

Cellular Senescence: Defining a Path Forward

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Cellular senescence is a cell state implicated in various physiological processes and a wide spectrum of age-related diseases. Recently, interest in therapeutically targeting senescence to improve healthy aging and age-related disease, otherwise known as senotherapy, has been growing rapidly. Thus, the accurate detection of senescent cells, especially *in vivo*, is essential. Here, we present a consensus from the International Cell Senescence Association (ICSA), defining and discussing key cellular and molecular features of senescence and offering recommendations on how to use them as biomarkers. We also present a resource tool to facilitate the identification of genes linked with senescence, SeneQuest (available at <http://Senequest.net>). Lastly, we propose an algorithm to accurately assess and quantify senescence, both in cultured cells and *in vivo*.

Cellular Senescence: Walking a Line between Life and Death

Cell states link both physiological and stress signals to tissue homeostasis and organismal health. In both cases, the outcomes vary and are determined by the signal characteristics (type, magnitude, and duration), spatiotemporal parameters (where and when), and cellular capacity to respond (Gorgoulis et al., 2018). In the case of potentially damaging stress, damage is reversed and the structural and functional integrity of cells restored. Alternatively, damage can be irreversible, and

cells activate death mechanisms mainly to restrict the impact on tissue degeneration. Between these extremes, cells can acquire other states, often associated with survival but also with permanent structural and functional changes. An example is the non-proliferative but viable state, distinct from G0 quiescence and terminal differentiation, termed cellular senescence (Rodier and Campisi, 2011). Formally described in 1961 by Hayflick and colleagues, cellular senescence, derived from the latin word *senex* meaning “old” (Hayflick and Moorhead, 1961), was originally observed in normal diploid cells that



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ceased to proliferate after a finite number of divisions (Hayflick limit), later attributed to telomere shortening (see section “Cell-Cycle Arrest”).

Cellular senescence has since been identified as a response to numerous stressors, including exposure to genotoxic agents, nutrient deprivation, hypoxia, mitochondrial dysfunction, and oncogene activation (Table 1). Over the last decade, improved experimental tools and the development of reporter-ablation mouse models have significantly advanced our knowledge about causes and phenotypic consequences of senescent cells. However, specific markers and a consensus on the definition of what constitutes senescent cells are lacking. Further, although a link to organismal aging is clear, aging and senescence are not synonymous (Rodier and Campisi, 2011). Indeed, cells can undergo senescence, regardless of organismal age, due to myriad signals including those independent of telomere shortening. Consequently, senescent cells are detected at any life stage from embryogenesis, where they contribute to tissue development, to adulthood, where they prevent the propagation of damaged cells and contribute to tissue repair and tumor suppression. Thus, cellular senescence might be an example of evolutionary antagonistic pleiotropy or a cellular program with beneficial and detrimental effects. Here, we clarify the nature of cellular senescence by: (1) presenting key features of senescent cells, (2) providing a comprehensive definition of senescence, (3) suggesting means to identify senescent cells, and (4) delineating the role of senescent cells in physiological and pathological processes, that altogether pave the way for developing new therapeutic strategies.

Definition and Characteristics of Cellular Senescence

Cellular senescence is a cell state triggered by stressful insults and certain physiological processes, characterized by a prolonged and generally irreversible cell-cycle arrest with secretory

features, macromolecular damage, and altered metabolism (Figure 1). These features can be inter-dependent (Figure 1) but for clarity are described here separately.

Cell-Cycle Arrest

One common feature of senescent cells is an essentially irreversible cell-cycle arrest that can be an alarm response instigated by deleterious stimuli or aberrant proliferation. This cell-cycle withdrawal differs from quiescence and terminal differentiation (He and Sharpless, 2017). Quiescence is a temporary arrest state with proliferation re-instated by appropriate stimuli; terminal differentiation is the acquisition of specific cellular functions accompanied by a durable cell-cycle arrest mediated by pathways distinct from those of cellular senescence (Figure 2). In turn, senescent cells acquire a new phenotype. Although the senescence cell-cycle arrest is generally irreversible, cell-cycle re-entry can occur under certain circumstances, particularly in tumor cells (Galanos et al., 2016; Milanovic et al., 2018; Patel et al., 2016; Saleh et al., 2019) (Figure 2).

In mammalian cells, the retinoblastoma (RB) family and p53 proteins are important for establishing senescent cell-cycle arrest (Rodier and Campisi, 2011). RB1 and its family members p107 (RBL1) and p130 (RBL2) are phosphorylated by specific cyclin-dependent kinases (CDKs; CDK4, CDK6, CDK2). This phosphorylation reduces the ability of the RB family members to repress E2F family transcription factor activity, which is required for cell-cycle progression (Sharpless and Sherr, 2015). In senescent cells, however, the CDK2 inhibitor p21^{WAF1/Cip1} (CDKN1A) and CDK4/6 inhibitor p16^{INK4A} (CDKN2A) accumulate. This accumulation results in persistent activation of RB family proteins, inhibition of E2F transactivation, and consequent cell-cycle arrest, which, in time, cannot be reversed by subsequent inactivation of RB family proteins or p53 (Beauséjour et al., 2003). This persistence is enforced by heterochromatinization of E2F target genes (Salama et al., 2014), the effects of cytokines secreted by senescent cells (Rodier and Campisi, 2011), and/or

Table 1. Selected List of Factors Triggering Senescence

Inducer		<i>In vivo</i> process
Telomere attrition	Inhibitors of telomerase activity (e.g., SYUIQ-5, pyridostatin, azidothymidine)	Aging; cancer
Genotoxic drugs	DNA replication stress inducers (e.g., hydroxyurea, bromodeoxyuridine); DNA-damaging agents including DNA topoisomerase inhibitors (e.g., doxorubicin, etoposide), DNA crosslinkers (e.g., cisplatin, mitomycin C), and drugs with complex effects (e.g., actinomycin D, bleomycin)	Cancer treatment and side effects
Irradiation	Ionizing and UV	Cancer treatment and side effects
Oncogenic stress		Tumor suppression and promotion
Loss of tumor suppressors		Tumor suppression and promotion
Replicative and/or mitotic stress		Aging; cancer treatment
Oxidative stress	Reactive oxygen species (ROS) inducers (e.g., hydrogen peroxide, paraquat)	Aging; tissue repair
Mitochondrial dysfunction		Aging?
Perturbed proteostasis	ER stress, mTOR, UPR	Aging, diet
Ribosomal stress		N/A (Aging?)
Inhibitors of cyclin-dependent kinases	p16/p21 up-regulation (activators of p53) (e.g., nutlin 3a); drugs (e.g., palbociclib, ribociclib)	Tumor suppression; cancer treatment
Cytokines	TGF- β	Aging?
Activators of protein kinase C	TPA/PMA, PEP005, PEP008	Aging?
Epigenetic modifiers	DNA methyltransferases inhibitors (e.g., 5-aza-2-deoxycytidine); histone deacetylases inhibitors (e.g., sodium butyrate, trichostatin A); histone acetyltransferases inhibitors (e.g., curcumin, C646); histone methyltransferases inhibitors (e.g., BRD4770)	Cancer treatment
Matricellular proteins	CCN1	Tissue repair
High-fat diet (hyperglycemia)		Diet; diabetes
Autophagy impairment		Aging and/or age-related diseases?
Lamin B1 silencing		Progeroid syndromes?
N/A, not available.		

enduring reactive oxygen species (ROS) production (Takahashi et al., 2006). Notably, in senescent murine cells, ARF—an alternate reading frame protein of the *p16^{INK4a}* gene locus that activates p53—also has an important role in regulating cell-cycle arrest (Sharpless and Sherr, 2015).

