Comment for Nature Cell Biology

The challenge of identifying senescent cells

Jesús Gil[1, 2,*]

[1]MRC Laboratory of Medical Sciences (LMS), Du Cane Road, London, W12 0NN, UK.
[2]Institute of Clinical Sciences (ICS), Faculty of Medicine, Imperial College London, Du Cane Road, London W12 0NN, UK.

*Corresponding author: jesus.gil@imperial.ac.uk

ABSTRACT

Reliable ways to identify senescent cells are a bottleneck for fully understanding the roles of senescence in physiology and disease and implementing effective clinical trials of senotherapeutics. This Comment examines the challenges of identifying senescent cells, revises the current recommendations for how to best assess senescence, and discusses how emerging technologies can address these issues.

MAIN TEXT

Senescence is a complex cellular stress response that limits cell proliferation by causing a stable cell cycle exit accompanied by multiple phenotypic changes, including alterations in cell and nuclear size, chromatin rearrangements, metabolic reprogramming and an immunomodulatory secretome known as the senescence-associated secretory phenotype (SASP) [1] (Figure 1a).

For many years, the physiological relevance of senescence was ignored and cellular senescence was regarded as an artefact associated with tissue culture. The contrast with apoptosis (a parallel cell fate that plays important roles in health and disease) was notable. The pathophysiological implications of apoptosis were evident from the 1990-2000s, thanks in part to the clarity provided for markers such as induction of caspase activity. At that time, the physiological relevance of senescence (or even whether senescence was not an artefact due to tissue culture) was hotly debated due to the difficulties in identifying senescent cells *in vivo*.

Senescence-associated ß-galactosidase (SA-ß-Gal) and the induction of the cyclin-dependent kinase inhibitor (CDKI) $p16^{INK4a}$ are two of the most widely used markers of senescence. Both were discovered in the 1990s: SA-ß-Gal was first described as a senescence marker in 1995 and $p16^{INK4a}$ shortly after [1]. However, different issues, discussed below, still complicated the identification of senescent cells *in vivo* and contributed to underappreciating the importance of senescence.

Excitingly, research in the last decade has clarified the wide impact that cellular senescence has on pathophysiology and ageing. Several seminal papers showed in 2005 how the presence of senescent cells in the early stages of tumorigenesis hampered tumour progression [2]. A few years later, the development of mouse models allowing for the regulated ablation of senescent cells made it possible to appreciate the pathophysiological relevance of senescence [3]. Senescent cells play an active role in cancer, ageing and age-related diseases and can be targeted therapeutically, which has resulted in extensive research in pre-clinical models that are starting to be translated into early-stage clinical trials. Still, the single question that those of us

working in the field of senescence are asked more frequently is, how do you identify senescent cells?

Current widely accepted methods to identify senescent cells

In 2020, the International Cell Senescence Association (ICSA) presented a consensus view on how to best identify senescent cells [1]. In short, they propose the use of multiple markers in a three-step process (Figure 1b), that would first asses senescence-associated ß-galactosidase (SA-ß-gal) activity or an alternative (such as lipofuscin accumulation) and later other senescent markers such as $p16^{INK4a}$ (or alternatives including $p21^{CIP1}$, lack of proliferation or loss of Lamin B1). A final layer of refinement comes from examining context-dependent senescence. The senescence community is aware of the need for improving and clarifying such guidelines and different initiatives to fill that gap are in progress. This commentary intends to discuss the reasons why identifying senescence is not straightforward, the possibilities to solve this problem and the opportunities that this will provide.

Why is it difficult to identify senescence in vivo?

There are multiple reasons why a single universal marker of senescence has not been identified and might not exist. For a start, senescence is an 'umbrella term' that defines many different cell types (including normal and cancer cells) undergoing a stable cell cycle arrest in response to a wide variety of stresses. In addition, senescence is a heterogeneous and dynamic process, with a phenotype that evolves with time. So, it could be argued that finding a single universal marker for senescence might be an impossible task, like finding a universal marker for cellular differentiation.

