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Heterogeneity of Cellular Senescence and its Implications for the Development of Markers

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Heterogeneity of Cellular Senescence and its Implications for the Development of Markers

Alejandra Hernández Segura

2019

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Heterogeneity of Cellular Senescence and its Implications for the Development of Markers

Phd thesis

to obtain the degree of PhD at the
University of Groningen
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Rector Magnificus prof. E. Sterken
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Friday 22 May 2019 at 11.00 hours

by

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About the cover

A few months ago I asked Nancy Halsema, my friend and colleague, to design the cover of this thesis. The only request I made was to represent the main topic of my research: cellular senescence. I suggested to depict the telomere shortening, the most studied senescence inducer. I had no further creative input. Later, Nancy asked me a bunch of questions about my taste in colour, my family, my home country (Mexico), its traditions and about my life there. I did not see a connection between our conversation and the request I had made earlier. Still, I was more than happy to talk about Mexico, which after 7 years abroad is still my true home country.

I already knew that Nancy was very talented, being that she has amazed everyone at the various scientific art competitions in ERIBA. Yet, I was amazed with the result. She came up with this brilliant design that included a bit of everything we talked about that night. Few months later the idea was transformed into this beautiful oil painting that I consider a very artistic, personal and yet scientific way of representing cellular senescence. I think it can use some further explanation though, so you can appreciate the creativity and work that Nancy put into it.

- *The Mexican pink*: the background colour is no coincidence or a simple matter of taste. This tone of dark pink or fuchsia is what in my country we call “Mexican pink”, the official colour of Mexico. Perhaps it is not the classical choice for a thesis book, but it is a display of pride for my country.
- *The cactus, the eagle and the snake*: anyone that has paid attention to the Mexican flag will notice the resemblance. The National Emblem at the centre of the Mexican flag has an eagle standing on a cactus devouring a snake. This symbolism comes from a legend in which the Aztec God Huitzilopochtli marked the Promised Land for his people with exactly that symbol. The Aztecs found the eagle in a small island in the lake of Texcoco, where they later built Tenochtitlan, what we now know now as Mexico City.
- *The telomere erosion*: The snakes also represent a chromosome (which explains why there are two instead of one, as in the flag) with the eagle representing the damage that the ends of the chromosomes (snake) suffer in this type of senescence.
- *The volcano*: On the back of the cover you can see a volcano named Popocatepetl, “The Smoking Mountain”. This is the volcano closest to my natal city, Puebla. My whole life I have woken up looking at it from the window of my parents’ bedroom and I have lived through tons of its small and bigger explosions.
- *The church*: Right in front of the volcano on the cover there is a barely perceptible shadow of the church called “Santuario de la Virgen de los Remedios”. This church is in the town where I went to university (Cholula). The church is very special because it was built on top of the broadest (known) pyramid of the whole world (known as Tlachihualtépetl, “The Hand-made hill”). People in my city are quite proud of this church and pyramid.

Anyone that knows me well, knows as well that I am very proud of my country. I am happy and honored to see that Nancy found a majestic way to transmit that. Thank you so much, Nancy.

An abstract painting with a vibrant pink background. On the right side, there are dark, textured brushstrokes in shades of black, grey, and brown. In the bottom left corner, there are two large, rounded green shapes with dark spots, resembling cacti or succulents. Several smaller, bright orange-red spots are scattered in the lower middle area.

Chapter 1

General Introduction and Thesis
Outline

Aging and its impact on health

López-Otín *et al* [1] defined aging as the functional decline that most organisms experience with time. Particularly in humans, it is recognized that some functional impairment appears with normal aging, for instance hearing loss, reduced visual acuity and loss of muscle strength [2]. Even more worrying is the association of aging to conditions that threaten the life quality even further or that threaten the life itself, such as cardiovascular and neurodegenerative diseases, diabetes mellitus, osteoarthritis and cancer [2], some of which are within the top causes of death (<http://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death>).

The prevalence of such diseases is likely to increase in the upcoming years due to the aging of the whole population. United Nations predicts that the population 60 years or over will double by 2050 and more than triple by 2100 [3]. The extension of life expectancy is viewed as a success of the modern medicine. However, the main challenge now is to ensure that people live not only longer but healthier for a longer time. It is no surprise then that aging has become a hot topic in the economic and political arena and that aging research has increased its impact.

Efforts are being made to understand and target the different aspects that characterize aging: stem cell exhaustion, altered intercellular communication, genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction and cellular senescence [1]. It is well known that all these features of aging are interconnected. However, cellular senescence has been in the spotlight lately as a potential target for therapy. Last years have been particularly prolific in the senescence field, as genetically modified mice that allowed targeting and selective elimination of senescent cells exhibited an extended health- and lifespan [4–6], giving clear evidence of the potential that senescence has as an anti-aging target.

Cellular Senescence and the challenges to use it as a target

Cellular senescence is a stable cell cycle arrest that normally appears as a cellular response to damaging stimuli. Unrepairable DNA damage, oxidative stress and oncogene activation all lead to senescence [7]. Thus, cellular senescence is viewed as an anti-tumor mechanism and at least in this context it exerts a positive function in the organism. Furthermore, senescence has other positive roles as well, by participating in wound healing and in development [8,9]. However, it is the accumulation of senescent cells in an organism what causes the negative effects that lead to a reduced ability to repair tissues, a change in the microenvironment and a continuous inflammation, all characteristics of aging and age-related diseases [10]. Therefore, the senescence field has made great efforts to find a senotherapy (a therapy targeting senescent cells) that works in humans, where no genetic modifications are ethically allowed. The ideal senotherapy would preserve the positive functions of senescent cells while reducing or eliminating the negative ones. Two main types of senotherapies have been developed, each one with different goals: 1) preventing the accumulation of senescent cells by eliminating them once they are generated (“senolytics”) [6,11,12] or 2) modifying the senescence phenotype in such a way that the negative effects are avoided (for instance, by targeting molecules secreted by senescent cells that damage their microenvironment) [13–16]. As of yet, none of these senotherapies have been approved for use in humans as anti-aging

therapies.

The lack of deep knowledge on the senescence phenotype is the main hurdle that needs to be overtaken. The senescence phenotype is very heterogeneous, with few markers being consistently present in every single senescent cell [17]. Even the features that are universal, such as cell cycle arrest and high lysosomal activity, are by no means specific for senescence [18,19]. Targeting something we cannot even identify properly and let alone fully understand baulks therapy efficiency. Furthermore, once targeted, the evaluation and prediction of the outcomes for any type of therapy also relies on the use of biomarkers. It is exactly on this niche where my research lies. The aim of my PhD project was to study the heterogeneity of the senescence phenotype and to analyze its impact on the search of senescence markers. This included pointing out the pitfalls of the current markers and to search for new ones.

Outline of the thesis

Chapter II gives a deeper introduction to senescence, the features that characterize it and the current markers of senescence. It reviews and summarizes our current knowledge on the molecular regulation of the hallmarks of senescence and it also emphasizes the challenges for the development of senescence markers and senotherapies.

As mentioned before, different stimuli can induce senescence and multiple markers are measured to validate the senescence phenotype. Chapter III is a collection of protocols with a detailed explanation on how to induce senescence *in vitro* and how to measure some of the well-known senescence markers. This chapter also touches upon the limitations of these markers and the influence of culturing conditions on the senescence phenotype.

The lack of universal and specific markers of senescence reflects the heterogeneity of the senescence phenotype. In Chapter IV we embarked on a deep study of the transcriptome of different “types” of senescence, unveiling a large heterogeneity among senescent samples that had not been systematically studied before.

Many of the senescence markers are measured using qPCR, a semi-quantitative technique that relies on the use of reference genes (genes that are stably expressed in the conditions being compared). However, there are no systematic studies available evaluating which genes are actually stably expressed in proliferating and senescent cells. This results in lack of reliability and reproducibility of the qPCR data. In Chapter V we evaluated the usage of appropriate reference genes in the field and used large amounts of RNAseq and qPCR data to propose the best candidates to be used as reference genes in senescence-related experiments.

In Chapter VI we update the findings of Chapter IV, but focusing mainly on entire pathways or biological functions important to senescence, instead of particular molecular effectors. We also discuss another important and under-studied aspect of senescence: its heterogeneity at the single-cell level. We demonstrate that even markers that are universally expressed at the population level, are not necessarily used in every single senescent cell.

Finally, in Chapter VII we analyze the results of the whole thesis and discuss important questions that derive from our results.

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Chapter 2

Hallmarks of Cellular Senescence

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Abstract

Cellular senescence is a permanent state of cell cycle arrest that promotes tissue remodeling during development and after injury, but can contribute to the decline of the regenerative potential and function of tissues, to inflammation, and to tumorigenesis in aged organisms. The identification, characterization and pharmacological elimination of senescent cells have therefore gained attention in the aging field. However, the non-specificity of current senescence markers and the existence of different senescence programs strongly limit these tasks. Here, we describe the molecular regulators of senescence phenotypes, and discuss the consequence of this regulation in identifying senescent cells *in vitro* and *in vivo*. We also highlight the importance that these levels of regulations have in the development of therapeutic targets.

The Complexity of the Senescence Phenotype

The functional decline of an organism throughout life affects multiple organs and is accompanied by the appearance of several diseases. This general decline of functional capabilities is known as aging (see **Glossary**) and is fairly conserved among species [1].

A main feature of aged organisms is the accumulation of cellular senescence [1] –a state of permanent cell cycle arrest in response to different damaging stimuli [2] (see **Box 1**). An excessive and aberrant accumulation of senescent cells in tissues can negatively affect regenerative capacities and create a pro-inflammatory milieu favorable to the onset and progression of various age-related diseases, including cancer [3,4]. However, senescent cells have several beneficial functions for the organism. Due to the activation of an irreversible proliferation arrest, cellular senescence is seen as a strong safeguard against tumorigenesis [3]. Moreover, senescent cells can act via both cell and non-cell autonomous mechanisms as positive regulators of tissue remodeling and repair during development and adulthood [5,6]. Deleterious functions of senescent cells are potentially powerful targets for anti-aging approaches [7], but the existence of beneficial senescence programs highly complicates the development of interventions without incurring toxicities.