Additional features of the senescent cell-cycle arrest include ribosome biogenesis defects and derepression of retrotransposons (De Cecco et al., 2019; Lessard et al., 2018). However, currently no specific marker of the senescent cell-cycle arrest has been identified (Hernandez-Segura et al., 2017). For example, RB and p53 activation also occurs in other forms of cell-cycle arrest (Rodier and Campisi, 2011). Even p16^{INK4A}, which is considered more specific to senescence, is expressed in certain non-senescent cells (Sharpless and Sherr, 2015) and is not expressed by all senescent cells (Hernandez-Segura et al., 2017). Thus, detecting a senescence-associated cell-cycle arrest requires quantification of multiple factors and features.

Secretion

Senescent cells secrete a plethora of factors, including pro-inflammatory cytokines and chemokines, growth modulators, angiogenic factors, and matrix metalloproteinases (MMPs), collectively termed the senescent associated secretory pheno-

type (SASP) or senescence messaging secretome (SMS) (Figure 1; Table 2) (Coppé et al., 2010; Kuilman and Peeper, 2009). The SASP constitutes a hallmark of senescent cells and mediates many of their patho-physiological effects. For example, the SASP reinforces and spreads senescence in autocrine and paracrine fashions (Acosta et al., 2013; Coppé et al., 2010; Kuilman and Peeper, 2009) and activates immune responses that eliminate senescent cells (Krizhanovsky et al., 2008a; Muñoz-Espín and Serrano, 2014). SASP factors mediate developmental senescence (Muñoz-Espín et al., 2013; Storer et al., 2013), wound healing (Demaria et al., 2014), and tissue plasticity (Mosteiro et al., 2016) and also contribute to persistent chronic inflammation (known as inflammaging) (Franceschi and Campisi, 2014). Thus, the SASP can explain some of the deleterious, pro-aging effects of senescent cells. Further, the SASP can recruit immature immune-suppressive myeloid cells to prostate and liver tumors (Di Mitri et al., 2014; Eggert et al., 2016) and stimulate tumorigenesis by driving angiogenesis and metastasis (Coppé et al., 2010).

While the senescent cell-cycle arrest is regulated by the p53 and p16^{INK4A}/Rb tumor suppressor pathways, the SASP is controlled by enhancer remodeling and activation of

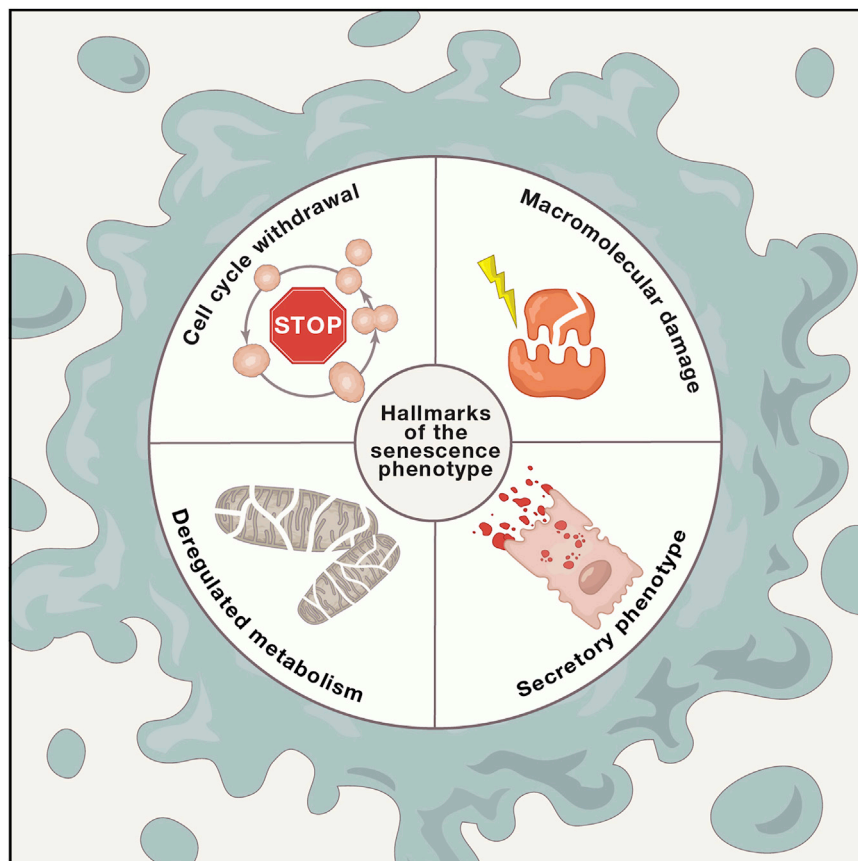


Figure 1. The Hallmarks of the Senescence Phenotype

Senescent cells exhibit the following four interdependent hallmarks: (1) cell-cycle withdrawal, (2) macromolecular damage, (3) secretory phenotype (SASP), and (4) deregulated metabolism (see also text).

(Takasugi et al., 2017). Overall, defining the senescent secretome in each biological context will help identify senescence-based molecular signatures.

Macromolecular Damage

DNA Damage. The first molecular feature associated with senescence was telomere shortening, a result of the DNA end-replication problem, during serial passages (Shay and Wright, 2019). Telomeres are repetitive DNA structures found in terminal loops at chromosomal ends and stabilized by the Shelterin protein complex. This organization renders telomeres unrecognizable by the DNA damage response (DDR) and double-strand DNA break (DSB) repair pathways (de Lange, 2018; Shay and Wright, 2019). Telomerase, the enzyme that maintains telomere length, is not expressed by most normal somatic (non-stem) cells but is expressed by most cancer cells that have overcome senescence. Moreover,

telomerase activity reconstitution in normal cells leads to telomere elongation, extending their replicative lifespan in culture (Bodnar et al., 1998; Shay and Wright, 2019).

Telomere shortening during proliferation culminates in telomeric DNA loop destabilization and telomere uncapping, generating telomere dysfunction-induced foci (TIFs) that activate the DDR, eventually causing cell-cycle arrest. This response can also be elicited by inhibiting or altering genes involved in telomere maintenance (d'Adda di Fagagna, 2008). Another form of DNA damage, termed telomere-associated foci (TAFs), can exist at telomeres due to oxidative DNA damage at telomeric G-rich repeats, irrespective of telomere length or shelterin loss (de Lange, 2018; Shay and Wright, 2019).

Although half of the persistent DNA damage foci in senescent cells localize to telomeres, other stressful subcytotoxic insults can trigger senescence by inducing irreparable DNA damage (Figure 1). Numerous genotoxic agents, including radiation (ionizing and UV), pharmacological agents (e.g., certain chemotherapeutics), and oxidative stress trigger senescence. Moreover, activated oncogenes can induce senescence (known as OIS) as a tumor-suppressive response, restricting the uncontrolled proliferation of potentially oncogenic cells. OIS is often mediated by the tumor suppressors p16^{INK4A} and ARF, both encoded by the *CDKN2A* locus, imposing a cell-cycle arrest (Kuilman et al., 2010; Serrano et al., 1997). However, the DDR also plays a major role in triggering OIS (Gorgoulis and

transcription factors, such as NF- κ B, C/EBP β , GATA4 (Ito et al., 2017; Kang et al., 2015; Kuilman and Peeper, 2009; Salama et al., 2014), mammalian target of rapamycin (mTOR) and p38MAPK signaling pathways (Freund et al., 2011; Ito et al., 2017; Kuilman and Peeper, 2009). Upstream signals triggering SASP activation are multiple and differ depending on the senescence inducer but include DNA damage, cytoplasmic chromatin fragments (CCFs) that trigger a type 1 interferon response, and damage-associated molecular patterns (DAMPs) that activate the inflammasome (Acosta et al., 2013; Davalos et al., 2013; Li and Chen, 2018).

