeutic peptide resulted in decreased viability of already senescent cells, and amelioration of age related phenotypes [93] (Fig. 3).

Apoptotic Signaling in Senescent Cells

Intrinsic apoptosis pathway

Although senescent cells are proposed to be resistant to apoptosis, an upregulation of pro-apoptotic "initiators" such as PUMA and BIM has been described, surprisingly coupled to a reduction of anti-apoptotic Bcl-2 [93]. These findings suggest that senescent fibroblasts may be primed to undergo apoptosis, but that the execution of the death program might be restrained. During the mitochondria-mediated intrinsic pathway of apoptosis, pro-apoptotic initiators (e.g., tBid, BIM, PUMA, etc.) activate Bax and Bak, which permeabilize the mithochondria and allow the release of cytochrome C. Cytochrome C and the apoptosis protease factor-1 (Apaf-1) assemble the apoptosome, an important structure capable of activating caspase signaling resulting in the rapid cleavage of multiple substrates within the cell (e.g., caspase-9, caspase-3, etc.). In contrast, anti-apoptotic Bcl-2 homolog proteins such as Bcl-2, Bcl-xL, Bcl-W, and Mcl-1 are primarily known for their inhibitory role in the intrinsic pathway of apoptosis by sequestering Bax and Bak and thereby preventing the leakage of cytochrome C and apoptosome formation (reviewed in Ref. [94]). Although the levels of pro-apoptotic factors in senescent cells are for the most part unclear, several articles report dependence of senescent cells on anti-apoptotic Bcl-2 homolog proteins [36,45,62]. Accordingly, targeting anti-apoptotic Bcl-2 homolog proteins using pharmacological inhibitors ABT-263, ABT-737, A1331852, and A1155463 resulted in apoptosis of various senescent cell types without affecting the viability of normal cells, confirming their potential uses as senolytic drugs [36,38,95,96]. However, these findings also highlighted that Bcl-2 homolog proteins may act in a redundant manner to protect senescent cells from apoptotic death [38], limiting the potential of senolytic drugs based on interfering with this pathway.

Extrinsic apoptosis pathway

Extrinsic apoptosis is a type of programmed cell death initiated extracellularly through the engagement of death receptor signaling on the target cell (reviewed in Ref. [97]). Death receptors are transmembrane proteins of the tumor necrosis factor (TNF) receptor superfamily that contain a cytoplasmic "death" domain capable of transducing an extracellular apoptotic signal into intracellular signaling pathways. The most studied death receptors are TNF receptor 1 (TNFR1), CD95 (Fas), and TNF-related apoptosis inducing ligand-receptor (TRAIL) receptors 1 and 2 (TRAIL-R1 and TRAIL-R2, also known as DR4, and DR5; Table 1).

Upon ligand binding, death receptors can trimerize and recruit specialized adaptor proteins via their death domain such as Fas-associated death domain (FADD). In turn, FADD can recruit pro-caspase 8 and form a death-inducing signaling complex (DISC) capable of generating mature caspase-8 through self-cleavage. Mature caspase-8 can next activate downstream effector caspases such as caspase-3, as well as reinforce apoptosis by engaging the intrinsic apoptosis pathway through proteolytic cleavage of the pro-apoptotic protein Bid into t-Bid. Caspase-8 therefore plays an essential role in apoptosis induction by connecting the extrinsic pathway of apoptosis with the intrinsic mitochondrial pathway. Although it was reported that senescent fibroblasts might increase their resistance to apoptosis via reducing capsase-3 activity [23], the