The senescence phenotype is often characterized by the activation of a chronic DNA damage response (DDR), the engagement of various cyclin-dependent kinase inhibitors (CDKi), enhanced secretion of pro-inflammatory and tissue remodeling factors, induction of anti-apoptotic genes, altered metabolic rates and endoplasmic-reticulum (ER) stress (**Figure 1**). As a consequence of these signaling pathways, senescent cells show structural aberrations, from enlarged and more flattened morphology, altered composition of the plasma membrane, accumulation of lysosomes and mitochondria, and nuclear changes (**Figure 2**).

The understanding of how the different hallmarks are regulated and how they overlap with non-senescence states are essential to choosing the right methods to measure them. However, there are two important problems for the identification, isolation and characterization of senescent cells. First, many of the senescence-associated molecular and morphological features are present in other cellular states and conditions. Second, the phenotype of senescent cells is highly heterogeneous and dynamic, possibly a consequence of various distinct senescence programs. These limitations have to be carefully taken into account for the generation of therapies targeting senescent cells.

Box 1: Types of Senescence

In vitro senescence can be induced by different stimuli [2]. Whether all these “types of senescence” actually occur in vivo is not known yet. Here we describe the main models of senescence used in research. At least two other biological events, namely wound healing and development, are known to rely on senescence [3]. However, these two types have been less described and thus they scope the scope of this review.

- *Replicative Senescence*: it refers to the decrease in proliferation potential observed after multiple cell divisions that ultimately leads to a total arrest [160]. The shortening of telomeres as a consequence of multiple cell divisions in non-transformed cells has been blamed for this type of senescence [3].
- *DNA damage-induced Senescence*: Irreparable DNA damage can induce either senescence or apoptosis, depending on the magnitude of the damage[2]. In vitro, multiple DNA damaging agents are used to induce this type of senescence including radiation (ionizing and UV) or multiple drugs (see chemotherapy-induced senescence) [2].
- *Oncogene-Induced Senescence (OIS)*: The activation of oncogenes -such as Ras or BRAF- or the inactivation of tumor suppressors -such as PTEN- can lead to senescence [2,3].
- *Oxidative Stress-Induced Senescence*: either oxidizing products of the cell metabolism or known oxidative agents (e.g. H₂O₂) can cause senescence [49]. Although oxidizing agents exert their effect partly through DNA-damage, other cellular components and processes get also affected.
- *Chemotherapy-induced Senescence*: Multiple anti-cancer drugs are able to induce senescence. Some of them (such as bleomycin or doxorubicin) induce DNA damage, while others can act through different mechanisms such as inhibition of CDKs (e.g. abemaciclib, palbociclib) [163].
- *Mitochondrial Dysfunction-associated Senescence (MiDAS)*: it was recently reported that induction of mitochondrial dysfunction also leads to senescence [56]. The phenotype, particularly the SASP, seems to be characteristic of this type of senescence [56].
- *Epigenetically-induced Senescence*: inhibitors of DNA-methylases (e.g. 5-aza-2'-deoxycytidine) or histone deacetylases (e.g. suberoylanilide hydroxamic acid and Sodium Butyrate) are also known to cause senescence [163].
- *Paracrine Senescence*: Senescence induced by the SASP produced by a primary senescent cell [70].

Here, we describe the main hallmarks of senescent cells, the methods used to measure them and the limitations for their use as markers. Finally, we discuss how the senescence-associated hallmarks are currently exploited for anti-senescence interventions.

Signaling pathways as Hallmarks of Senescence**DNA damage Response (DDR)**

In the presence of DNA damage, cells activate a robust response, the DDR. Double strand DNA breaks (DSBs) are powerful activators of DDR, and can lead to cellular senescence when unresolved. DSBs promote the recruitment and binding of ATM kinase to the DNA damage site [8,9]. This recruitment drive the phosphorylation of the histone H2AX, which facilitates the assembly of specific DNA-repair complexes

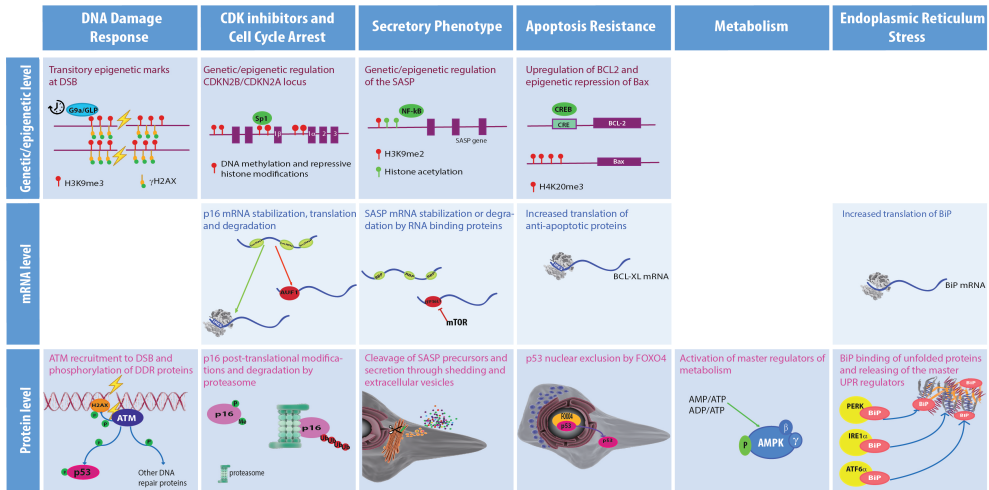


Figure 1. Hallmarks of Senescence: Regulation of Signaling Pathways. The figure 1 depicts the main regulation steps for key molecular players and signaling pathways of senescence. The regulation is described at three different levels: 1) genetic/epigenetic level that includes mechanisms that modify transcription (for instance, histone modifications, DNA methylation and recruitment of key transcription factors), 2) mRNA level that includes mechanisms such as mRNA stabilization or degradation and even recruitment of ribosomes, all of them affecting translation, 3) protein level that includes regulatory mechanisms that occur after translation (for instance, post-translational modifications, protein degradation, protein transport). DSB=double strand breaks; DDR=DNA damage response; CDK=Cyclin-dependent kinases; SASP=Senescence-associated Secretory Phenotype; UPR=Unfolded Protein Response

(Figure 1) [10]. Histone methylation can also contribute to the assembly of damage response components; a complex of kap-1, HP1, and H3K9 methyltransferase SUV39H1 are loaded directly onto the chromatin at DSBs, leading to the methylation of H3K9. H3K9me3 functions as a binding site and to activate the acetyltransferase Tip60, and subsequently acetylate and activate ATM [11]. Therefore, H3K9 methylation is required for ATM-mediated DNA damage signaling at early stages of the response, but H3K9 methylation has to be later reversed to promote the repair process. DDR provokes the degradation of G9a/GLP methyltransferase, which causes a global reduction in H3K9 dimethylation, including that of IL-6 and IL-8 promoters, two components of the Senescence-Associated Secretory Phenotype (SASP; discussed later) [12]. Many substrates are phosphorylated by ATM, including the two essential kinases CHK1 and CHK2, which propagate the signal by further phosphorylating their substrates [13,14]. The persistence of DDR induces the phosphorylation of p53 at multiple serine residues, which enhances its ability to induce the transcription of many genes [15].

Inductions of γ -H2AX nuclear foci or phosphorylated p53 are commonly used as markers of senescence. However, the DDR is activated by a variety of DNA damaging stimuli that do not lead cells into a senescent state. Moreover, not all the senescence programs are a consequence of DNA damage responses.

Cyclin-dependent kinase inhibitors (CDKi) and cell cycle arrest

Cyclin-dependent kinases (CDKs) phosphorylate and regulate multiple proteins

involved in cell cycle progression (**Figure 1**). Main drivers of the cell cycle arrest in senescence are the CDKi encoded in the CDKN2A (p16INK4a, hereafter p16), CDKN2B (p15INK4b, hereafter p15) and CDKN1A (p21CIP, hereafter p21) loci.

P16 consists of a 16kDa protein that directly interacts and inhibits CDK4/6. P16 is often used as a unique and specific marker for senescence (see **Box 2**), and its transcriptional activation has been extensively used to report the presence of senescent cells in vivo [6,16,17]. Experimental evidences suggest that main inducers of P16 levels are epigenetic changes, but other regulators from promoter accessibility to protein stability have been described.

The methyl-transferase DNMT3b is responsible for the de novo methylation of the p16 promoter [18], while DNMT1 maintains existing methylation. Inhibitors of DNMT1 cause demethylation of the p16 promoter and a senescence-like phenotype [19–21]. However, methylation levels do not always correlate with p16 gene expression [22]. The Polycomb group Repressive Complexes (PRC1 and PRC2) are also responsible for deposition of repressive histone modifications at the CDKN2A locus [23], and can be recruited to the p16 promoter by the antisense long non-coding RNA for p16, ANRIL [24]. Other epigenetic marks, such as the repressive histone variant macroH2A1, are enriched in the inactive but depleted in the active p16 locus [25].

Transcription factors, such as Sp1, Ets, AP1 (particularly JunB subunit) and PPAR γ [26–29], bind to the p16 promoter and trigger its transcription, while repressor mechanisms such as the INK4A transcription silence element (ITSE), YB1, ID1 and AP-1 (c-Jun subunit) balance the activation of p16 [23,27,30,31].

The RNA binding proteins hnRNP A1 and A2 promote the stability of p16 transcripts [32], while the ribonuclear protein AUF1 binds p16 mRNA and promotes its degradation [33]. Interestingly, there are also hints that p16 can suppress the expression of AUF1 [34]. Translation of the p16 mRNA can be modulated through a region on its 5'UTR end, which contains an Internal Ribosome Entry Site (IRES) [35], and the affinity of p16 for CDK4 can be modulated by Ser140 phosphorylation and Arg138 methylation [36]. Finally, p16 protein is degraded by N-terminus polyubiquitination and ultimately, the proteasome and this elimination is favored upon conditions of sub-confluence [37,38].