The SASP composition and strength varies substantially, depending on the duration of senescence, origin of the pro-senescence stimulus, and cell type (Childs et al., 2015). Further, single-cell RNA sequencing (scRNA-seq) reveals considerable cell-to-cell variability of SASP expression (Wiley et al., 2017). For example, transition from an early transforming growth factor β (TGF- β)-dependent secretome to a pro-inflammatory secretome is governed by fluctuation of Notch1 activity (Ito et al., 2017). Moreover, an interferon type 1 response occurs as a later event and is driven in part by derepression of LINE-1 retrotransposable elements (De Cecco et al., 2019). Senescent cells also communicate with their microenvironment through juxtacrine NOTCH/JAG1 signaling (Ito et al., 2017), release of ROS (Kuilman et al., 2010), cytoplasmic bridges (Video S1) (Biran et al., 2015), and extracellular vesicles, such as exosomes

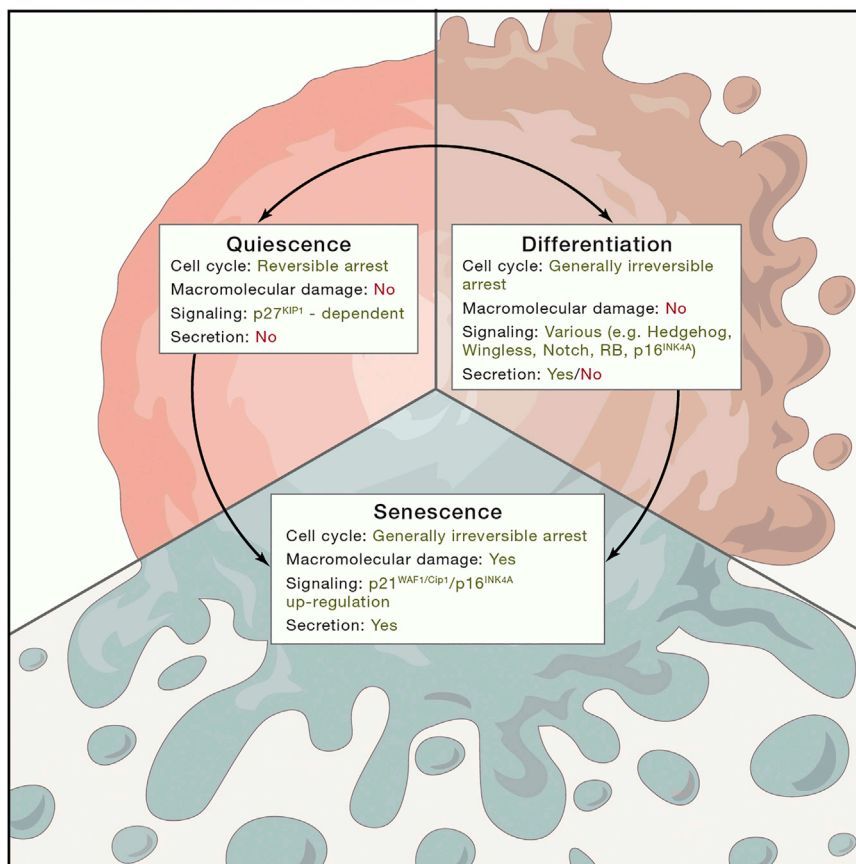


Figure 2. Cell-Cycle Withdrawal in Senescent, Quiescent, and Terminally Differentiated Cells

Depicted are differences in cell-cycle-arrest reversibility, activated signals (see text), secretory functions, and macromolecular damage that allow discrimination between these cellular states. Macromolecular damage is a common feature of senescence. Secretion is another common feature of senescence and is sometimes (context-dependently) found in the differentiated state. Cell-cycle arrest is generally considered irreversible during senescence and terminal differentiation, although cell-cycle re-entry can occur under certain conditions. Green color: active and/or present, red color: inactive and/or absent. Arrows depict connections between the cellular states.

damaged proteins help identify senescent cells (Figure 1). A prominent source of protein damage is ROS, which oxidize both methionine and cysteine residues and alter protein folding and function (Höhn et al., 2017). Many protein tyrosine phosphatases (PTPs) contain cysteine residues in their active sites that can be inactivated by oxidation. This inactivation can trigger senescence by hyperactivating ERK signaling, similar to the effect of activated oncogenes (Deschênes-Simard et al., 2013). High phospho-ERK levels were detected in pre-neoplastic lesions, rich in senescent cells such as mel-

Halazonetis, 2010; Gorgoulis et al., 2018; Halazonetis et al., 2008). In this case, the damage signal originates at collapsed replication forks as a result of oncogene-driven hyperproliferation. Recently, it was shown that the DDR and ARF pathways can act in concert during OIS with the former requiring a lower oncogenic load than the latter (Gorgoulis et al., 2018).

Senescent cells harbor persistent nuclear DNA damage foci termed DNA-SCARs (DNA segments with chromatin alterations reinforcing senescence). DNA-SCARs are distinct from transient damage foci; unlike transient foci, they specifically associate with promyelocytic leukemia (PML) nuclear bodies, lack the DNA repair proteins RPA and RAD51 as well as single-stranded DNA (ssDNA), and contain activated forms of the DDR mediators CHK2 and p53 (Rodier et al., 2011). DNA-SCARs are dynamic structures with the potential to regulate multiple aspects of the senescent cells, including growth arrest and SASP (Rodier et al., 2011). However, as not all senescence-inducing stimuli generate a persistent DNA damage response, DNA-SCARs are not a global feature of the senescent cells. CCFs are another type of DNA damage in senescent cells (Ivanov et al., 2013). These CCFs activate a proinflammatory response, mediated by the cGAS-cGAMP-STING pathway (Ivanov et al., 2013; Li and Chen, 2018), that can serve as another non-inclusive senescence-associated marker.

Protein Damage. Proteotoxicity is a hallmark of aging and cellular senescence (Kaushik and Cuervo, 2015). Hence,

anocytic nevi and benign prostatic hyperplasia (BPH) (Deschênes-Simard et al., 2013), and are a characteristic of therapy-induced senescence (Haugstetter et al., 2010). The PTP oxidation pattern can be revealed by a monoclonal antibody that recognizes oxidized cysteine (Karisch et al., 2011).

ROS, in the presence of metals, can carbonylate proline, threonine, lysine, and arginine residues. Protein carbonylation exposes hydrophobic surfaces, leading to unfolding and aggregation, and protein carbonyl residues can be specifically detected using antibodies (Nyström, 2005). Moreover, carbonyl residues can react with amino groups to form Schiff bases, contributing to protein aggregation. Subsequent cross linking with sugars and lipids forms insoluble aggregates, termed lipofuscin from the Greek “lipo” meaning fat and “fuscus” meaning dark. Lipofuscin can be visualized in lysosomes by light microscopy or a histochemical method using a biotinylated Sudan Black B (SBB) analog (GL13) (Evangelou et al., 2017). The latter is emerging as another indicator of senescent cells in culture and *in vivo* (Evangelou et al., 2017; Gorgoulis et al., 2018; Myrianthopoulos et al., 2019). It should be noted that damage accumulation continues, even when cell division ceases, and can continue for months or even years.