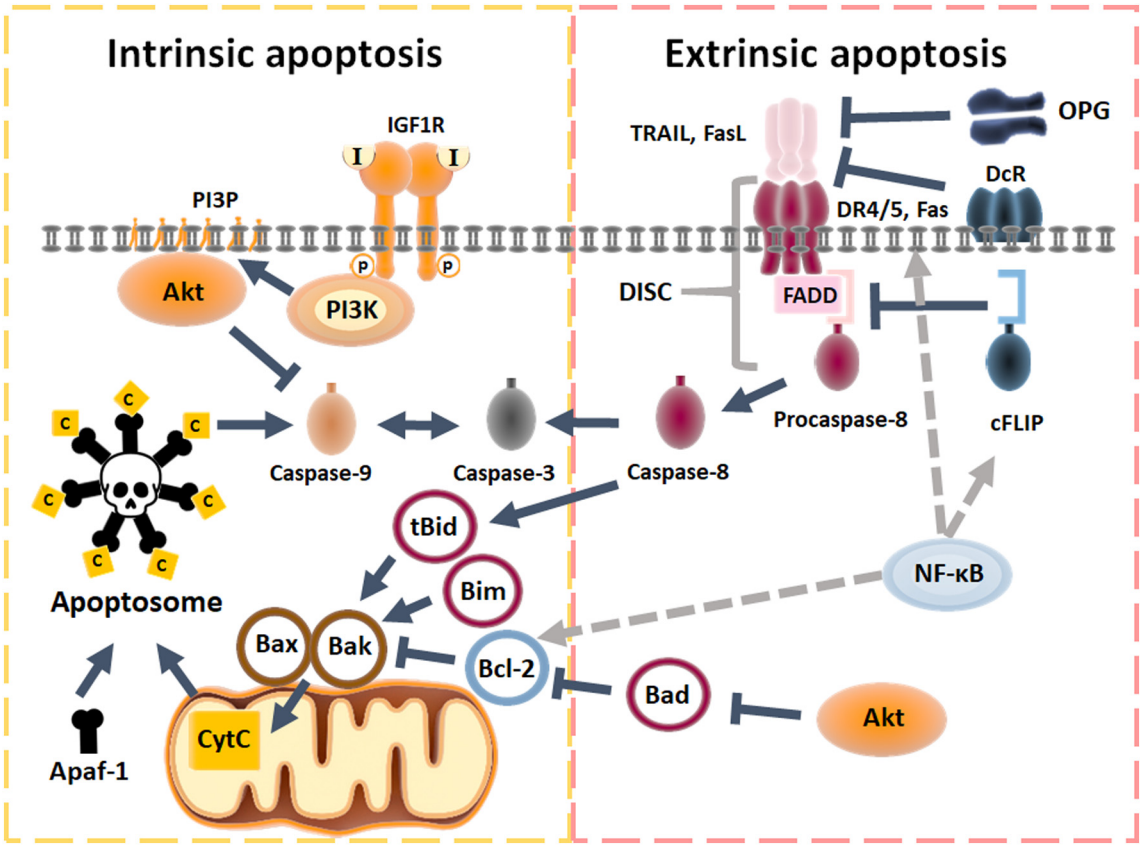

Fig. 4. Intrinsic and extrinsic pathways of apoptosis in senescent cells. The extrinsic pathway of apoptosis is initiated upon binding of apoptotic ligands (TRAIL, FasL) to their respective death receptors (DR4/5, Fas). Activated death receptors recruit adaptor protein FADD, which binds to pro-caspase 8 to form a DISC capable of generating mature caspase-8 through self-cleavage. Caspase-8 can next activate downstream effector caspases such as caspase-3 and reinforce apoptotic signaling by engaging the intrinsic apoptosis pathway through proteolytic cleavage of Bid into t-Bid. tBid and other proapototic proteins such as Bim activate Bax and Bak, which permeabilize the mithochondria and allow the release of cytochrome C. Cytochrome C together with Apaf-1 assembles the apoptosome, which activates caspase-9 and effector caspase-3 resulting in the rapid cleavage of multiple substrates within the cell. In contrast, anti-apoptotic Bcl-2 homolog proteins sequester Bax and Bak, thereby preventing cytochrome C leakage. Pro-apoptotic Bad can bind to anti-apoptotic Bcl-2 proteins, indirectly promoting apoptosis. However, PI3K-Akt signaling may inhibit pro-apoptotic components such Bad or caspase-9.

development of genetic mouse models of inducible death of senescent cells suggest differently. Genetic mouse models of senescent cell clearance include p16-3MR mice (p16 inducible three modality reporter [16]) and INK-ATTAC mice (apoptosis through targeted activation of caspases [35]). Although mechanistically different, treatment with an exogenous drug in both models result in caspase 8 activation and downstream signaling, suggesting that senescent cells are readily capable of caspase-mediated apoptosis, at least downstream of caspase 8 (Fig. 4).