Moreover, there are many reasons with even the most widely used senescent markers are not universal or cannot be used in isolation (Table 1). No senescence markers are unique to senescent cells. SA-ß-Galactosidase activity is elevated in senescent cells because they upregulate lysosomal biogenesis and have more lysosomes (and their associated enzymes such as ß-Galactosidase). However, there are cell types, such as macrophages, that have high lysosomal content and stain positive in SA-ß-Gal assays, regardless of their senescent status. Similarly, $p16^{INK4a}$ is one of the most used markers for senescence, but cyclin-dependent kinase inhibitors (CDKI) other than $p16^{INK4a}$, such as $p21^{CIP1}$, might be upregulated in some types of senescence [1].

Moreover, cells with deletions or mutations in the RB pathway (a common occurrence in cancer) will display high levels of $p16^{INK4a}$ but will not be senescent. Conversely, *CDKN2A*, the gene encoding for $p16^{INK4a}$ is often mutated in cancer (https://portal.gdc.cancer.gov/genes/ENSG00000147889), and cancer cells can still undergo therapy-induced senescence despite not expressing $p16^{INK4a}$. In addition, markers of cell cycle arrest will not distinguish senescent post-mitotic cells from their normal counterparts (that have already exited the cell cycle) in vivo. The heterogeneity of the SASP makes it difficult to come up with a unique universal SASP signature encompassing all senescence types [1]. And DNA damage can happen in the absence of senescence and vice versa.

The identification of senescent cells *in vivo* presents additional challenges. For a start, senescent cells are often rare, representing at the most a small percentage of the cells present, even in old or diseased tissues. In part, this is due to the active process of immune surveillance that eliminates them, further obscuring their rate of appearance and turnover and potentially biasing the subset of senescent cells that we can detect at a given time (i.e., those that linger in the tissue and might be resistant to immune-mediated clearance).

Finally, there are technical issues associated with the detection of senescent cells. For example, senescence-associated ß-galactosidase activity is an enzymatic assay. To which extent cells become 'positive' depends on incubation times and factors such as confluency. In addition, SA-ß-Gal staining only works in cryosection (preferably fresh), which limits the reach of this assay. Detection of $p16^{INK4a}$ by IHC or IF in mouse tissues, although possible has been regarded as unreliable, due to a combination of issues that might include expression levels, the exposure of the antigen and the existing antibodies. Moreover, due to the different characteristics of senescent cells when compared with non-senescent cells (difference in size, adhesion, etc.), technologies that rely on the dissociation and isolation of cells from a tissue might be inherently biased against senescent cells.

Alternative approaches to detect senescence

Importantly, an array of complementary and in some cases novel approaches are being used to enable the detection of senescent cells (Figure 2). Analysis of

transcriptional profiles of senescent cells has served to define novel senescence markers, such as uPAR, as a membrane and secreted biomarker of senescence [4]. Transcriptional signatures have the advantage that they can be combined with analytical methodologies, such as gene set expression analysis (GSEA), to detect senescence and avoid pitfalls associated with individual markers. Given the heterogeneity of senescence, signatures might be limited in the types of senescence that they detect, although efforts have aimed to derive signatures identifying multiple senescent types by concentrating on commonly regulated genes [5] or using more *ad hoc* approaches, as for establishing the SenMayo gene set [6]. Other methods have been designed with a specific type of senescence in mind, such as the SENCAN classifier, which uses bulk RNA-Seq data and machine learning to identify senescence in cancer cells [7]. While most transcription-based signatures can equally work in bulk and single cell (sc) RNA-Seq, others (such as the senescence index tool, SIT) [8] have been specifically designed with scRNA-Seq data in mind.

ScRNA-Seq is particularly useful for identifying senescent cells in heterogeneous tissues *in vivo*. For example, different cell types, including endothelial cells, pericytes, astrocytes and Mueller cells become senescent during retinopathy, but treatment with Bcl2-family inhibitors selectively depletes a population of senescent endothelial cells causing pathological angiogenesis [9]. scRNA-Seq has also been used in combination with reporter mice to identify the heterogeneity of senescent responses with age and disease. Indeed, senescent reporter models combined with more traditional approaches have been important in identifying senescence in liver sinusoidal endothelial cells, which might help explain some side effects of senolysis [10].