Most protein oxidative damage is not reversible, and degradation by the ubiquitin proteasome system (UPS) or autophagy often eliminates these proteins. As the UPS (Deschênes-Simard et al., 2013) and autophagy are active in senescent cells, they

Table 2. Senescence-Associated Secretory Phenotype (SASP) Components

Class	Component
Interleukins	IL-6; IL-7; IL-1; IL-1b; IL-13; IL-15
Chemokines	IL-8; GRO-a, -b, -g; MCP-2; MCP-4; MIP-1a; MIP-3a; HCC-4; eotaxin; eotaxin-3; TECK; ENA-78; I-309; I-TAC
Other inflammatory molecules	TGF β ; GM-CSF; G-CSF; IFN- γ ; BLC; MIF
Growth factors; regulators	Amphiregulin; epiregulin; heregulin; EGF; bFGF; HGF; KGF (FGF7); VEGF; angiogenin; SCF; SDF-1; PIGF; NGF; IGFBP-2, -3, -4, -6, -7
Proteases and regulators	MMP-1, -3, -10, -12, -13, -14; TIMP-1; TIMP-2; PAI-1, -2; tPA; uPA; cathepsin B
Receptors; ligands	ICAM-1, -3; OPG; sTNFR1; sTNFR2; TRAIL-R3; Fas; uPAR; SGP130; EGF-R
Non-protein molecules	PGE2; nitric oxide; ROS
Insoluble factors	Fibronectin; collagens; laminin

Data are based on [Coppé et al. \(2010\)](#).

could prove to be useful in characterizing the senescent state ([Ogrodnik et al., 2019a](#)). Similarly, PML bodies act as sensors of ROS and oxidative damage ([Niwa-Kawakita et al., 2017](#)) and can also be non-exclusive biomarkers of cellular senescence ([Vernier et al., 2011](#)).

Lipid Damage. Lipids are essential for cell membrane integrity, energy production, and signal transduction. Some age-related diseases are characterized by altered lipid metabolism, resulting in lipid profile changes ([Ademowo et al., 2017](#)). Although senescent cells are marked by changes in lipid metabolism, it is unclear how this contributes to the senescent phenotype ([Figure 1](#)).

Mitochondrial dysfunction during senescence can result in ROS-driven lipid damage, lipid deposits ([Correia-Melo et al., 2016; Ogrodnik et al., 2017](#)), and lipofuscin accumulation ([Gorgoulis et al., 2018](#)). Apart from oxidation, lipid-derived aldehyde modifications (e.g., 4-hydroxy-2-nonenal [4-HNE]) have been reported in senescent cells ([Ademowo et al., 2017; Jurk et al., 2012](#)).

Lipid accumulation in senescent cells can be visualized using various commercial dyes and assays ([Ogrodnik et al., 2017](#)) or immunostaining for lipid associated proteins such as perilipin 2 ([Ogrodnik et al., 2017](#)). Importantly, genetic or pharmacological clearance of senescent cells in obese and aging mice reduced lipid deposits in liver ([Ogrodnik et al., 2017](#)) and brain ([Ogrodnik et al., 2019b](#)).

Despite the association with lipid accumulation, our knowledge about specific lipid metabolite composition in senescent cells is sparse. Fatty acids, their precursors, and phospholipid catabolites—such as eicosapentaenoate (EPA), malonate, 7- α -hydroxy-3-oxo-4-cholestenoate (7-HOCA), and 1-stearoylglycerophosphoinositol—increase in senescent fibroblasts, whereas linoleate, dihomo-linoleate, and 10-heptadecenoate decline ([James et al., 2015](#)). Moreover, free cholesterol rises, accompanied by reduced phospholipids and cholesteryl esters derived from acetate, while fatty acid synthase and stearoyl-CoA desaturase-1 decline ([Maeda et al., 2009](#)). Several methods are available to detect lipid changes in tissues and cells, but their use as a senescence biomarker remains limited due to high variability of the senescence-associated lipid profile. For example, lipid metabolites vary significantly between oncogene-induced senescence and replicative senescence ([Quijano et al., 2012](#)).

Deregulated Metabolic Profile

Mitochondria. Senescent cells exhibit several changes in mitochondrial function, dynamics, and morphology. Mitochondria in senescent cells are less functional, showing decreased membrane potential, increased proton leak, reduced fusion and fission rates, increased mass, and abundance of tricarboxylic acid (TCA) cycle metabolites ([Kaplon et al., 2013; Passos et al., 2010](#)). While mitochondria are more abundant, it appears their ability to produce ATP is compromised ([Birch and Passos, 2017; Korolchuk et al., 2017](#)). In contrast, senescent cells often produce more ROS, which can cause protein and lipid damage, as discussed in previous sections (see “Protein Damage” and “Lipid Damage”), but also telomere shortening and DDR activation ([Passos et al., 2007](#)). Targeting aspects of mitochondrial biology, such as the electron transport chain (ETC), complex I assembly, mitochondrial fission rates, and biogenesis, mitochondrial sirtuins and/or disruption of the TCA cycle can trigger senescence ([Correia-Melo et al., 2016; Jiang et al., 2013; Kaplon et al., 2013; Miwa et al., 2014; Moiseeva et al., 2009; Park et al., 2010; Wiley et al., 2016](#)). Altered AMP:ATP and ADP:ATP ratios during senescence contribute to cell-cycle withdrawal by activating AMPK (AMP-activated protein kinase), a main sensor of energy deprivation ([Birch and Passos, 2017](#)).

Mitochondrial dysfunction during senescence is also implicated in SASP regulation. Mitophagy (mitochondrial clearance) in senescent cells appears to suppress the SASP ([Correia-Melo et al., 2016](#)). Genetic or pharmacological inhibition of the ETC can induce senescence even though cells lack expression of key pro-inflammatory SASP factors, such as IL-6 and IL-8 ([Wiley et al., 2016](#)). NAD⁺/NADH ratios are reduced in senescent cells ([Wiley et al., 2016](#)), which could alter the activity of poly-ADP ribose polymerase (PARP) and sirtuins, both involved in activation of the SASP regulator NF- κ B ([Birch and Passos, 2017](#)).

While substantial data support a role for mitochondria in senescence in culture, less is known *in vivo*. Mouse models of mitochondrial dysfunction and enhanced oxidative stress show increased senescence ([Wiley et al., 2016](#)), but a detailed characterization of mitochondrial function in senescent cells *in vivo* is lacking. Because mitochondrial dysfunction characterizes other cellular processes ([Eisner et al., 2018](#)), it is not a consistent biomarker of senescence. Finally, it is not clear whether senescent cells contribute to declining mitochondrial function during aging and age-related diseases ([Srivastava, 2017](#)).

Lysosomes. Secretion requires simultaneous activation of anabolic and catabolic processes (see [Secretion](#)) ([Salama et al., 2014](#)). Increased catabolism provides energy and raw materials, and is favored by the lysosome, the end-degradation compartment of phagocytosis, endocytosis, and autophagy ([Settembre and Ballabio, 2014](#)). Lysosome biogenesis is transcriptionally driven and depends on the cellular energetic or degradative needs ([Settembre and Ballabio, 2014](#)). Intriguingly, when amino acid levels in the lysosomal lumen are high, mTOR1 is recruited and activated and *vice versa* ([Settembre and Ballabio, 2014](#)). Additionally, lysosomes interact with mitochondria to preserve mitochondrial homeostasis (see “Mitochondria”) ([Park et al., 2018](#)).