P16 induction is often measured by mRNA levels. However, the complex nature of the CDKN2A locus hinders the design of specific primers. CDKN2A encodes not only p16 but also p14, p12 and p16- γ , all of which have a similar nucleotide and/or protein sequence [23]. Co-amplification of p16- γ or p12 is less problematic because p16- γ is speculated to have the same CDKi function than p16, and p12, despite acting as a transcription factor instead of a CDKi, is only expressed in pancreas [23]. However, p14 is ubiquitously expressed and it has a completely different function promoting p53 activation [39]. Reports that base their conclusions on the expression of the whole locus or in assays co-amplifying p16 and p14 should be carefully interpreted. Even when the appropriate assay is used, it has been observed that changes in mRNA levels do not always reflect protein levels [40]. Moreover, measuring p16 protein levels can be challenging, particularly in mouse, where the lack of antibodies is a major complication.

p15 function and protein structure are similar to those of p16, but less attention has

been paid to p15 in the context of senescence [23]. p15 is downstream of the Raf-Mek-Erk and the PI3K/AKT/FOXO3 pathways [41,42], mainly regulated epigenetically by PRC complexes and histone modifications and transcriptionally by Sp1 and Miz-1 [43–45]. The latter is in turn negatively controlled by Myc and positively regulated by Arnt, a key effector in hypoxia-induced signaling. Regulation of p15 at the post-transcriptional level is less studied. However, it is long known that p15 is at least partially regulated at the translational level, by stabilization of its mRNA [46]. Moreover, p15 protein can be stabilized by TGF- β [47].

p21 is capable of inhibiting a broad range of CDKs but paradoxically, is also necessary for cell cycle progression [48]. Although p21 is consistently regulated in response to different senescence-inducing stimuli [49], its expression is part of a more generic DDR and mainly regulated by direct transactivation via p53, which makes difficult to use it as a unique senescence marker. P21 can also be activated in a p53-independent manner, guided by pathways such as TNF- β and using Sp1 as a main transcription factor [43,48,50]. Other mechanisms of p21 regulation include: transcriptional repression via c-Myc, ID1, CTIP-2, CUT and retinoid X receptor; inhibition of transcriptional elongation by disassembling elongation factors via Chk1; control of mRNA stability through binding of miRNAs (miR-17-92, miR-106a-363 and miR-106b-25) or RNA binding proteins (HuD, HuR, RBM28, Msi-1, PCBP1/CP1/hnRNP E1, TAX), or through phosphorylation (via Akt1/PKB, PKA, PKC, PIM-1, GSK β); proteasomal degradation (through ubiquitination by the E3 ubiquitin ligases) [48]. AUF1 also targets p21 mRNA directly or indirectly by promoting degradation of some of its modulators (e.g. p53 and c-Myc). Therefore, p16 might indirectly regulate p21 expression via downregulation of AUF1 [34].

A general block of proliferation could be measured via EdU-incorporation or colony-formation assays. However, lack of proliferation is not a specific mark of senescent cells and is impractical for measuring senescence in post-mitotic cells or inadequate for *in vivo* experiments.

Secretory Phenotype

Senescent cells secrete cytokines, chemokines and proteinases which are positively or negatively implicated in a number of biological processes such as wound healing and cancer progression [6,51,52]. The SASP is highly heterogeneous [49] and regulated at many levels, making the identification of more general regulatory mechanisms a challenge (**Figure 1**).

The SASP is to large extent a transcriptional program mediated by the pro-inflammatory transcription factor NF- κ B [53]. The major trigger for NF- κ B activation is the DDR, but more recently the cGAS/STING pathway (see section “Nuclear Changes”) has also been implicated [54,55]. In accordance with the hypothesis that DNA damage is an essential driver of the SASP, mitochondrial dysfunction-associated senescence (MiDAS) is associated with mild or no transcriptional induction of several SASP factors [56]. Additional transcription factors involved in the regulation of SASP genes are GATA4 and C/EBP β [57,58]. Interestingly, NOTCH signaling is dynamically expressed upon senescence [59], which may explain the dynamic composition of the SASP [49], and counteracts C/EBP β induction [59]. Notably, although NF- κ B mainly induces the expression of inflammatory cytokines, other pathways such as the Jak2/

Stat3 pathway upregulates a different set of cytokines, mainly immunosuppressive ones, such as CXCL1/CXCL2, GM-CSF, M-CSF, IL10 and IL13 [61]. In Pten-null prostate tumors, the Jak/Stat3 pathway is active and therefore it is hypothesized that inhibition of Jak/Stat3 would help tumor clearance [60].

Transcription of SASP genes can also be dependent on epigenetic changes. Repressive marks such as H3K9me2 are reduced at the promoters of IL6 and IL8, two major SASP components, probably due to the degradation of the methyltransferase G9a via proteasomal degradation downstream of an ATM-dependent DDR signal [12]. In contrast, sirtuin 1 (SIRT1), a known histone deacetylase, is downregulated during senescence and leads to increased expression of IL-6 and IL-8 via histone acetylation of the promoter regions [61]. The histone variant macroH2A1 has an interesting role in SASP expression. In one hand, macroH2A1 is depleted from SASP-containing loci during senescence and yet it is required for the expression of a number of SASP factors, including IL1A, IL1B, IL6, IL8 and MMP1, among others [62]. This apparent contradiction is explained by a feedback loop in which SASP expression causes ER-stress, which in turn activates ROS production and DDR. The DDR (through the activity of ATM), promotes macro-H2A1 mobilization in an attempt to reduce SASP expression and therefore ER-stress [62]. As mentioned, the DDR (and particularly ATM) are known activators of NF- κ B, which still poses a contradictory function for ATM and the DDR. Another histone variant, H2A.J also positively regulates the transcription of SASP factors [63]. Finally, the three-dimensional arrangement of the chromatin also influences the transcription of SASP genes [64], while chromatin-bound HMGB2 fine tunes SASP expression by avoiding heterochromatin spreading [65].

At the post-transcriptional level, mTOR regulates the SASP via two mechanisms: i) it promotes translation of IL1A, in turn, activating NF- κ B and C/EBP β [57,66]; ii) it indirectly inhibits the RNA binding protein ZFP36L1, preventing it from degrading mRNA encoding SASP factors [67]. In accordance, senescent endothelial cells that do not upregulate the mTOR pathway do not activate a SASP [68]. Moreover, the production of Reactive Oxygen Species (ROS) can trigger the p38/MAPK pathway, which in turn phosphorylates and activates other RNA binding proteins responsible for the stabilization of SASP-dependent mRNA [69].

Once synthesized some SASP factors undergo post-translational modifications. For instance, the inflammasome –a protein complex formed by caspase 1 and accessory proteins- plays an important role in the activation of the IL1-signaling pathway, by cleaving and activating IL1 β [70]. Moreover, inhibition of either caspase-1 or IL1-receptor reduced the expression of SASP and partially prevented OIS through inhibition of paracrine senescence [70]. SASP factors such as IL1 and IL6 can play cell autonomous functions and re-inforce the senescence state [71]. However, many SASP factors exert a non-cell autonomous function and can alter the behavior of neighboring cells [72]. SASP factors have been shown to promote the reorganization of embryonic structures, participate in tissue remodeling and repair, and enhance immunosurveillance [5,6]. In contrast, the chronic presence of some of the pro-inflammatory and tissue remodeling factors, such as interleukins and MMPs, have been associated to disease states and aging phenotypes [52]. Many SASP members are produced as soluble proteins that can be directly transported to the extracellular environment, but some of the SASP factors are initially expressed as transmembrane proteins and need to be released in the

extracellular space by ectodomain shedding [73]. ADAM17, a sheddase, is upregulated in OIS and cancer and regulates the ectodomain shedding of several SASP factors from the cell membrane [74,75]. Furthermore, some SASP members are secreted via small extracellular (exosome-like) vesicles that once released by senescent cells, can exert a more distal function, for instance enhancing proliferation of cancer cells [76]. Notably, the biogenesis of these vesicles is p53-dependent [77].

It is clear that the SASP plays an important role in the pathophysiological activity of senescent cells, but it is too unspecific and heterogeneous to be used as an unequivocal marker for senescence [49,52]. However, quantification of SASP composition could be potentially used for the definition of different senescence programs. For example, senescent cells associated to tissue repair express a number of MMPs and growth factors, such as PDGF-A and VEGF [6,78], while age-associated or therapy-induced senescent cells are mainly associated to inflammatory factors [79,80].

Apoptosis resistance

Senescent cells activate a number of pro-survival factors and become resistant to apoptosis [81]. Upon treatment with apoptosis inducers, senescent cells are unable to downregulate the anti-apoptotic protein BCL-2 due to chronic activation of the transcription factor cAMP response element-binding protein (CREB), which prevents BCL-2 inhibition [82]. In addition, the pro-apoptotic gene Bax, was shown to be enriched with the repressive histone mark H4K20me3 [83] (**Figure 1**).

More recently, additional pro-survival networks have been associated with senescence [84]. Key nodes of these networks include ephrins, PI3K, p21, BCL-XL, and plasminogen activated inhibitor-2 [84]. Members of the BCL-2 family, specifically anti-apoptotic BCL-XL and BCL-W, are essential for the survival of senescent cells [85]. BCL-W is transcriptionally upregulated during senescence, whereas BCL-XL shows a higher rate of translation mediated by an IRES motif [49,85]. FOXO4 is overexpressed at the mRNA and protein level in senescent cells, and prevents cell death by sequestering p53 in the nucleus [86]. p21 protects senescent cells from death by restricting JNK and caspase signaling under persistent DNA damage [87]. Finally, HSP90 was shown to be a key protein for the survival of senescent cells via stabilization of P-AKT [88].

Induction of expression of various BCL2 family members is a promising method to identify senescent cells. However, certain non-senescent cells types, particularly blood cells, also show upregulation of these anti-apoptotic regulators [89].

Metabolism

Senescent cells are metabolically active, and increases in the AMP/ATP and ADP/ATP ratios have been reported during senescence (**Figure 1**) [90]. AMP protects AMP-activated protein kinase (AMPK) from dephosphorylation and causes its allosteric activation [91]. AMPK thus acts as a sensor of the reduced energetic state, further activating catabolic pathways while inhibiting biosynthetic ones, and regulating p53 and other targets [91].

p53 can further regulate cellular metabolism by leading to inhibition of glucose uptake and glycolysis, and promoting the tricarboxylic acid (TCA) cycle, oxidative phosphorylation and fatty-acid oxidation [56,92]. Indeed, the high TCA cycle activity is

Box 2: Current methods to detect markers of senescence

Multiple techniques have been used to identify senescent cells. Given the heterogeneity of senescent cells and the lack of specificity of the markers, a combination of different techniques is often used and encouraged for the detection of senescent cells.