There are technical issues associated with scRNA-Seq, such as a bias towards specific cell types depending on the protocols used to isolate cells. This can result in the underrepresentation of senescent cells. In addition, the expression of senescence markers, such as $p16^{INK4a}$ or $p21^{CIP1}$ is not high and their mRNAs are relatively short, making them underrepresented due to the amplification methods utilized during library preparations. Isolating specific cell types before scRNA-Seq might help with this issue by reducing the contribution of highly expressed genes to the variability of the cell population. Despite these limitations, scRNA-Seq is already revolutionizing our

understanding of the role of senescent cells *in vivo* and can result in the discovery of novel markers of signatures for specific senescent cell populations. Senescent markers identified using scRNA-Seq can be combined with spatial transcriptomics (or spatial proteomics or metabolomics) to provide a better picture of how senescent cells reshape tissue architecture and influence their microenvironment, as summarized in a recent review [11]. Another approach to identifying senescent cells is based on their characteristic morphological changes. An advantage of these approaches is that they do not rely on the identification and detection of specific markers. Machine-learning algorithms can classify and evaluate either morphological or nuclear changes occurring in senescent cells. These classifiers based on senescent morphology not only have the potential to identify senescent cells in culture but can also be used to assess senescence in H&E-stained tissue sections [11].

Towards the identification of senescent cells in patients

The above methods can be applied in a wide range of experimental and preclinical settings, but to evaluate senescence in patient cohorts and clinical trials, we need methodologies that ideally are non-invasive and can be applied longitudinally – such as potential biomarkers of senescence that can be detected in the blood. A combination of cytokines, which includes SASP components, has been shown to correlate with age and medical risk [12]. The soluble form of uPAR is produced by senescent cells and could also be considered a biomarker of their presence [9]. Certain prostaglandins are specifically produced by senescent cells and their presence in blood indicates senolysis [13]. A recent study observes how mitochondrial DNA (mtDNA) is released by senescent cells and cell-free mtDNA increases in the plasma of older mice and humans [14]. In addition, other approaches, such as detecting senescent biomarkers associated with extracellular vesicles (whose release is enhanced in senescent cells) or combining diagnostics measuring circulating tumour cells (CTCs) and senescence profiling, could be exploited to evaluate senescence in the blood.

Visualization of senescence burden with non-invasive imaging techniques such as magnetic resonance imaging (MRI) or positron emission tomography (PET) is another powerful approach that could be used to monitor longitudinally senescent patients. In this regard, tracers that inform on SA-ß-Gal activity are being trialled to image senescence. For example, ß-galactose-coated nanoparticles can be loaded with

probes to monitor senescence [15]. Similarly, [^{18}F]FPyGal, a PET tracer reporting ß-galactosidase activity, can measure senescence in patients and its safety is currently being assessed in a clinical trial (NCT04536454, https://classic.clinicaltrials.gov/ct2/show/NCT04536454). Alternative ways to monitor senescence could be adapted for use in medical imaging.

Real-time monitoring of senescence holds immense promise for translating senotherapies to the clinic but has limitations. The above-described approaches are based on the detection of individual senescent markers (e.g. SA-ß-Galactosidase, iron), which are not universal and could be elevated in other circumstances. Moreover, MRI or PET will need the presence of a mass of senescent cells, which might make them more useful for monitoring senescence in tumours, but perhaps not in other circumstances where senescent cells might be interspersed in the tissue and in more reduced numbers. Finding a universal marker is the holy grail of senescence research and there are plenty of initiatives to identify senescent cells. The Cellular Senescence Network (SenNet), funded by the National Institutes of Health has been established to answer these questions and has the potential to be transformative (https://sennetconsortium.org). Others are employing complementary methods that include sophisticated uses of AI, detection methods such as Raman microspectroscopy or the development of more complex, probes. A combination of different markers or approaches might be eventually more accurate for monitoring senescent cells *in vivo*.