Upstream of caspase-8, cFLIP is a negative regulator of extrinsic apoptosis, and it competes with procaspase-8 for binding to FADD and thus prevents DISC formation. cFLIP is partially responsible for TRAIL resistance in normal cells [98] but is downregulated in response to DNA-damage [99], as well as with the activation of the Myc oncogene in pre-transformed and fully transformed fibroblasts [100]. Moreover, cFLIP expression was shown to decrease with progression into senescence in primary (thymic) epithelial cells, where its expression is regulated via NF-κB activation [101]. These findings suggest that cFLIP may not play a role in senescent cell resistance, while supporting the notion senescent cells may be instead primed to apoptosis.

At the cytoplasmic membrane level, decoy receptors (DcRs) may play a role in resistance to extrinsic apoptosis. DcRs are extracellularly similar to death receptors but lack an intracellular death domain. Consequently, DcRs compete for ligand binding but are incapable of signal transduction, serving as inhibitory mechanisms of extrinsic apoptosis. The upregulation of DcR1 [1,8,102] (TNFRSF10C) and DcR2 [49,103–106] (TNFRSF10D) has been proposed as marker of senescence. Interestingly, the SASP also

includes soluble DcRs such as osteoprotegerin, which binds both to TRAIL and to Receptor activator of nuclear factor kappa-B ligand (RANKL) [1,107].

So far, a protective role has only been demonstrated for DcR2 in human senescent fibroblasts, where silencing of DcR2 using siRNA or shRNA increased susceptibility to TRAIL-induced extrinsic apoptosis [49]. However, non-transformed (normal) cells are already resistant to TRAIL-induced apoptosis due to multiple redundant pathways [98]. An increased resistance of senescent cells in a previously sensitive population, such as cells that have undergone malignant transformation and that were induced into senescence by genotoxic stress, remains to be described. Evidence suggests that chemotherapy and ionizing radiation may instead sensitize cancer cells to extrinsic apoptosis via the upregulation of DR5 and the downregulation of c-FLIP, thereby promoting DISC formation and overcoming DcR2 upregulation [99,108].

Despite an increased resistance of senescent fibroblasts to treatment with all extrinsic apoptosis inducing ligands (Fas [49], TRAIL [49], and TNF-alpha [36]), an increased resistance across cell types seems unlikely. For instance, replicative senescent endothelial cells are more sensitive than early passage cells to both FasL [109] and TNF-alpha [110]. Furthermore, recent evidence suggests that human embryonic stem cells, as well as induced pluripotent stem cells, express all the canonical components of the extrinsic apoptotic signaling pathway, irrespective of their origin [111]. These cells were found to be initially resistant to TRAIL, but sensitization was induced upon stress derived from proteosynthesis inhibition. Accordingly, an increased susceptibility to extrinsic cell death via TRAIL was observed in human embryonic stem cells following treatment with the chemotherapeutic drug cisplatin, a known inducer of senescence [112]. However, cisplatin-treated human embryonic stem cells were not characterized as senescent and treatment with TRAIL was performed only 24 h after treatment with cisplatin, suggesting that a senescent response had not yet been fully established and matured. Nevertheless, these results are similar to the synergistic effects of TRAIL-mediated targeting of DR5 in combination with cisplatin [113] or with ER stress inducers [114], as reported in tumor context. Together, these findings suggest that an increased resistance to extrinsic apoptosis is not a conserved feature of senescent cells.

Immunogenicity of senescent cells

Immune-mediated clearance

The role of the innate immune system in immune-mediated clearance of senescent cells via phagocytes, such as macrophages, has been described for developmental senescence, tissue-repair senescence, and oncogene induced senescence. However, most forms of cellular senescence are proposed to be cleared through the degranulation of natural killer (NK) cells [49]. NKG2D stress ligands are reportedly upregulated in replicative, oncogene-induced, and therapy-induced premature senescence, where they interact with the NKG2D receptor on NK cells and trigger the release of perforin-filled granules [115]. Upon degranulation, perforin-formed pores enable granzyme penetration and caspase activation to induce apoptosis of the target cell. Accordingly, perforin knockout mice accumulate more senescent cells in their tissues with age and display chronic inflammation and increased tissue fibrosis [116]. Similarly, a dependence on the NKG2D receptor–ligand interaction has been recently observed in the removal of senescent cells by uterine NK cells in cycling human endometrium [117].