- *DNA damage response*: the presence of γ -H2AX foci measured by Immunostaining demonstrates continuous and unrepaired DNA damage [3]. Measurement of the level of phosphorylated p53, a key signaling player for the DDR, can be used [2].
- *Cell cycle arrest*: two types of assays are normally used to prove that cells have exited the cell cycle. The first one includes a direct measure of the proliferation potential of the cells via the measurement of the colony-formation potential or of the DNA synthesis rate by BrdU/EdU-incorporation assays. The second type includes measuring the expression level of the CDKIs p16 and p21 [3,162].
- *Secretory Phenotype*: cytokines (e.g. IL1a, IL6, IL8), chemokines (e.g. CCL2) and metalloproteinases (e.g. MMP1, MMP3) are used as markers [3,51]. It is also common practice to use either immunostainings or ELISAs to measure the protein expression and secretion of some of these factors, particularly of IL6 [84].
- *Apoptosis Resistance*: the upregulation of the BCL-proteins Bcl-2, Bcl-w or Bcl-xL has been used as a marker of senescence [81,85]. However, it is not yet regularly used.
- *Metabolism*: due to the lack of information and consensus about the metabolic changes that cells undergo upon senescence induction, this feature is not often used as a marker of senescence.
- *Endoplasmic-Reticulum Stress*: perhaps due to the inconsistency on the particular branch of the Unfolded Protein Response (UPR) that is activated in different senescence-inducing stimuli, this feature is not often used as a marker of senescence [106–109].
- *Cell Size*: in vitro, the enlarged cell body and the irregular shape of senescent cells is easily evaluated by regular bright-field microscopy [3]. Immunofluorescence targeting vimentin, actin or another cytoplasmic protein can be used to measure changes in cell shape [107].
- *Composition of Plasma Membrane*: rarely measured in senescent cells and not used as a regular marker of senescence. Recently, an oxidized form of vimentin present at the plasma membrane and other membrane proteins such as DEP1 and DPP4 have been proposed as markers [127–129].
- *Increased Lysosomal Content*: senescence-associated β galactosidase (SA- β gal) is the most common marker of lysosomal activity and one of the first tests used to assess senescence [131]. Alternatively, Sudan Black B (SBB) or its biotin-labeled analog (GL13) can be used to detect the lipofuscins from old lysosomes and LysoTrackers or orange acridine can reveal the high lysosomal content [137,144].
- *Accumulation of Mitochondria*: the use of mitotracker to measure their membrane potential and electronic microscopy to evaluate their cell shape (fusion/fission) has been used in some studies [143].
- *Nuclear Changes*: senescence-associated heterochromatin foci (SAHF) are observed as darker regions within the nucleus of senescent cells after DAPI staining and are enriched in markers of heterochromatin such as H3K9me3 and HP1 γ . However, this feature is not shared by all types of senescence and not apparent in mouse cells [3]. Alternatively, downregulated levels of LaminB1 have become a common marker [157].

essential for the tumor suppressing action of senescent cells [93]. Many other proteins besides AMPK directly or indirectly regulate p53, for instance via post-translational modifications or direct interactions, including MDM2 and loss of PTEN [94]. Mitochondrial dysfunction and mTOR activation also generate metabolic changes. MiDAS activates AMPK, and causes a reduction in the TCA cycle and in NAD⁺ [95], a key cofactor for many other proteins [96], both important players during senescence. Meanwhile, mTOR decreases autophagy and therefore influences protein homeostasis [97]. However, despite much evidence on the upregulation of mTOR during senescence [66,67,97], the autophagy status of senescent cells is less understood [98].

Finally, Rb, an essential player in cell cycle arrest during senescence, can also induce metabolic changes. Particularly, it can increase oxidative phosphorylation by promoting the conversion of pyruvate into acetyl-coA via a PDP2-mediated activation of the pyruvate dehydrogenase (PDH) [99]. Rb also inhibits the generation of deoxyribonucleotides through regulation of key enzymes via the transcription factor E2F1 [100].

It is currently a difficult task to use metabolic changes as markers for senescence. First, these changes [101,102] can be either a cause or a consequence of several other hallmarks of senescent cells. Second, only few studies have focused on the whole metabolome of senescent cells [103,104]. Additional characterization could help to identify specific metabolites for use as senescence markers.

Endoplasmic Reticulum (ER) stress

Multiple factors such as oxidative stress, mutations, infections and lack of chaperones can cause ER-stress, leading to accumulation and aggregation of proteins. To cope with the stress, the ER initiates the Unfolded Protein Response (UPR) that leads to a reduction in protein synthesis, enlargement of the ER and export of misfolded proteins [105]. Indeed, senescent cells have an increased UPR possibly in response to the increased protein synthesis demanded by the SASP [106,107].

Notably, the UPR seems to influence many other hallmarks of senescence although not always using the same effectors (Figure 1) [106–109]. Indeed, the UPR is composed by three pathways regulated by PERK, IRE1 α and ATF6 α respectively [105]. BiP – an ER protein - is known to bind these three master regulators and inhibit their functions [110]. Upon ER-stress, BiP binds to the misfolded/unfolded proteins instead, releasing its former partners and allowing UPR activation. Thus, BiP plays a central role in the UPR and possibly in senescence. Unsurprisingly, BiP is tightly regulated, mainly at the translational level: it was demonstrated that the translational efficiency of BiP is largely increased upon UPR activation [111].

Remarkably, PERK and IRE1 α are both transmembrane proteins with kinase activity that are also stabilized by heat-shock proteins (mainly HSP90 and HSP72) and whose activation is also influenced by the fluidity of the membrane [110]. ATF6 α is a cAMP-dependent transcription factor that, when inactive, localizes to the ER as a transmembrane protein. Upon activation, ATF6 α traffics to the nucleus where it is cleaved to generate an active transcription factor. Therefore, it is also regulated by its cleaving enzymes S1P and S2P [110]. Although some studies have monitored the ER-stress in senescence by qPCR of different downstream genes (ATF4, GRP78,

GADD153 or spliced XBP1) [62], this is not a common practice in the senescence field and therefore there are no consensual markers.

Morphological alterations as Hallmarks of Senescence

Cell Size and Shape

A key feature of *in vitro* senescence is the enlarged and irregularly shaped cell body. Activation of mTOR pathway is necessary for the enlargement of the cell body of senescent endothelial cells [68]. mTORC1 is known to integrate various stress signals and to modulate cell growth accordingly [112], and mTORC1 activation occurs in response to senescence-inducing stimuli [113]. In normal aging, the decline in growth factors –such as GDF11– might also contribute to activation of mTORC1 and the hypertrophy observed particularly in cardiac cells [114]. mTORC1 is mainly modulated at the post-translational level by activation of its catalytic site by the GTP-ase Rheb and by a reconfiguration of the whole mTORC1 complex, which favors interaction with downstream effectors [115].

A contributor to the senescence-associated altered cell shape is rearrangement of the cytoskeleton, mainly of vimentin filaments [106,107,116]. When decoupling the changes in size and shape in senescent cells, it was discovered that the ATF6 α signaling pathway, –one of the three branches of the UPR, (see section “Endoplasmic Reticulum”)– can control the size of the ER and the changes in cell shape during senescence [106,107]. Although the Cyclooxygenase-2/Prostaglandin-E2 pathway is a downstream effector of ATF6 α that influences cell size [107], specific targets of ATF6 α responsible for modifying cell shape are not entirely known yet. ATF6 α , as well as the other UPR branches, are able to activate NF- κ B [110,117,118], and can directly bind on the vimentin promoter (**Figure 2**) [112].

Changes in size and morphology are easily measured with either normal or fluorescent microscopy, but difficult to detect and quantify *in vivo* or *in situ*.

Composition of plasma membrane (PM)

The PM has a central role in communication with neighboring cells and the extracellular space. The most consistent change in the composition of the PM in senescent cells is the upregulation of caveolin-1 (**Figure 2**), an important component of cholesterol-enriched microdomains called caveolae [119]. A possible explanation for the senescence-promoting effects of caveolin-1 is its functional cooperation with the MAP kinase signaling pathway, a downstream effector of multiple senescence-inducing stimuli [120]. As a positive feedback loop, the p38 MAPK pathway seems to upregulate caveolin-1 both at the transcriptional level, by inducing Sp1 [121], and at the post-translational level, by direct phosphorylation [122].

In addition, caveolin-1 influences the morphology and the adherence of senescent cells [123,124]. It also contributes to an increase in p53 activity via the downregulation of SIRT1, activation of ATM and inhibition of MDM2 [125]. The function of caveolin-1 during senescence is likely dependent on its localization within caveolae instead of within other cellular compartments, as inhibition of nuclear caveolin-1 failed to inhibit senescence in IMR90 fibroblasts [126].

Other PM proteins have also been reported to change their expression in senescence. Althubiti *et al* [127] performed a proteomic screening of plasma membrane-associated proteins, identified 107 of them as being specific for senescence and developed a staining protocol using two of these proteins (DEP1 and B2MG) to detect senescent cells. Further optimization and mechanistic studies exploring the functional role of these proteins in senescence are still needed. Also, an oxidized form of vimentin is specifically expressed at the PM of senescent fibroblasts [128]. Remarkably, the surface protein DPP4 was demonstrated to sensitize senescent cells to elimination by natural killer cells [129].

Further validation of PM proteins is a promising strategy for the identification of novel markers with the unique advantage of allowing sorting of senescent cells.