Lysosomes in senescent cells increase in number and size, evident by the cytoplasmic granularity seen microscopically ([Robbins et al., 1970](#)) [Video S1](#); for non-senescent cells see [Video S2](#)). The increased lysosomal number might reflect an attempt to balance the gradual accumulation of dysfunctional lysosomes by producing more new lysosomes. Thus, the balance between anabolism and catabolism, vital for secretion, is extended. This balance is maintained during OIS through the TOR-autophagy spatial-coupling compartment (TASCC), which coordinates the production of SASP factors ([Salama et al., 2014](#)).

The elevated lysosomal content does not necessarily reflect increased activity, as the degradation stage of autophagy also declines ([Park et al., 2018](#)). Thus, the lysosome-mitochondrial axis degrades, leading to decreased mitochondrial turnover that increases ROS production. Subsequently, ROS targets cellular structures, including lysosomes, which forms a vicious feedback loop that induces more damage ([Park et al., 2018](#)). The increased lysosomal mass has been linked to senescence-associated beta-galactosidase (SA- β -gal) activity ([Hernandez-Segura et al., 2018](#)), a senescence biomarker. However, although the SA- β -gal is prominent in senescent cells ([Dimri et al., 1995](#); [Hernandez-Segura et al., 2018](#)), it is neither required nor a determinant of the senescent phenotype ([Hernandez-Segura et al., 2018](#)). From a therapeutic viewpoint, the enlarged lysosomal compartment offers an increased capacity to trap drugs that can be protonated, such as the selective CDK4/6 inhibitors palbociclib, ribociclib, and abemaciclib. This capacity reduces their effective concentration in the cytosol and nucleus but is counteracted by the slow release of the drugs from the lysosomes, thereby increasing drug exposure time ([Llanos et al., 2019](#)). Another senescence trait, related to lysosomal malfunction, is the intra-lysosomal accumulation of lipofuscin aggresomes (see “Protein Damage” and “Lipid Damage”) (as reviewed in [Gorgoulis et al., 2018](#)). Interestingly, lipofuscin was reported to stimulate expression of the anti-apoptotic factor Bcl-2, conferring resistance to apoptosis, another characteristic of senescent cells ([McHugh and Gil, 2018](#)). Lysosomes in senescent cells also participate in chromatin processing (CCFs) (see “DNA Damage” and [Secretion](#)) ([Ivanov et al., 2013](#)).

Senescence-Associated (Epi-)genetic and Gene Expression Changes

The features listed above are associated with changes in gene expression, determined by transcriptional regulation of coding and non-coding RNAs, which can be exploited for senescence

detection. Here, we discuss such major alterations and describe a novel database that can aid the identification of genes associated with senescence, termed SeneQuest (<http://Senequest.net>) (see Supplementary Information and [Table S1](#)).

Chromatin Landscape

Epigenetic modifications occur during senescence but are mostly context dependent ([Cheng et al., 2017](#)). For example, replicative senescence has been correlated with global loss of DNA methylation at CpG sites ([Cheng et al., 2017](#)). In addition to the global loss of DNA methylation, cellular senescence entails focal increases in DNA methylation at certain CpG islands ([Cruickshanks et al., 2013](#)). Interestingly, this DNA methylation profile somewhat resembles the cancer- and aging-associated methylome patterns ([Cruickshanks et al., 2013](#); [Xie et al., 2018](#)). Cells undergoing OIS fail to show such alterations in DNA methylation ([Xie et al., 2018](#)), reinforcing the diverse nature of epigenetic alterations during senescence.

Senescent cells also exhibit a global increase in chromatin accessibility, but the genome-wide profile varies depending on the stimulus ([De Cecco et al., 2013](#)). Individual histone modifications and variants ([Cheng et al., 2017](#); [Hernandez-Segura et al., 2018](#); [Rai et al., 2014](#)) demonstrate alterations during senescence. For instance, H4K16ac is often enriched at active promoters in senescent, but not proliferating, cells ([Rai et al., 2014](#)). Its accumulation correlates closely with histone variant H3.3, which is deposited into chromatin in a DNA-replication-independent manner by the HIRA/UBN1/CABIN1 and ASF1a chaperones ([Rai et al., 2014](#)). Notably, N terminus proteolytic cleavage of H3.3 correlates with gene repression in a different subset of genes during senescence ([Ivanov et al., 2013](#)). Global loss of linker histone H1 is another senescence feature ([Funayama et al., 2006](#)). Certain histone modifications are crucial for senescence, such as elevated H4K20me3 and H3K9me3, which contribute to the proliferation arrest ([Cheng et al., 2017](#); [Di Micco et al., 2011](#); [Salama et al., 2014](#)), whereas elevated H3K27ac at gene enhancers promotes a SASP ([Hernandez-Segura et al., 2018](#)).

Senescence is also associated with chromatin morphological changes. Senescence-associated heterochromatin foci (SAHFs), visualized as DAPI-dense foci, are enriched in heterochromatin protein (HP) 1. SAHFs derive from chromatin factors—including RB, histone variant macroH2A, high mobility group A proteins, the HIRA/UBN1/CABIN1, and ASF1a chaperones—and increased nuclear pore density ([Boumendil et al., 2019](#); [Salama et al., 2014](#)). SAHFs were initially hypothesized to contribute to gene regulation ([Salama et al., 2014](#)). However, SAHFs were since shown to comprise of largely late-replicating gene poor heterochromatic regions, even in proliferating cells, suggesting a small role in senescence-associated gene expression ([Salama et al., 2014](#)). Senescence is also correlated with global loss of linker histone H1 ([Funayama et al., 2006](#)). Notably, SAHFs seem to be cell type and stimulus dependent, as they are not seen in all senescent cells ([Di Micco et al., 2011](#); [Kennedy et al., 2010](#); [Sharpless and Sherr, 2015](#)), rendering them useful for senescence identification, while the functional significance remains to be elucidated.

Another chromatin feature, termed senescence-associated distension of satellites (SADSs), corresponds to de-compaction of (peri-)centric constitutive heterochromatin ([Cruickshanks](#)

et al., 2013; De Cecco et al., 2013; Swanson et al., 2013). SADSs precede SAHFs formation and might be widely linked to senescence (Swanson et al., 2013). Retrotransposable elements are another type of constitutive heterochromatin related to senescence. The normally repressed LINE-1 (L1) retrotransposons are activated, stimulating the cGAS-STING pathway that elicits a type 1 interferon response (see *Secretion*) (De Cecco et al., 2013). Therefore, in addition to triggering genomic instability, these elements fuel the SASP (Criscione et al., 2016).