The role of the adaptive immune system is less understood but is proposed to likewise contribute to senescent cell surveillance, at least in tumorigenic contexts such as oncogene induced senescence. In this case, the mounting of an adaptive response against senescent cells is proposed to occur via (CD4) T cells and to be dependent on the upregulation of MHC class II molecules on the surface of senescent cells [118,119]. The search for additional senescence-associated cell-surface antigens is still ongoing, but a recent study identified a majority of poly-reactive antibodies capable of discriminating senescent cells [120]. Said antibodies were of the IgM isotype of the innate immune system and bound to a surface exposed oxidative adduct of the intermediate filament vimentin. These findings suggest oxidized products resulting from increased ROS in senescent cells may result in the generation of neoantigens recognizable by the immune system as additional “eat-me” signals. Markedly, the complex dynamics of “eat-me” and “don't eat me” signals on the surface of senescent cells remain to be fully elucidated.

Immune checkpoints

The display of “eat-me” signals such as phosphatidylserine is required but not sufficient for the immune clearance of apoptotic cells. Instead, “eat-me” signals need to be paired to the downregulation of “don't eat me” and immunosuppressive signals normally expressed in healthy cells, such as CD47 (reviewed in Ref. [121]). A recent study suggests that healthy cells display CD47 in high-density surface clusters capable of suppressing phagocytosis. In contrast, apoptotic cells lose CD47 clustering, and dispersed CD47 becomes unable to protect from phagocytosis [122]. It is unclear whether senescent cells reach the stage of CD47 signaling inhibition.

NF-κB-dependent pro-inflammatory signaling appears to directly upregulate CD47, at least in some cancers, facilitating their escape from immune surveillance [123]. As NF-κB is considered a master regulator of the SASP, it is tempting to speculate senescent cells may continue to display sufficient levels of CD47. Also, the treatment of endothelial cells with thrombospondin-1, a CD47 ligand, results in cell cycle stalling and senescence induction [124,125]. Recently, Casey *et al*. [126] described that the downregulation of CD47 and of the immune checkpoint inhibitor PD-L1 in cancer cells led to senescence through the inactivation of Myc oncogene. Later evidence revealed that DNA-damaged cancer cells upregulate the immune checkpoint inhibitor PD-L1 in response to DNA double-strand breaks, but PD-L1 levels returns back to normal in the few surviving cells that have entered senescence [127]. The authors proposed a transient upregulation of PD-L1 may therefore prevent an overactivation of the immune system, but PD-L1 overexpression in non-transformed primary fibroblasts following DNA damage was not observed [127]. Put together, these findings suggest that the senescent program does not involve the upregulation of immune checkpoint inhibitors in response to DNA damage but may instead play a role in their downregulation, thereby facilitating senescent cells surveillance.

Similarly, treatment of tumor cells with cyclin-dependent kinases 4/6 (CDK4/6) inhibiting drugs was shown to induce cell cycle arrest and to activate the expression of endogenous retroviral elements, which stimulated the production of type III interferons, leading to enhanced tumor antigen presentation and enhanced anti-tumor immunity [128]. Therefore, in addition to promote cell cycle progression, CDK4/6 may also promote the upregulation of immune checkpoint inhibitors, and their inhibition via CDKis may enhance their susceptibility to immune checkpoint blockades and immune surveillance.

Implications for Senescence Interventions

Increasing evidence suggests that senescent cells are primed to apoptosis due to unresolved chronic stresses, and this might favor the efficacy of known senolytic drugs. In oncology, two-step therapeutic strategies aim to first induce cancer cells into senescence via cytotoxic drugs and then to exploit the vulnerability of senescent cancer cells to apoptosis by using senolytics (reviewed in Ref. [129]). However, given the deleterious roles of senescent cells and the negative systemic side effects associated with chemotherapy, these strategies should be best approached with caution. Recently, the use of genetic screens and compound libraries has yielded aurora kinase inhibitors as powerful inducers of senescence in cancer cells (independent of p53) [130]. Importantly, senescent cancer cells also acquired vulnerability to the anti-apoptotic Bcl-2 inhibitor ABT-263 regardless of how senescence was induced. Further research is needed to assess the effects of aurora kinase inhibition in normal cells, as opposed to chemotherapy, in combination with senolytic drugs.