Increased lysosomal content

The senescence state is characterized by upregulation of many lysosomal proteins and increased lysosomal content (**Figure 2**) [130]. The activity of the lysosomal enzyme senescence-associated beta-galactosidase (SA- β gal) activity is used as a surrogate marker for the enhanced lysosomal content of senescent cells. Since SA- β gal is upregulated during senescence, its residual activity can be measured at a sub-optimal pH of 6.0 [131,132]. SA- β gal is one of multiple transcripts encoded by the GLB1 gene [131]. Transcriptional and post-translational regulation of this enzyme include the presence of Sp1 binding sites on its promoter, the regulation of alternative splicing, and the cleavage

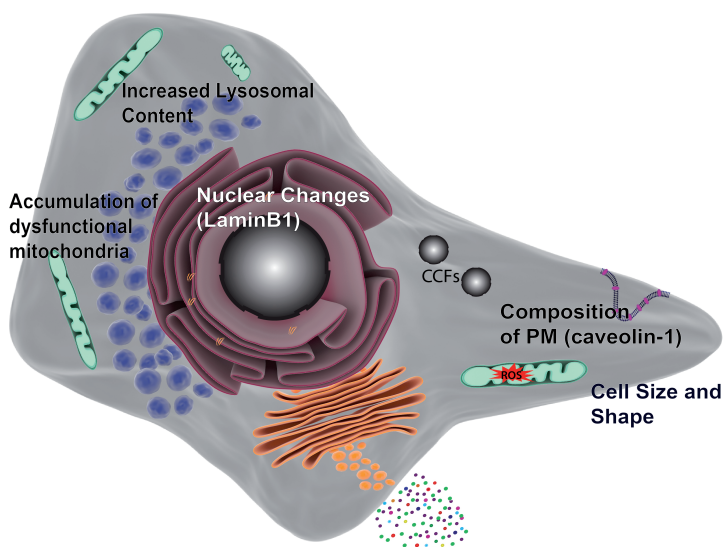


Figure 2. Hallmarks of Senescence: Morphological Alterations. The molecular pathways of senescence result in morphological alterations. Senescent cells are enlarged and have an irregular shape, the nuclear integrity is compromised due to loss of laminB1 which also leads to the appearance of Cytoplasmic Chromatin Fragments (CCFs), they have an increased lysosomal content that is often detected as high b-galactosidase activity, they have large but dysfunctional mitochondria that produce high levels of Reactive Oxygen Species (ROS) and their plasma membrane changes its composition (for instance, upregulating caveolin-1).

of the translated peptide, resulting in two smaller peptides that associate to each other and conform the active enzyme [133–136]. However, the mechanisms governing its overexpression in senescence are surprisingly unexplored. Only recently, it was shown [59] that GLB1 is negatively regulated by the NOTCH1-pathway at the transcriptional level. SA- β gal staining is arguably the most common marker of senescence. However, it cannot be used for paraffin-embedded tissue sections and live cells, which strongly limit its application.

Enhanced lysosomal content during senescence could be the result of accumulating old lysosomes or of enhanced lysosomal biogenesis. Residual bodies, namely lipofuscins, support the lack of lysosomal removal [137]. Lysosomal biogenesis is largely controlled by Transcription factor EB (TFEB), known for coordinately regulating multiple lysosomal proteins and being a downstream effector of the mTOR signaling pathway. It has been shown that TFEB can bind the promoter of β -hexosaminidase, another lysosomal enzyme, in response to oncogene-induced senescence (OIS) [138]. However, claims that TFEB is either up- or down-regulated in senescence or aging have also been reported, which makes its use as a senescence marker very difficult [130,139]. An alternative marker for the detection of accumulating lysosomes is Sudan Black B (SBB). SBB selectively binds lipofuscins and demonstrates reduced lysosomal degradation. Importantly, it can be used in paraffin-embedded tissue sections [137] and lately, a biotin-labelled analog (GL13) was synthesized helping an enhanced detection [140].

Nevertheless, high lysosomal activity is not a specific senescence marker, and the constitutive expression of SA- β gal has been identified in non-senescent cells [141].

Accumulation of mitochondria

Senescent cells show an increased number of mitochondria (**Figure 2**) [142]. However, the membrane potential of these mitochondria is decreased, leading to the release of mitochondrial enzymes like EndoG and intensified ROS production [143,144].

The main source of the extra mitochondrial content is accumulation of old and dysfunctional mitochondria due to reduced mitophagy [145]. This is, at least partly, a consequence of reduced mitochondrial fission and increased fusion [146], possibly as a mechanism to protect mitochondria from mitophagy and senescent cells from apoptosis [95].

A key step for triggering mitochondrial fission is the recruitment and translocation of Parkin to damaged mitochondria. PINK1 and Mfn2 recruit Parkin to the mitochondrial membrane, and deficiency in Mfn2 causes mitochondrial dysfunction in mouse embryonic fibroblasts and cardiomyocytes [147]. In contrast, cytoplasmic p53 interacts with Parkin, inhibiting its translocation to damaged mitochondria in doxorubicin-induced senescence in cardiomyocytes [148]. Translational regulation of Parkin cannot be excluded, since its expression is upregulated at the protein level [149].

It is unlikely that mitochondrial biogenesis plays a significant role in the increase in mitochondrial content during senescence, as senescent cells show only a transient upregulation of PGC-1 α and PGC-1 β -two important regulators of mitochondrial biogenesis [150]- followed by downregulation [146].

Nuclear changes

A common mark of senescent cells is the loss of LaminB1, a structural protein of the nuclear lamina (**Figure 2**) [49,151]. The destabilization of the nuclear integrity caused by reduced LaminB1 results in other nuclear changes such as loss of condensation of constitutive-heterochromatin and the appearance of Cytoplasmic Chromatin Fragments (CCFs) enriched in epigenetic marks associated with DNA-damage [152]. These CCFs can also be secreted to the extracellular environment via exosomes and activate DDR in other cells [12]. Moreover, senescence-associated heterochromatin foci (SAHF) –4',6-diamidino-2-phenylindole (DAPI) intense nuclear foci enriched in repressive epigenetic marks [153]– have been proposed as a compensatory mechanism to keep constitutive-heterochromatin repressed [154]. However, SAHFs are not universal markers and are mainly observed in OIS [3].

LaminB1 is downregulated at different levels. Transcriptomics studies demonstrate that downregulation of LaminB1 mRNA is a widespread marker of senescence [49]. It was also reported that miRNA-23a, which is upregulated in senescent cells, can target LaminB1 mRNA, reducing its translation [155]. Interestingly, LaminB1 is exported to the nucleus together with the CCFs and aided by the autophagy protein LC3. Once in the nucleus, it is submitted for degradation by the lysosomes [156].

LaminB1 downregulation depends on p53 and p16 but is independent from other senescence-associated pathways such as p38-MAPK, NF- κ B, DDR and ROS [157].

During irradiation-induced senescence, a sub-G1 population of cells with lower DNA content progressively appears [144]. Reduction of DNA content could be due to CCFs, but also to the release of the mitochondrial endonuclease G (EndoG) and its translocation to the nucleus as a response to loss of mitochondrial potential [144].

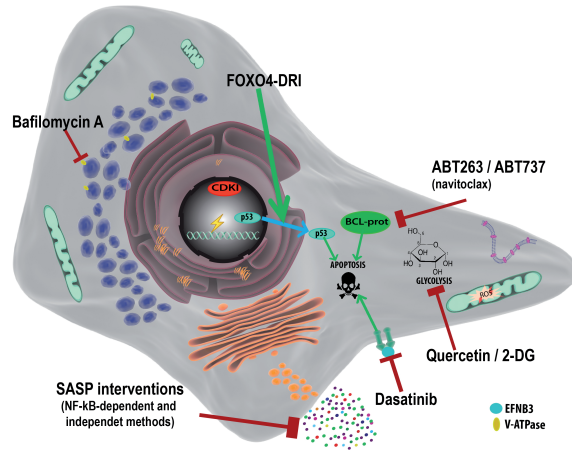
Finally, it was recently reported that senescent cells express many markers of “stemness” [158]. For instance, the gene signature of Bcl2-lymphomas submitted to therapy-induced senescence resembles the one of adult tissue stem-cells. Moreover, senescent lymphoma cells were positive for other markers such as Sca1 and H3K9me3, among others. Notably, if these senescent lymphoma cells manage to escape senescence by decreasing the expression of key senescence genes, their growth ability becomes larger than the one of the never senescent counterparts. However, this is a very recent finding that needs further confirmation in a wider type of samples to be considered a senescence hallmark.

Implications for Senescence Interventions

Among the various biological functions where cellular senescence is involved, their role in diseases such as cancer and aging has made them attractive therapeutic targets. Strategies to interfere with senescent cells are mostly based on the markers listed above (**Figure 3**). Two main approaches are currently under development: 1) specific elimination of senescent cells; 2) inhibition of the SASP.

The first approach focuses on identifying compounds that can specifically induce senescent cells to die – also defined as ‘senolytics’. As apoptosis-resistance is a main feature of senescent cells, mechanisms involved in conferring this resistance are a preferential target of senolytics. Indeed, some of the compounds discovered so far are:

Figure 3. Senescence Interventions. The different therapies that target senescent cells are depicted showing their main molecular targets. The so-called senolytics induce cell death specifically by targeting the Bcl-2 family of anti-apoptotic proteins (e.g. ABT263 and ABT737), by promoting nuclear exclusion of p53 (FOXO4-DRI), by targeting glycolysis (quercetin, 2-DG) or other pathways that lead to apoptosis (dasatinib). Many other therapies target either one or multiple members of the SASP by targeting NF- κ B or other pathways controlling the secretory phenotype.



ABT263 and ABT737, inhibitors of different members of the Bcl-2 family of anti-apoptotic proteins; FOXO4-DRI, a peptide that forces the nuclear exclusion of p53; Quercetin, a polyphenol with various functions including inhibition of Pi3K; Dasatinib, an anti-cancer drug with various functions including inhibition of EFNB1 and B3; 2-deoxyglucose (2-DG), a false substrate for the glucose exokinase which saturates the glycolytic flux [7]. Many of these drugs have intrinsic toxicities which could limit their use for human purposes. Moreover, they target only subsets of senescent cells without discriminating among beneficial and deleterious senescence programs.

The second approach aims at reducing the negative effects of the SASP. As NF κ B is a major driver of the SASP, inhibiting its function or neutralizing some of its members is an effective strategy to lower the expression of several pro-inflammatory factors in senescent cells [7]. Interestingly, major life-extending compounds such as rapamycin, resveratrol and metformin have been shown with variable degree of confidence to reduce the SASP [97,159]. A major limitation of SASP inhibitors is the non-specificity for senescent cells, as most of these compounds are known anti-inflammatory drugs with severe toxicities when supplied for long-term. Moreover, the SASP can have beneficial functions, and current SASP modulators are unable to spare these positive programs from being inhibited.

Concluding remarks

Since the discovery of senescent cells by Hayflick and Moorhead in 1961 [160], the scientific community has struggled to identify universal and unequivocal markers characterizing the senescence state. The difficulty to identify such markers reflects the complexity of the senescence phenotype and the existence of highly heterogeneous senescence programs. At the moment, the only possibility resides in combining the measurement of multiple hallmarks in the same sample [3]. For example, qPCR is a method of preference to measure many markers at the same time with relatively low costs. However, three problems arise with this choice. First, standard qPCR experiments use relative quantification methods relying on reference or housekeeping genes, such as cytoskeleton proteins (e.g. actin or tubulin) or metabolic enzymes (e.g. GAPDH),

Glossary

Aging: Functional decline of an organism throughout life [1].

Alternative splicing: process that allows a gene to encode different mRNA products by differentially using exons and excluding introns in a primary transcript to give rise to different processed mRNAs.

Apoptosis: normal physiological form of cell death [82].