Downregulation of lamin B1, a major component of the nuclear lamina, is another key feature of senescence (Dou et al., 2015; Freund et al., 2012; Shah et al., 2013; Shimi et al., 2011). Lamin B1 loss correlates with epigenetic profiles (Salama et al., 2014), as well as senescence-associated chromatin structures (SAHFs and SADSs) (Salama et al., 2014; Swanson et al., 2013). Its reduction occurs predominantly at H3K9me3-rich regions, a process that appears to liberate H3K9me3 from the nuclear lamina promoting spatial rearrangement of H3K9me3 heterochromatin to form SAHFs (Salama et al., 2014). Hi-C analysis (genome-wide mapping of chromatin contacts) in OIS revealed a reduction in local connectivity at regions enriched for H3K9me3 and lamin B1, perturbing these long-range interactions (Chandra et al., 2015). Replicative senescence, on the other hand, showed loss of long-range and gain of short-range interactions within chromosomes (Criscione et al., 2016), implying that the nature of senescence-associated high-order chromatin interactions is stimulus and context dependent (Zirkel et al., 2018). Furthermore, lamin B1 loss and reduced nuclear integrity is suggested to fuel the SASP by contributing to CCF formation (Dou et al., 2015; Ivanov et al., 2013), thereby stimulating the cGAS-STING pathway and interferon response (see *Secretion*) (Li and Chen, 2018). Autophagy-mediated CCF formation (Dou et al., 2015) together with reduced histone synthesis (O'Sullivan et al., 2010) might also lead to a global loss of core histones during senescence, affecting the chromatin landscape (Chan and Narita, 2019; Ivanov et al., 2013).

Transcriptional Signatures

Several genes linked to the cell-cycle arrest and SASP are frequently interrogated in combination with other biomarkers to validate the senescence phenotype or type of senescence (Figure 1). For example, increased expression of the cyclin-dependent kinase inhibitors, CDKN1A (p21^{WAF1/Cip1}), CDKN2A (p16^{INK4A}), and CDK2B (p15^{INK4B}), and a subset of SASP genes, along with decreased expression of cyclins CCNA2 and CCNE2 and LMNB1 should be determined. In addition, the transcriptome of putative senescent cells should be established, which can then be compared with the increasing number of existing senescence transcriptomes (Hernandez-Segura et al., 2018).

Whole-transcriptome studies have been instrumental in defining major signaling pathways involved in establishing senescence phenotypes, and in some cases, predicting drug targets (Zhu et al., 2015). A set of 13 genes was differentially regulated in several cell types undergoing distinct forms of senescence, including oncogene-, replicative-, and DNA-damage-induced senescence (Hernandez-Segura et al., 2017). More recently, a similar study, which considered only fibroblasts and endothelial cells, also attempted to define senescence-associated transcriptome signatures (Casella et al., 2019). Due

to the current paucity of transcriptome data sets, and the availability of more single-cell studies that allow evaluation of intra-population variability (Wiley et al., 2017; Zirkel et al., 2018), these gene signatures will likely change in coming years. But ultimately a senescence-gene-expression signature will prove valuable for identifying senescence under many conditions in culture and *in vivo*.

miRNAs and Non-coding RNAs

Non-coding RNAs, particularly microRNAs (miRNAs), can influence the senescence program, alone or in concert. Functional studies revealed several miRNAs that directly or indirectly modulate the abundance of key senescence effectors, including p53, p21^{WAF1/Cip1}, and SIRT1 (Suh, 2018). miR-504 targets the p53 3'UTR, reducing p53 abundance and activity (Hu et al., 2010). Also, Gld2-mediated stabilization of miR-122 enables its binding to the CBEP 3' UTR, resulting in decreased p53 mRNA polyadenylation and translation (Burns et al., 2011). Conversely, miR-605 targets MDM2, triggering p53-mediated senescence (Xiao et al., 2011), and multiple miRNAs downregulate p21^{WAF1/Cip1}, including 28 miRNAs that block OIS (Borgdorff et al., 2010). Likewise, miR-24 suppresses p16^{INK4a} in cells (Lal et al., 2008) and disease models, including osteoarthritis (Philipot et al., 2014). Intricate miRNA feedback loops can modulate senescence programs. For example, a p53/miRNA/CCNA2 pathway drives senescence independently of the p53/p21^{WAF1/Cip1} axis (Xu et al., 2019). Similarly, p53-dependent upregulation of miR-34a/b/c downregulates cell proliferation and survival factors (Hermeking, 2010). Non-coding RNAs also regulate the SASP (Panda et al., 2017). MiR-146a/b, for example, increases weeks after senescence induction and dampens a proinflammatory arm of the SASP (Bhaumik et al., 2009). miRNAs also downregulate repressors of senescence, including polycomb group (PcG) members CBX7, EED, EZH2, and SUZ12 (miR-26b, 181a, 210, and 424), leading to p16^{INK4a} derepression and senescence initiation (Overhoff et al., 2014). Finally, the role of miRNAs in senescence extends beyond their classical functions. For example, Argonaute 2 (AGO2) binds let-7f in the nucleus, forming a complex with RB1 (pRB), resulting in repressive chromatin at CDC2 and CDCA8 promoters (Benhamed et al., 2012). Silencing these E2F target genes is required for senescence initiation.

Long non-coding RNAs (lncRNAs) (>200 nt) can bind RNA, DNA, or proteins to regulate senescence. For example, ANRIL, a 30-40kb antisense transcript encoded by the CDKN2A locus, binds CBX7 to repress INK4b/ARF/INK4a expression (Kim et al., 2017). Likewise, the lncRNA PANDA recruits PcG complexes, suppressing senescence-promoting genes (Kim et al., 2017), whereas silencing of GUARDIN, a p53-responsive lncRNA, causes senescence or apoptosis (Hu et al., 2018). By contrast, following OIS induced by RAF, the lncRNA VAD preserves senescence by decreasing repressive H2A.Z deposition at INK promoters (Kim et al., 2017). Also, lncRNA UCA1 disrupts association of the RNA-binding protein hnRNP A1 with p16^{INK4A}, but not p14^{ARF}, transcripts (Kim et al., 2017). In addition, non-coding RNA profiling, with a focus on miRNAs, provides a senescence signature (Suh, 2018). Intriguingly, the miRNA content of small extracellular vesicles released by senescent cells varies, evolving over time (Terlecki-Zaniewicz et al., 2018).

Immune-Regulation and Anti-apoptotic Proteins

The search for senescent protein markers started in OIS. In addition to identifying known cell-cycle regulators, these studies identified DCR2 as a common marker of senescence (Collado et al., 2005), later shown to characterize other types of senescence. DCR2 is a decoy death receptor that protects senescent cells from immunity-mediated apoptosis, thus blocking immune surveillance of senescent cells (Sagiv et al., 2013). Similarly, the natural killer (NK) cell activating receptor (NKG2D) ligands MICA and ULBP2 increase upon replicative-, OIS-, and DNA-damage-induced senescence (Krizhanovsky et al., 2008b; Sagiv et al., 2016). Cell surface markers are of special interest because they should allow quantification, isolation, and single cell transcriptional analysis of senescent cells extracted from tissues. However, DCR2 and NKG2D ligands are not conserved among species, making mouse-to-human comparisons not possible. Recently, two additional upregulated cell surface markers, Notch1 in OIS and DPP4 in replicative and OIS, were identified (Hoare et al., 2016). Both proteins have roles in regulating the SASP. Furthermore, an oxidized form of membrane-bound vimentin was identified as a senescence marker that could be used to target these cells by the adaptive immune system (Frescas et al., 2017). Finally, senescent cells are resistant to apoptosis, which can be mediated by increased expression of anti-apoptotic BCL-2 family members (Yosef et al., 2016).