Overall, the pressing need to develop strategies for the detection of senescent cells and senescence burden to stratify and monitor clinical trials for senotherapeutics should result in better ways to detect senescence in clinical cohorts, preclinical and experimental samples, which in turn should contribute to the progress of senescence research and our understanding of senescence.

ACKNOWLEDGEMENTS

I thank the reviewers for their thoughtful suggestions and apologise to the authors whose work could not be cited due to space limitations. Core support from MRC (MC_U120085810) and a grant from CRUK (C15075/A28647) funded research in J. Gil's laboratory.

COMPETING INTERESTS

J. G. has acted as a consultant for Unity Biotechnology, Geras Bio, Myricx Pharma Ltd., and Merck KGaA; owns equity in Geras Bio and share options in Myricx Pharma Ltd. and is a named inventor in MRC And Imperial College patents related to senolytic therapies. J.G. currently receives funding from Pfizer. Unity Biotechnology funded research on senolytics in J.G.'s laboratory in the past.

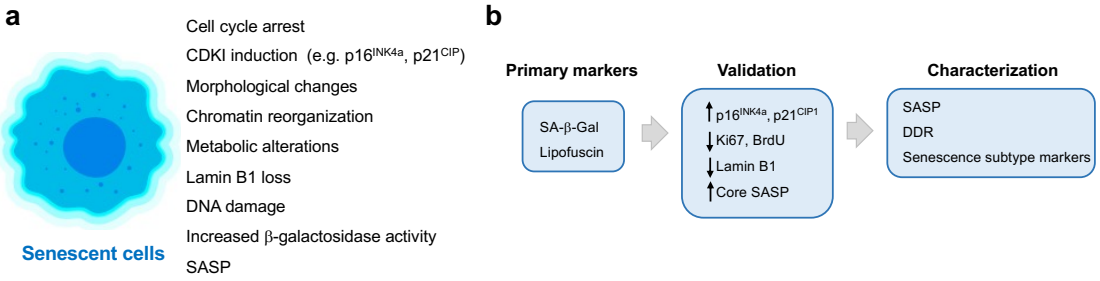

Figure 1. Cellular senescence and current recommendations to detect it. a, Senescent cells exit the cell cycle and display several characteristics, including induction of cyclin-dependent kinase inhibitors (CDKI), changes in their morphology, chromatin reorganization, metabolic alterations, downregulation of Lamin B1, DNA damage, increase in levels of lysosomal ß-galactosidase activity (referred to as senescence-associated ß-galactosidase activity, SA-ß-Gal) and induction of a secretome referred as the senescence-associated secretory phenotype (SASP). **b,** Current guidelines to detect senescent cells, as explained in detail in [1] The workflow suggests an initial assessment of primary markers, followed by verification with additional markers and finally characterizing other factors that might vary depending on the type of senescence examined. Figure partially generated with Biorender (Biorender.com).

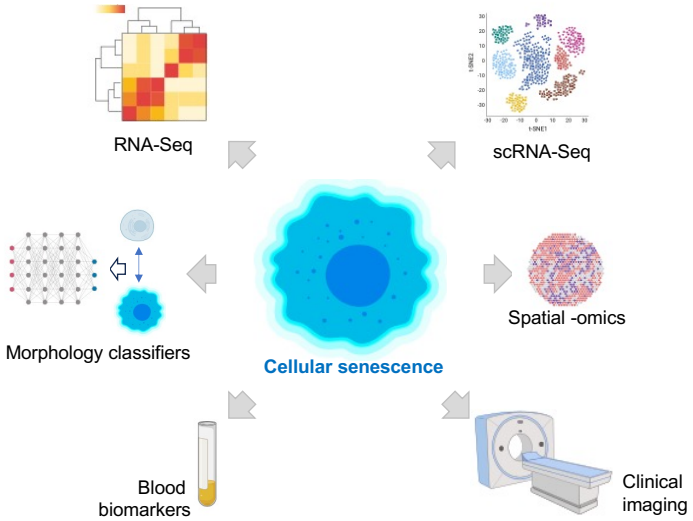

Figure 2. Alternative methodologies to detect senescence. An example of different approaches to detect senescence. These include expression-based analysis of either bulk or single cells (sc) RNA-Seq, spatial-omic approaches, machine-learning-based morphology classifiers, detection of biomarkers in blood or other biological fluids and clinical imaging. Figure partially generated with Biorender (Biorender.com).