Autophagy: Intracellular degradation system. It can degrade nonspecific (general autophagy) or specific (selective autophagy) targets [149].

Caveolae: cholesterol-enriched microdomains of the plasma membrane [119].

Cellular Senescence: State of permanent cell cycle arrest in response to different damaging stimuli [2].

DNA damage response: robust response of the cells to the presence of DNA damage.

Epigenetic landscape: regulatory mechanism of genes that affects their transcription. It includes methylation of DNA, post-translational modification of histones and other chromatin-remodeling events [1].

Heterochromatin: chromatin enriched in repressive marks.

Inflammasome: protein complex formed by caspase 1 and adaptor proteins

Metabolism: Set of chemical reactions that occur in an organism to obtain energy and building materials or to use them to build different and more complex structures.

Mitochondrial dysfunction: Condition in which the regulation of mitochondrial homeostasis, the production of mitochondrial metabolites, the mitochondrial membrane potential and the Reactive Oxygen Species generation are altered [95,143].

Mitophagy: Selective autophagy of mitochondria (see “autophagy” in Glossary) [146].

Nuclear lamina: protein structure surrounding the interior of the nuclear membrane that supports the structural integrity and shape of the nucleus [154].

Omics techniques: High throughput techniques including genomics, proteomics, transcriptomics, metabolomics, etc.

Post-translational regulation: regulatory mechanism in which a protein suffers modifications (such as methylations, phosphorylations, acetylations, shedding, etc) that affect their function.

qPCR: Molecular biology technique used to amplify and detect small amounts of mRNA from a particular protein and to quantify them [161].

Reactive Oxygen Species (ROS): By-products of mitochondrial respiration [143].

Senescence-Associated- β galactosidase: activity of the β -galactosidase enzyme detectable at pH6.0

Senescence-Associated Heterochromatic Foci (SAHF): 4',6-diamidino-2-phenylindole (DAPI) intense nuclear foci enriched in repressive epigenetic marks [153].

Secretory Phenotype: set of molecules secreted by Senescent Cells. It includes extracellular matrix remodeling enzymes and inflammatory molecules [3].

Shedding: proteolytic release of the extracellular domain of transmembrane proteins [75].

Transactivation: increased expression of a gene requiring the presence of another protein called “transactivator”.

Transcriptional regulation: regulatory mechanism in which the transcription of the gene is either activated or repressed, for instance by transcription factors or repressors.

Translational regulation: regulatory mechanism in which the translation of an mRNA into protein is either enhanced or reduced.

Unfolded Protein Response: response of the ER against accumulation and aggregation of proteins that happens as a consequence of multiple factors such as oxidative stress, mutations, infections and lack of chaperons [105].

which expression might vary in senescence [161,162]. Second, some of the hallmarks of senescence are regulated at the translational or post-translational level, making the qPCR an inadequate method. Third, the use of qPCR is currently performed on whole populations, while the engagement of single cell techniques aimed at analyzing any cell in a certain tissue or biopsy would be preferable.

The use of -omics techniques to quantify various macromolecules, possibly at the single-cell level to include intra-populations variability, is then a preferred avenue for the discovery of novel markers [162]. Currently, the low sensitivity and high cost of such techniques are not suitable for the study of senescence, particularly for the characterization of senescent cells in tissues.

An alternative strategy is to systematically define the different senescence programs by identifying program-specific traits. This approach would not only offer a fresh and more complex understanding of the heterogeneity of cellular senescence, but also provide better hits for the design of therapeutic approaches aimed at interfering with detrimental senescence features while maintaining the beneficial components.

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Chapter 3

Induction and Validation of Cellular Senescence in Primary Human Cells

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Abstract

Cellular senescence is a state of permanent cell cycle arrest activated in response to different damaging stimuli. Activation of cellular senescence is a hallmark of various pathophysiological conditions including tumor suppression, tissue remodeling and aging. The inducers of cellular senescence *in vivo* are still poorly characterized. However, a number of stimuli can be used to promote cellular senescence *ex vivo*. Among them, most common senescence-inducers are replicative exhaustion, ionizing and non-ionizing radiation, genotoxic drugs, oxidative stress, and demethylating and acetylating agents. Here, we will provide detailed instructions on how to use these stimuli to induce fibroblasts into senescence. This protocol can easily be adapted for different types of primary cells and cell lines, including cancer cells. We also describe different methods for the validation of senescence induction. In particular, we focus on measuring the activity of the lysosomal enzyme Senescence-Associated β galactosidase (SA- β gal), the rate of DNA synthesis using 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay, the levels of expression of the cell cycle inhibitors p16 and p21, and the expression and secretion of members of the Senescence-Associated Secretory Phenotype (SASP). Finally, we provide example results and discuss further applications of these protocols.

Introduction

In 1961 Hayflick and Moorhead reported that primary fibroblasts in culture lose their proliferative potential after successive passages [1]. This process is caused by the sequential shortening of telomeres after each cell division. When telomeres reach a critically short length they are recognized by the DNA-Damage Response (DDR) which activates an irreversible arrest of proliferation –also defined as replicative senescence. Replicative senescence is currently one of the many stimuli that are known to induce a state of permanent cell cycle arrest that renders cells insensitive both to mitogens and to apoptotic signals [2,3]. The senescence program is normally characterized by additional features including high lysosomal activity, mitochondrial dysfunction, nuclear changes, chromatin rearrangements, endoplasmic reticulum stress, DNA damage and a senescence-associated secretory phenotype (SASP) [2,4]. Senescent cells have multiple functions in the body: development, wound healing and tumor suppression [3]. Equally, they are known to play an important role in aging and, paradoxically, in tumor progression [5]. The negative, and partially contradictory, effects of senescence are often attributed to the SASP [6].

Recently, it was shown that elimination of senescent cells from mice leads to lifespan extension and to elimination of many of the aging features [7–12]. In the same way, multiple drugs have been developed to either eliminate senescent cells (senolytics) or to target the SASP [13,14]. The anti-aging therapeutic potential has recently attracted more attention to the field.

The study of mechanisms associated to cellular senescence and the screenings for pharmacological interventions heavily rely on *ex vivo* models, particularly on human primary fibroblasts. While there are some common features activated by diverse senescence inducers, a large variability in the senescence phenotype is observed and dependent on various factors including cell type, stimulus and time point [2,15–17]. It is imperative to consider the heterogeneity for studying and targeting senescent cells.

Therefore, this protocol aims to provide a series of methods used to induce senescence in primary fibroblasts by using different treatments. As it will be explained, the methods can easily be adapted to other cell types.

Apart from replicative senescence, we describe five other senescence-inducing treatments: ionizing radiation, ultraviolet (UV) radiation, doxorubicin, oxidative stress and epigenetic changes (namely promotion of histone acetylation or DNA demethylation). Both, ionizing radiation and UV-radiation cause direct DNA damage and, at the appropriate dose, trigger senescence [18,19]. Doxorubicin also causes senescence mainly through DNA damage by intercalating into the DNA and disrupting topoisomerase II function and thus halting DNA repair mechanisms [20]. The expression of genes essential for senescence is normally controlled by histone acetylation and DNA methylation. As a consequence, histone deacetylase inhibitors (e.g. sodium butyrate and SAHA) and DNA demethylating (e.g. 5-aza) agents trigger senescence in otherwise normal cells [21,22].

Finally, four of the most common markers associated to senescent cells will be explained: activity of the senescence associated- β galactosidase (SA- β gal), rate of DNA synthesis by EdU incorporation assay, overexpression of the cell cycle regulators and cyclin-dependent kinase inhibitors p16 and p21, and overexpression and secretion of members of the SASP.

Protocol

General preparation

- D10 medium: Supplement DMEM medium-Glutamax with 10% FBS and 1% penicillin/streptomycin (Final concentration: 100U/ml)
- Sterile PBS: Dissolve the tablets in water according to manufacturer's instructions. Sterilize by autoclave.
- Trypsin 1x: Dilute 5ml Trypsin-Versene EDTA/10x 1:10 in 45ml sterile PBS.

Note: Throughout the protocol we use cell culture conditions that are closer to the physiological conditions for primary fibroblasts. This means that we incubate cells at 37 °C and 5% CO₂ as is normally done, but using 5% O₂ instead of the “standard” 20% O₂.

Note 2: All handling of samples should be done in sterile conditions by using a laminar flow hood, lab coat and gloves. Cells are kept at 20% O₂ (room conditions) only while being handled.

Induction of senescence

1. *Replicative Senescence*

Protocol

- 1.1 While handling cells or any material that will be in contact with them (pipets, flasks, media, etc) work under sterile conditions, by using a laminar flow hood,

lab coat and gloves. Cells are kept at 20% O₂ (room conditions) only while being handled.

- 1.2 Seed 7×10^5 viable primary fibroblasts in a low population doubling (PD) in a T75 flask ($\sim 9.3 \times 10^3$ cells/cm²) containing 10 ml D10.
- 1.3 Grow the cells in a cell incubator at 37 °C with 5% CO₂ and 5% O₂ until they reach 70-80% confluence (3-4 days for proliferating cultures in a low PD).
- 1.4 Detach the cells using 3 ml Trypsin 1x and incubating for ~ 5 -7 minutes in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂. Monitor the cells regularly with a cell culture microscope to check the detaching process.
- 1.5 When cells are spherical stop the Trypsin by adding 9 ml of D10.

Note: Never incubate longer than 10 minutes.

- 1.6. Spin the cells for 5 min at 300xg. Cells will form a pellet at the bottom of the tube, while debris and smaller particles will remain in the supernatant.
- 1.7. Remove the supernatant and dissolve the cell pellet in 1 ml D10 and perform a cell count using an automated cell counter according to manufacturer's instructions or a Neubauer chamber for manual counting. While counting, include an assay to check the viability of the cells (e.g. via trypan blue exclusion [23]).
- 1.8. Calculate the cumulative PD using this formula:

$$PD_{\text{new}} = 3.32 * (\text{LOG}(\text{cell number total}) - (\text{LOG}(\text{cell number seeded})) + PD_{\text{old}}$$

Cell number total= all cells counted: dead and alive.

Cell number seeded= number of viable cells seeded (8×10^5 cells).

PD_{old}= population doubling at the moment of seeding.

PD_{new}=population doubling at the moment of counting (after incubation).