In Vivo Models to Study Cellular Senescence Senescence Reporter Mice

Several transgenic mice were developed to estimate $p16^{INK4A}$ expression *in vivo* or *ex vivo* using luciferase or fluorescent protein reporters. Measuring luciferase activity longitudinally revealed an increase in $p16^{INK4A}$ expression as mice age, as well as an age-dependent increase in inter-animal variability, whereas isolation of fluorescent $p16^+$ cells allowed phenotyping (Liu et al., 2019; Ohtani et al., 2010). This approach allows the endogenous $p16^{INK4A}$ promoter to drive signals but causes $p16$ hemizygosity. Another mouse ($p16$ -3MR) used a luciferase (rLUC), monomeric red fluorescent protein (mRFP) and herpes simplex virus thymidine kinase (HSV-TK)-fusion protein driven by the $p16^{INK4A}$ promoter present on a bacterial artificial chromosome and integrated into the mouse genome (Demaria et al., 2014). This approach allows detection and killing of senescent cells and does not perturb the endogenous CDKN2A locus. Finally, INK-ATTAC mice express a FKBP-Caspase 8 fusion-protein and enhanced green fluorescent protein (eGFP) reporter to kill and detect $p16^+$ cells, driven from a 1.6 kb fragment of the $p16^{INK4A}$ promoter (Baker et al., 2011; Folgueras et al., 2018). Despite differences between these mice, they have been valuable in showing that senescent cells contribute to a wide range of age-related pathologies (Calcinotto et al., 2019). Mice expressing luciferase and eGFP from $p21^{WAF1/Cip1}$ promoter are also available (Ohtani et al., 2007).

Murine Models of Accelerated Senescence and Aging

Several progeric mouse models have been developed to mimic human progeric syndromes, including DNA repair and genome-integrity deficiencies (Folgueras et al., 2018). Progeroid mice with accelerated senescence and shortened lifespans are also useful for assessing the role of cellular senescence in aging

and testing senotherapeutics. For example, the demonstration that ablation of $p16^{INK4A}$ expressing cells slowed age-related declines in progeroid $BubR1^{H/H}$ mice provided the first evidence that senescent cells are causal for certain aging phenotypes (Baker et al., 2011; Folgueras et al., 2018). BUBR1 is important for the mitotic spindle assembly checkpoint (Guo et al., 2012). $BubR1^{H/H}$ mice, which express 10% of the normal level of BUBR1, have increased aneuploidy, several progeroid features, and increased expression of senescence markers in several organs (Folgueras et al., 2018). Selective removal of $p16^{INK4A+}$ cells from $BubR1^{H/H}$ INK-ATTAC mice delays kyphosis, cataracts, and muscle atrophy, but it does not delay cardiac arrhythmias and arterial wall stiffening nor does it extend lifespan (Baker et al., 2011; Folgueras et al., 2018).

Similarly, $Ercc1^{-/-}$ progeroid mice, harboring a DNA-repair defect, prematurely develop multiple morbidities associated with age, driven in part by accelerated accumulation of senescent cells in numerous tissues (Folgueras et al., 2018). $Ercc1^{-/-}$ mice (Folgueras et al., 2018) express 5% of the normal level of the endonuclease ERCC1-XPF, important for nucleotide excision, inter-strand crosslink, and double-strand break repair. These mice develop numerous age-related histopathologic lesions in virtually every tissue (Folgueras et al., 2018) and accumulate oxidative DNA damage faster than wild-type mice (Wang et al., 2012). Treatment of $Ercc1^{-/-}$ mice with senolytic drugs reduces senescence markers and extends health span (Fuhrmann-Stroissnigg et al., 2017; Yousefzadeh et al., 2018; Zhu et al., 2015). Crossbreeding of these models with the $p16^{INK4A}$ reporter transgenes permits monitoring senescent cell burden longitudinally in live animals (Robinson et al., 2018; Yousefzadeh et al., 2018).

Hutchinson-Gilford progeria syndrome (HGPS) is a segmental- or tissue-specific progeria, caused by mutations that compromise lamin A processing (Cau et al., 2014). Mice with altered or deleted LMNA develop HGPS-like phenotypes. They also accumulate senescent cells, as determined by SA- β -gal staining and mRNA levels of senescence markers, in skeletal muscle and heart, consistent with sites of age-related pathology and disease (Folgueras et al., 2018). Similarly, in a mouse model of HGPS that recapitulates the pathogenic LMN-splicing mutation ($Lmna^{G609G/G609G}$ mice), senescence in the liver and kidney was observed (Osorio et al., 2011). However, senescent cells have not yet been shown to be causative for HGPS pathology.

A mouse model of trichothiodystrophy (TTD) (Andressoo et al., 2006), caused by a specific mutation in the Xpd gene, also indicated a role for senescent cells in premature aging. Here, the role of senescence in driving aging in the $Xpd^{TTD/TTD}$ was clearly documented by the fact that treatment with a D-retro inverso (DRI)-isoform peptide of FOXO4 was able to disrupt FOXO4 interaction with p53. Treatment with the FOXO4-DRI peptide-reduced lethargy in $Xpd^{TTD/TTD}$ mice and improved fur density, running wheel activity, and physical responses to stimuli (Baar et al., 2017).

Loss of Cu-Zn-superoxide dismutase ($Sod1$) in mice accelerates aging (Zhang et al., 2017). $Sod1^{-/-}$ mice show increased oxidative DNA damage, senescence ($p16^{INK4A}$, $p21^{WAF1/Cip1}$), SASP factors ($Il1\beta$, $Il6$), SA- β -gal $^+$ cells, and age-associated pathology in kidneys (Zhang et al., 2017). To date, senescence has not been demonstrated to drive pathology in $Sod1^{-/-}$ mice.

Deletion of the repressive NFKB1 subunit of the transcription factor NF- κ B induces premature aging in mice. These mice have been shown to experience chronic, progressive low-grade inflammation that contributes to a wide spectrum of aging phenotypes and early mortality; however, in contrast to some of the widely used progeria mouse models, these mice have a maximum lifespan of approximately 20 months. Furthermore, these mice show increased incidence of senescent cells in multiple tissues (Jurk et al., 2014).

Finally, the selective inbreeding of AKR/J mice resulted in numerous senescence-accelerated mouse (SAMP) strains including SAMP1-3 and SAMP6-11 (Takeda et al., 1997). Although these mice have increased senescence and thus can be used for testing senotherapeutics, it remains unclear which mutant genes drive senescence in these strains.

Identification of Cellular Senescence *In Vivo* A Simplified Algorithm for Detecting Senescent Cells *In Situ*

In vivo, senescent cells reside within tissues. Their impact on tissue function can be local or global due to the SASP (Xu et al., 2018). To understand how senescence affects tissue function, tissue remodeling, and aging, we need tools to identify senescent cells in tissues.

Single cell analyses can be performed on most tissues. Common techniques include immunostaining, *in situ* hybridization, and multicolor (imaging) flow cytometry. Even higher numbers of markers can be assessed by mass cytometry (cytometry by time-of-flight [CYTOF]) (Abdelaal et al., 2019). Although promising, limitations include loss of information about spatial associations and variable efficiency of isolation of different cell types, including senescent versus non-senescent cells. Therefore, microscopic imaging remains a preferred method for *in situ* senescence detection.

As mentioned, there is currently no single marker with absolute specificity for senescent cells. Marker specificity varies, depending on cell type, tissue, organismal developmental stage, species, and other factors. However, some markers have more universal validity while others are related to specific senescence types. Therefore, we advise a multi-marker approach, combining broader and more specific markers for more robust detection of senescent cells *in situ* (Figure 3).