Table 1. Examples of known issues with different markers of senescence

Senescence marker	Potential issues
SA-ß-Galactosidase	Unspecific staning (e.g. with high confluency)
	Non-senescent cell types positive (e.g. macrophages)
	Work in cryosections but not in paraffin
$p16^{INK4a}$	Often deleted in cancer cells
	Highly expressed in RB-mutant tumours
	Problems detecting $p16^{INK4a}$ in mouse tissues
	Other CDKIs might be induced instead of $p16^{INK4a}$
Lack of proliferation	Not relevant for post-mitotic senescent cells
DNA damage	DNA damage can occur in the absence of senescence
	Senescence can happen in absence of DNA damage
SASP	Heterogeneous composition of SASP

REFERENCES

1 Gorgoulis, V. *et al.* Cellular Senescence: Defining a Path Forward. *Cell* **179**, 813-827, doi:10.1016/j.cell.2019.10.005 (2019).

2 Collado, M. & Serrano, M. Senescence in tumours: evidence from mice and humans. *Nature reviews. Cancer* **10**, 51-57, doi:10.1038/nrc2772 (2010).

3 Baker, D. J. *et al.* Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature* **530**, 184-189, doi:10.1038/nature16932 (2016).

4 Amor, C. *et al.* Senolytic CAR T cells reverse senescence-associated pathologies. *Nature*, doi:10.1038/s41586-020-2403-9 (2020).

5 Hernandez-Segura, A. *et al.* Unmasking Transcriptional Heterogeneity in Senescent Cells. *Curr Biol* **27**, 2652-2660 e2654, doi:10.1016/j.cub.2017.07.033 (2017).

6 Saul, D. *et al.* A new gene set identifies senescent cells and predicts senescence-associated pathways across tissues. *Nat Commun* **13**, 4827, doi:10.1038/s41467-022-32552-1 (2022).

7 Jochems, F. *et al.* The Cancer SENESCopedia: A delineation of cancer cell senescence. *Cell Rep* **36**, 109441, doi:10.1016/j.celrep.2021.109441 (2021).

8 Troiani, M. *et al.* Single-cell transcriptomics identifies Mcl-1 as a target for senolytic therapy in cancer. *Nat Commun* **13**, 2177, doi:10.1038/s41467-022-29824-1 (2022).

9 Crespo-Garcia, S. *et al.* Pathological angiogenesis in retinopathy engages cellular senescence and is amenable to therapeutic elimination via BCL-xL inhibition. *Cell Metab* **33**, 818-832 e817, doi:10.1016/j.cmet.2021.01.011 (2021).

10 Grosse, L. *et al.* Defined p16(High) Senescent Cell Types Are Indispensable for Mouse Healthspan. *Cell Metab*, doi:10.1016/j.cmet.2020.05.002 (2020).

11 Gurkar, A. U. *et al.* Spatial mapping of cellular senescence: emerging challenges and opportunities. *Nat Aging* **3**, 776-790, doi:10.1038/s43587-023-00446-6 (2023).

12 Schafer, M. J. *et al.* The senescence-associated secretome as an indicator of age and medical risk. *JCI Insight* **5**, doi:10.1172/jci.insight.133668 (2020).

13 Wiley, C. D. *et al.* Oxylipin biosynthesis reinforces cellular senescence and allows detection of senolysis. *Cell Metab* **33**, 1124-1136 e1125, doi:10.1016/j.cmet.2021.03.008 (2021).

14 Iske, J. *et al.* Senolytics prevent mt-DNA-induced inflammation and promote the survival of aged organs following transplantation. *Nat Commun* **11**, 4289, doi:10.1038/s41467-020-18039-x (2020).

15 Munoz-Espin, D. *et al.* A versatile drug delivery system targeting senescent cells. *EMBO Mol Med* **10**, doi:10.15252/emmm.201809355 (2018).