Redundant mechanisms aid cell death prevention in both senescent and cancer cells, as observed with anti-apoptotic Bcl-2 family homologs [38]. Nevertheless, as senescent cells may rely more on anti-apoptotic players compared to normal cells that are free of intracellular stressors, targeting anti-apoptotic players may still represent a viable therapeutic strategy. Moreover, different apoptotic mechanisms exist across different cell types and senescent programs, and these differences may be exploited to allow preferential elimination of a specific subtype of senescent cells. In this respect, targeting a defined senescent subtype that is relevant to a specific pathology may be more desirable and with less side effects than simultaneously targeting all types of senescent cells.

It is important to note that senescent cells rely on multiple levels of regulation in order to achieve apoptosis resistance. The concurrent targeting of multiple and indirectly related anti-apoptotic pathways (SCAPs) may therefore result in increased sensitivity of senescent cells without incurring in toxicities for normal proliferating or quiescent cells. A combinatorial approach to senescent cell clearance is exemplified by the concomitant treatment of dasatinib and quercetin [62]. Targeting SCAP networks, as opposed to single targets, may enable lowering the therapeutic dosage of each drug, therefore decreasing off- and on-target side effects associated with single drugs. This is also demonstrated in tumor contexts, where the combination of metformin, a known inhibitor of the SASP, was able to decrease the dose of chemotherapy required for prolonged tumor remission [131]. Combined treatment at lower doses may also allow the repurposing of known drugs with senolytic potential that were previously discarded due to undesirable side effects, therefore improving their chance of success in clinical trials.

Despite an increased resistance to certain apoptotic stimuli, senescent cells may be more susceptible to various forms of metabolic targeting. Senescent cell hypercatabolism can be pharmacologically exploited for the elimination of senescent cells by means of synthetic lethal approaches such as glycolysis inhibition [82], autophagy inhibition [82], and mitochondrial targeting [87]. Synthetic lethal metabolic targeting could therefore be used alone or in combination with SCAP inhibitors for increased selectivity.

Finally, additional strategies alternative to apoptosis induction may be employed to alleviate the deleterious phenotypes associated with senescent

cells. For instance, the use of SASP modulators may prevent the establishment of a chronic SASP and dampen the negative side-effects of senescent cell persistence without the need for their removal from tissues. Similarly, the use of selective inhibitors for specific SASP components, such as neutralizing antibodies, may allow a tailoring of the SASP by only targeting SASP components thought to play a negative role in the tissue micro-environment while preserving the beneficial ones. Lastly, enhancing the natural clearance of senescent cells by the immune system could be another way of overcoming apoptosis resistance. The use of immune modulators or artificially increasing the number of immune effector cells may effectively restore senescence surveillance and decrease the senescent cell burden. These strategies have been recently described to more detail elsewhere (reviewed in Refs. [132],[133]).

Concluding Remarks

Senescent cells possess several adaptations in order to prevent cell death, and various features of the senescent phenotype directly contribute to an increased survival. Major efforts have been spent to identify and target pathways conferring resistance to apoptosis. However, most findings on apoptosis resistance have been reported in human fibroblasts, and underlying mechanisms are not necessarily conserved throughout cell types or across the various senescence programs. An important concept, supported by preliminary evidence, is that senescent cells may have an increased susceptibility to alternative forms of regulated cell death, but the molecular mechanisms remain poorly understood. Furthermore, it is unclear which senescent cell types contribute the most to their deleterious effects and thus warrant preferential targeting. Additional research into the mechanisms of apoptosis resistance in other cell types as well as their forms of cell death is required in order to identify exploitable differences for selective targeting.

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SAGA, senescence-associated growth arrest; SASP, senescence-associated secretory phenotype; SCAP, senescent cell anti-apoptotic pathway; UPR, unfolded protein response; TKR, tyrosine kinase receptor; ROS, reactive oxygen species; ER, endoplasmic reticulum; MiDAS, mitochondrial dysfunction-associated senescence; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; DcR, decoy receptor; mTORC, mTOR complex; FoxO, forkhead box O.

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