Challenges to Detect Senescent Cells in Humans

The role of senescence in human disease is clear from cellular studies, while *in vivo* evidence is only now catching up (Childs et al., 2015; He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014). OIS, initially described in culture, was the first type of senescence validated in humans (Serrano et al., 1997). OIS or senescence induced by loss of a tumor suppressor was verified *in vivo* in human and murine preneoplastic lesions (Collado et al., 2005; Gorgoulis and Halazonetis, 2010; Kuilman and Peeper, 2009) and primary or treated neoplasias (Haugstetter et al., 2010). Later reports on the diverse activities of the senescence secretome (see Secretion) led to the recognition of its pro-tumorigenic properties, establishing what is now accepted as the dual role of senescence in carcinogenesis (Lee and Schmitt, 2019). Evidence linking senescence to other common age-associated human diseases has recently emerged. These diseases include

neurodegenerative disorders, glaucoma, cataract, atherosclerosis and cardiovascular disease, diabetes, osteoarthritis, pulmonary, and renal and liver fibrosis (Childs et al., 2015; He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014) (Table S2).

In most studies, senescence is assessed in *ex vivo* cultures or fresh samples by SA- β -gal staining or indirect markers in formalin-fixed tissues (Haugstetter et al., 2010; He and Sharpless, 2017; Kuilman and Peeper, 2009; Muñoz-Espín and Serrano, 2014; Serrano et al., 1997). Since SA- β -gal is not suitable for fixed tissues, analyzing senescence in human samples is challenging. The histochemical dye SBB interacts with lipofuscin, another hallmark of senescent cells (Georgakopoulou et al., 2013). Lipofuscin is preserved in fixed material (Georgakopoulou et al., 2013) and is resilient, as it was isolated from a 210,000-year-old human fossil (Harvati et al., 2019; Myrianthopoulos et al., 2019). A recently developed reagent (GL13) is amenable to immunohistochemistry (Evangelou et al., 2017) and identified senescent Hodgkin and Reed-Sternberg (HRS) cells in Hodgkin lymphomas (cHL) where they predicted poor prognosis (Myrianthopoulos et al., 2019). These cells are giant in size, have a large and occasionally multilobular nucleus (an indication of an abortive cell cycle), have increased secretory activities, are embedded within an inflammatory milieu, and show a histological pattern strongly reflecting features of the senescence phenotype (Küppers et al., 2012) (Figure 1). Another method for identifying and quantifying senescent cells *in vivo* is SA- β -gal staining combined with ImageStream X analysis (Biran et al., 2017).

Despite promising results that each marker provides, no marker is completely senescence specific (Sharpless and Sherr, 2015) (Sharpless and Sherr, 2015). We recommend combining cytoplasmic (e.g., SA- β -gal, lipofuscin), nuclear (e.g., p16^{INK4A}, p21^{WAF1/Cip1}, Ki67) and SASP, context and/or cell-type-specific markers (Childs et al., 2015) (Figure 3).

Conclusions, Open Questions, and Perspectives

From the first description of cellular senescence by Hayflick and colleagues almost 60 years ago, significant progress has been made in understanding the characteristics and functions of senescent cells. A limitation, particularly for studying biospecimens, remains the absence of specific markers. To overcome this obstacle, we propose a multi-marker approach (Figure 3). This strategy could also be used to evaluate the efficacy of senolysis, an emerging therapeutic approach that recently entered clinical trials for treatment of various age-related pathologies (Myrianthopoulos et al., 2019).

Conceptually, senescence can be considered a non-linear, multivariable $[F(x,y)=z]$ function where the dependent variable (outcome), z , depends on the independent variables, x (stimulus) and y (environment). The non-linear processing is dictated by dynamic genetic and epigenetic processes that can lead to reprogramming cycles until a steady state is achieved. At first glance, the outcomes appear to be cell-cycle withdrawal and secretion of bioactive molecules. However, recent evidence suggests that the cell-cycle arrest is not always a necessary outcome, as post-mitotic cells, already unable to proliferate, can assume senescence-like features, and under certain conditions senescent cells can re-enter the cell cycle. The SASP appears a common senescence-associated feature, but it is

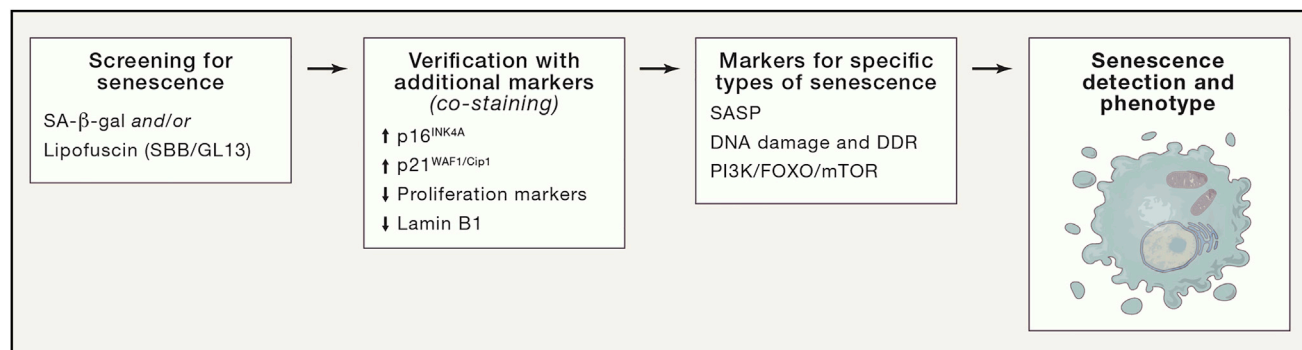


Figure 3. A Multi-Marker, Three-Step Workflow for Detecting Senescent Cells

The first step of the proposed workflow includes assessing senescence-associated beta-galactosidase (SA-β-gal) activity and/or lipofuscin accumulation (SBB or GL13 staining). Second, co-staining with other markers frequently observed in (p16^{INK4A}, p21^{WAF1/Cip1}) or absent from (proliferation markers, lamin B1) senescent cells. Third, identification of factors anticipated to be altered in specific senescence contexts should be identified. This multi-marker workflow can lead to the recognition of senescent cells with the highest accuracy.

highly heterogeneous. Thus, to understand the pleiotropic phenotypes of senescent cells, a shift from traditional reductionism to more systematic, multi-parametric approaches is needed. The development of sophisticated high-throughput methods and machine learning tools that can handle multi-omics data will help achieve this goal (Vougas et al., 2019). Although “old” and “new” have pros and cons, we can combine the best to achieve a “*de profundis*” analysis of senescent phenotypes. This approach will likely unveil more specific senescence-associated signatures to address important unanswered questions: what causes and regulates the SASP, how do genetic and epi-genetic determinants interact with triggering stimuli and cellular microenvironments? Which genomic repair systems act in different senescence scenarios, what causes cells to evade the growth arrest, and what phenotypes do “escaped” senescent cells acquire? Answers to these and other questions will help develop specific panels of markers for each senescence subtype (see step 3 in Figure 3) and guide the evolving field of senotherapy (van Deursen, 2019), thus achieving the best outcome within the spirit of precision medicine.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.cell.2019.10.005>.

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DECLARATION OF INTERESTS

M.D. is a cofounder and shareholder of Cleara Biotech and holds patents related to senolytics. A.A. is a cofounder and shareholder of Oncosence. J.C. is a cofounder and shareholder of Unity Biotechnology and holds patents related to senolytics. D.Z. is a cofounder and shareholder of Unity Biotechnology and holds patents related to senolytics. J.G. owns equity in and has acted as a consultant for Unity Biotechnology; is a scientific founder of, owns equity in, and acts as a consultant for Geras Bio; and holds patents related to senolytics. T.v.Z. is a member of the advisory boards of the Mayo Clinic Robert and Arlene Kogod Center on Aging and of Nuchido.

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