Example: If 7×10^5 primary fibroblasts (PD 35.2) were seeded on a T-75 flask and, after 4 days they reach 80% confluence and are split again, counting now 1.3×10^6 total cells (dead+alive).

$$PD_{\text{new}} = 3.32 * (\text{LOG}(1,300,000 \text{ cells}) - \text{LOG}(700,000)) + 35.2$$

$$PD_{\text{new}} = 36.1$$

- 1.9. Reseed 8×10^5 cells in a new T75, repeating steps 1.2–1.8.
- 1.10. After multiple consecutive passages, the culture will take longer to get confluent until cells stop dividing at all. Once cells stop dividing, test for senescence markers and/or harvest for downstream applications.

Note: Consider that the bigger size of senescent cells may cause the culture to appear full and yet have a low cell count. Thus, senescence can be assumed when the PD is stable and other senescence markers appear in the culture (see protocols 7-10).

Note: Use proliferating primary fibroblasts as control.

2. Ionizing Radiation-induced Senescence

Protocol

- 2.1. While handling cells or any material that will be in contact with them (pipets, flasks, media, etc) work under sterile conditions by using a laminar flow hood, lab coat and gloves. Cells are kept at 20% O₂ (room conditions) only while being handled.
- 2.2. Seed 7×10^5 viable primary fibroblasts in a low PD in a T75 flask ($\sim 9.3 \times 10^3$ cells/cm²) containing 10 ml of D10.
- 2.3. Incubate the cells overnight in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂.
- 2.4. Expose the cells to 10 gray of gamma irradiation according to the instructions of the machine in use.
- 2.5. Aspirate the medium from the cells and replace with 10 ml D10.
- 2.6. Incubate the cells in 10 ml D10 in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂ for another 10 days replacing the medium regularly, approximately every 3 days.
- 2.7. After 10 days, test for senescence markers and/or use the cells for downstream applications.

Note: Use proliferating primary fibroblasts of the same PD (before irradiation) as control.

3. Ultraviolet (UV) radiation-Induced Senescence

Protocol

- 3.1. While handling cells or any material that will be in contact with them (pipets, flasks, media, etc) work under sterile conditions by using a laminar flow hood, lab coat and gloves. Cells are kept at 20% O₂ (room conditions) only while being handled.
- 3.2. Seed $1.5\text{--}2 \times 10^5$ viable primary fibroblasts in a low PD in one well of a 6-well plate ($1.5\text{--}2.0 \times 10^4$ cells/cm²), add 2ml of D10 medium.
- 3.3. Place the cells in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂ and allow them to adhere to the plastic for at least five hours.
- 3.4. Take off the medium from the cells. Place the 6-well plate in the middle of the UV-radiation chamber and take the plastic lid off. Irradiate with UVB, 20-30mJ/cm².
- 3.5. Add 2 ml of medium per well.
- 3.6. Incubate the cells in 2ml D10 in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂ for another 7 days replacing the medium regularly, approximately every 3 days.
- 3.7. After 7 days, cells can be tested for senescence markers and used for downstream applications.

Note: Use proliferating primary fibroblasts of the same PD (before irradiation)

as control.

4. Doxorubicin-induced Senescence

Preparation

- 1000x Doxorubicin stock solution: Make a 250 μ M stock of Doxorubicin in PBS, filter-sterilize the solution and aliquot in 500 μ l per sterile Eppendorf tube. Store the doxorubicin stock at -80 °C.

Protocol

- 4.1. While handling cells or any material that will be in contact with them (pipets, flasks, media, etc) work under sterile conditions by using a laminar flow hood, lab coat and gloves. Cells are kept at 20% O₂ (room conditions) only while being handled.
- 4.2. Seed 7×10^5 viable primary fibroblasts in a low PD in a T75 flask ($\sim 9.3 \times 10^3$ cells/cm²) containing 10 ml D10.
- 4.3. Incubate the cells overnight in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂.
- 4.4. Dilute 11 μ l of the 1000x doxorubicin stock solution in 11 ml of D10 to a final concentration of 250 nM.

Note: Optimize the treatment for the cell type of interest by, for instance, making a curve dose-response to evaluate toxicity.

- 4.5. Aspirate the medium from the cells and replace with 10 ml D10 + doxorubicin.
- 4.6. Incubate the cells for exactly 24 hours in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂.
- 4.7. Aspirate the medium from the cells and carefully wash once with 10 ml D10.
- 4.8. Incubate the cells in 10 ml D10 for another 6 days replacing the medium regularly, approximately every 3 days.
- 4.9. At day 7 test for senescence markers and/or use for downstream applications.

Note: As control, use proliferating primary fibroblasts of the same PD treated for 24 hours with vehicle (PBS) 1:1000 in D10.

5. Oxidative Stress-induced Senescence

Protocol

- 5.1. While handling cells or any material that will be in contact with them (pipets, flasks, media, etc) work under sterile conditions by using a laminar flow hood, lab coat and gloves. Cells are kept at 20% O₂ (room conditions) only while being handled.
- 5.2. Seed 7×10^5 viable primary fibroblasts in a low PD in a T75 flask ($\sim 9.3 \times 10^3$ cells/cm²) containing 10 ml D10.
- 5.3. Incubate the cells overnight in a cell incubator at 37 °C with 5% CO₂ and 5% O₂.
- 5.4. Prepare a solution of $\sim 200 \mu$ M hydrogen peroxide in D10 medium by adding

22.6µl of 30% hydrogen peroxide in 11ml of D10.

Note: Optimize the treatment for the cell type of interest by making a curve dose-response to evaluate toxicity.

- 5.5. Aspirate the medium from the cells and add 10 ml of the freshly prepared D10 medium+hydrogen peroxide. Incubate for 2 hours at 37 °C with 5% CO₂ and 5% O₂.
- 5.6. Aspirate the medium from the cells and wash once with fresh D10 without hydrogen peroxide.
- 5.7. Add 10ml of D10 without hydrogen peroxide.
- 5.8. Incubate for 48 hours in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂.
- 5.9. Repeat steps 5.4-5.8 two times more for a total of three treatments.
- 5.10. Check for senescence markers or harvest for downstream applications.

Note: As control, use proliferating cells of the same PD treated with 22.6 µl sterile water in D10 for two hours.

6. Epigenetically-induced Senescence

Preparation

- Prepare stock and working solutions for the small molecule(s) to use according to **Table 1**. Filter-sterilize the solutions. Aliquot in sterile Eppendorf tubes. Store at -20 °C.

Table 1. Solutions needed for Epigenetically-Induced Senescence

	Diluent	Stock solution	Working solution	Dilution for Treatment	Final Concentration
SAHA	DMSO	100mM	1mM	1:1000	1µM
Sodium butyrate	Sterile water	---	1M	1:250	4mM
5-aza-2'-deoxycytidine (5-aza)	DMSO	100mM	10mM	1:1000	10µM

Protocol

- 6.1. While handling cells or any material that will be in contact with them (pipets, flasks, media, etc) work under sterile conditions, for instance, by using a laminar flow hood, lab coat and gloves. Cells are kept at 20% O₂ (room conditions) only while being handled.
- 6.2. Seed 7x10⁵ viable primary fibroblasts in a low PD in a T75 flask (~9.3x10³ cells/cm²) containing 10 ml D10.
- 6.3. Incubate the cells overnight in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂.
- 6.4. Prepare 11 ml of D10 medium + working solution for the desired treatment. The exact dilution per treatment can be seen in **Table 1**.
 - 6.4.1. 11 µl of 1mMSAHA working solution in 11 ml of D10.
 - 6.4.2. 44 µl of 1M sodium butyrate working solution in 11 ml of D10.

6.4.3. 11 μ l of 10mM 5-aza working solution in 11 ml of D10.

Note: Optimize the treatments for the cell type of interest by, for instance, making a curve dose-response to evaluate toxicity.

- 6.5. Add D10 medium + working solution for the desired treatment to the culture.
- 6.6. Incubate for 24 hours in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂.
- 6.7. Repeat steps 6.4-6.6 two times more, for a total of three treatments.
- 6.8. Change medium for simple D10 without drug.
- 6.9. After 3 days more, cells become senescent and ready for testing of senescence markers and downstream applications.

Note: As control, use proliferating primary fibroblasts of the same PD treated for three days with D10 medium+vehicle. The D10 medium+vehicle needs to be refreshed every 24 hours during those three days. The vehicle depends on the treatment used: 1:1000 DMSO for SAHA and 5-aza and 1:250 sterile water for Sodium Butyrate.

Markers of senescence

1. Senescence-associated β -galactosidase Staining

Preparation

- 20mg/ml X-gal: Dissolve 20mg X-gal in 1ml of dimethylformamide or DMSO. Store at -20 °C protected from light.
- 0.1M citric acid solution: Dissolve 2.1g citric acid monohydrate in 100ml water. Store at room temperature.
- 0.2M sodium phosphate solution: Dissolve 2.84g sodium dibasic phosphate or 3.56g sodium dibasic phosphate dehydrate in 100ml water. Store at room temperature.
- 0.2M citric acid/sodium phosphate pH 6.0: Dissolve 36.85ml of 0.1M citric acid solution and 63.15ml 0.2M sodium phosphate. Adjust exactly to pH 6.0! Store at room temperature.
- 100mM potassium ferrocyanide: Dissolve 2.1g of potassium ferrocyanide in 50ml water. Store at 4 °C protected from light.
- 100mM potassium ferricyanide: Dissolve 1.7g of potassium ferricyanide in 50ml water. Store at 4 °C protected from light.
- 5M sodium chloride: Dissolve 14.6g of sodium chloride in 50ml water. Store at room temperature.
- 1M magnesium chloride: Dissolve 4.8g of magnesium chloride in 50ml water. Store at room temperature.
- 2% formaldehyde + 0.2% glutaraldehyde in PBS: Dissolve 800 μ l of 25% glutaraldehyde and 12.5 μ l of 16% formaldehyde in 100 μ l of PBS. Store at room temperature protected from light.
- Staining solution: Prepare fresh according to **Table 2**

Protocol

- 1.1. For each sample, seed 1×10^4 cells in at least one well of a 24-well plate (5.2×10^3 cells/cm²) containing 500 μ l of D10 so that the cells are sparse. Treatments (if applicable) can be performed directly on this plate or, alternatively, cells treated already can be re-seeded into a 24-well plate.
- 1.2. Incubate the cells overnight at 37 °C with 5% CO₂ and 5% O₂.

1.3. Wash cells

Table 2. Staining solution Senescence-associated β -galactosidase Staining

Component	Volume	Final concentration
20mg/ml X-gal	1ml	1mg/ml
0.2M citric acid/sodium phosphate buffer pH=6.0	4ml	40mM
100mM potassium ferrocyanide	1ml	5mM
100mM potassium ferricyanide	1ml	5mM
5M sodium chloride	0.6ml	150mM
1M magnesium chloride	0.04ml	2mM
Water	12.4ml	-
Total	20ml	

- two times with 500 μ l of PBS.
- 1.4. Fix 3-5 minutes at room temperature using 500 μ l/well of 2% formaldehyde + 0.2% glutaraldehyde in PBS.
 - 1.5. Wash cells two times with 500 μ l of PBS.
 - 1.6. Prepare fresh staining solution, according to the number of samples to stain.
 - 1.7. Add staining solution (500 μ l/well) and seal the plate with parafilm to avoid evaporation.

Note: Evaporation may cause crystals to form and hinder the observation under the microscope.

- 1.8. Incubate cells in the dark (e.g. covered in aluminium foil) at 37 °C in a dry incubator (without CO₂) for 12-16 hours.

Note: CO₂ may affect the pH and therefore modify the results. Some cell types may require shorter incubation times.

- 1.9. Wash two times with 500 μ l of PBS.
- 1.10. Assess the results. Positive cells present a blue (mostly) perinuclear staining under a normal light microscope (**Figure 1A**).
- 1.11. For quantification, observe at least 100 cells per sample and count the number of positive cells. Since senescent cells change shape, it is often difficult to define the cell boundaries and to count the cells. Counterstain with DAPI to facilitate visualization and quantification of individual cells (**Figure 1B**). Quantify the samples (percentage of positive cells versus total amount of cells) using a fluorescent microscope (**Figure 1C**). Take multiple pictures of the same sample so that at the end you can count at least 100 single-cells and

evaluate the percentage of SA- β gal positive cells in them.

- 1.12. Compare results of senescent cells versus their appropriate control for the treatment used.

Note: a co-staining with EdU on the same sample is possible. Consider that cells should be then seeded on coverslips.

2. EdU Incorporation Assay

Protocol

- 2.1. Put a coverslip/well in a 24-well plate according to the number of samples to assess.
- 2.2. Seed 1×10^4 cells in at least one well of a 24-well plate (5.2×10^3 cells/cm²) per condition containing 500 μ l of D10 so that the cells are sparse. Treatments (if applicable) can be performed directly on this plate or, alternatively, already treated cells can be re-seeded into a 24-well plate.
- 2.3. Incubate the cells overnight at 37 °C with 5% CO₂ and 5% O₂.
- 2.4. Make a 20 μ M solution of EdU in D10 (1:250) according to the number of samples to treat (250 μ l per sample).
- 2.5. Remove half of the medium (250 μ l) from each well to treat and replace it with the D10 + EdU solution that was just prepared. Final EdU concentration is 10 μ M.
- 2.6. Incubate 18-24 hours in a cell culture incubator at 37 °C with 5% CO₂ and 5% O₂. Use the same incubation time in control and senescent cells.
- 2.7. Wash 2x with 500 μ l of PBS.
- 2.8. Fix the cells for 10 minutes with 500 μ l of 4% Formaldehyde in PBS.
- 2.9. Incubate 5 minutes in 500 μ l of 100 mM Tris (pH 7.6).
- 2.10. Permeabilize the cells for 10 minutes in 500 μ l of 0.1% Triton X-100 in PBS.
- 2.11. Wash 3x with PBS.
- 2.12. Prepare 50 μ l of label mix for each coverslip, adding the components in the following order:

PBS.....44.45 μ l

Cu(II)SO₄.....0.5 μ l

sulfo-Cy3-azide.....0.05 μ l

Sodium-Ascorbate 5 μ l

- 2.13. Put 50 μ l of the label mix on a piece of parafilm. Lift the coverslip with the aid of a pair of tweezers and a needle and let it rest on top of the label mix, with the surface containing the cells facing downwards. Ensure that there are no bubbles and that the whole surface of the coverslip is touching the label mix. Incubate for 30 min in the dark.
 - 2.14. Put the cells back in the wells of the 24-well plate and wash them three times with PBS.
 - 2.15. Mount with mounting media (including DAPI to visualize nuclei) onto glass
-

- slides and let them dry overnight
- 2.16. Visualize the EdU incorporation using a fluorescent microscope. Use a filter appropriate for Cy3 (excitation / emission: 552 / 570 nm)
 - 2.17. Make multiple pictures of the cells so that you can later quantify at least 100 cells per condition (Cy3 and DAPI).
 - 2.18. Quantify the percentage of cells that incorporated EdU by using the following formula:
$$\text{EdU positive cells (\%)} = (\text{EdU positive cell count (Cy3)} / \text{total cell count (DAPI)}) * 100$$
 - 2.19. Compare results of senescent cells versus their appropriate control for the treatment used.

Note: a co-staining with SA- β gal on the same sample is possible. Consider that cells should still be seeded on coverslips.

3. Gene Expression of p16, p21 and SASP

Special materials

- Primer-sets of Genes of Interest (50 μ M each primer).

A qPCR of p16, p21 and some relevant SASP factors is informative of the senescence status. The protocol described here makes use of the Universal Probe Library (UPL) system (Roche) for relative quantification using a real time-PCR. **Table 3** shows an overview of the primers used for detection of the CDK inhibitors p16 and p21 and of the most relevant SASP members, as well as for Tubulin and Actin, which serve as reference genes for the assay. Last column of **Table 3** enlists the particular UPL probe to be used for each assay.

Protocol

- 3.1. Prepare separate qPCR reaction mix for the desired assays. Always include as well the reference gene. According to **Table 4**.
 - 3.1.1 Run all samples in duplicates or triplicates.
- 3.2. Load 7.5 μ l/well of the qPCR reaction mix on a 384-well plate.
- 3.3. Add ~5ng of cDNA dissolved in 2.5 μ l of RNase-free water.

Note: it is preferable to have similar amounts of cDNA for all the samples to be compared. This can be achieved by using equal amounts of RNA for the reverse transcription.

- 3.4. Cover the plate with a seal and make sure that it sticks correctly covering evenly all the wells on the plate.
- 3.5. Spin the plate for 1 minute at 2000xg.
- 3.6. Run the plate in the Lightcycler480 for 40 cycles using the following protocol:
 - 1) 95 $^{\circ}$ C for 7 minutes
 - 2) 95 $^{\circ}$ C for 5 seconds

Table 3. Primers used to Measure Gene Expression of p16, p21 and SASP

Target	Forward Primer (5'→3')	Reverse Primer (5'→3')	UPL probe
Tubulin	CTTCGTCTCCGCCATCAG	CGTGTTCAGGCAGTAGAGC	#40
Actin B	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG	#64
P16	GAGCAGCATGGAGCCTTC	CGTAACTATTCGGTGC GTT	#67
P21	TCACTGTCTTGTACCTTGTGC	GGCGTTTGGAGTGGTAGAAA	#32
IL6	CAGGAGCCAGCTATGAACT	GAAGGCAGCAGGCAACAC	#45
IL8	GAGCACTCCATAAGGCACAAA	ATGGTTCCTTCCGGTGGT	#72
IL1a	GGTTGAGTTTAAAGCCAATCCA	TGCTGACCTAGGCTTGATGA	#6
CXCL1	CATCGAAAAGATGCTGAACAGT	ATAAGGGCAGGGCCTCCT	#83
CXCL10	GAAAGCAGTTAGCAAGGAAAGGT	GACATATACTCCATGTAGGGAAGTGA	#34
CCL2	AGTCTCTGCCGCCCTTCT	GTGACTGGGGCATTGATTG	#40
CCL20	GCTGCTTTGATGTCAGTGCT	GCAGTCAAAGTTGCTTGCTG	#39
PAI1	AAGGCACCTCTGAGAACTTCA	CCCAGGACTAGGCAGGTG	#19
MMP1	GCTAACCTTTGATGCTATAACTACGA	TTTGTCGCATGTAGAATCTG	#7
MMP3	CCAGGTGTGGAGTTCCTGAT	CATCTTTTGGCAAATCTGGTG	#72
MMP9	GAACCAATCTCACCGACAGG	GCCACCCGAGTGTAAACCATA	#53

- 3) 60 °C for 30 seconds
- 4) Repeat step 2-3 40 times
- 5) 37 °C for 1 minute

3.7. For the analysis of the results, use the method proposed for Livak and colleagues to analyze qPCR data [24]. Use either Tubulin or Actin as reference genes to calculate the ΔC_t value and use the appropriate control to calculate the for the $\Delta\Delta C_t$ value for the specific senescence-inducing treatment.

Table 4. Contents of the qPCR Reaction Mix

Component	Volume/sample
Sensifast Probe Lo-Rox mix	5 μ l
Primer-set (50 μ M)	0.1 μ l
UPL probe	0.1 μ l
Nuclease-free water	2.3 μ l
cDNA (~4ng)	2.5 μ l
Total	7.5μl

4. Protein expression and secretion of IL6

Protocol

- 4.1. Seed 5-10x10⁴ cells in at least one well of a 6-well plate (5.2-10.5x10⁴ cells/cm²) per condition containing 2 ml of D10. Treatments (if applicable) can be performed directly on this plate or, alternatively, cells treated already can be re-seeded into a 24-well plate. Let it stand overnight after seeding.
- 4.2. Remove the medium and replace for 2ml of DMEM medium WITHOUT FBS. Incubate in normal conditions for 24 hours.
- 4.3. Collect the medium in a 15-ml tube.
- 4.4. Centrifuge the sample 5 min at 300xg. Medium can be stored at -80C until

- processed.
- 4.5. Follow manufacturer's instructions to perform the ELISA (https://www.rndsystems.com/products/human-il-6-duoset-elisa_dy206).
 - 4.6. Compare results of senescent cells versus the appropriate control for the specific senescence-inducing treatment

Representative results

Enrichment of SA- β gal staining in senescent fibroblasts

β galactosidase (β gal) is a lysosomal enzyme that is expressed in all cells and that has an optimum pH of 4.0 [25,26]. However, during senescence, lysosomes increase in size and, consequently, senescent cells accumulate β gal. The increased amounts of this enzyme make it possible to detect its activity even at a suboptimal pH 6.0 [25,27]. **Figure 1A** shows representative images of the SA- β gal staining in proliferating versus senescent primary fibroblasts. Cells also look enlarged and with an irregular cell body. As mentioned, it can be hard to distinguish individual cells, so that a co-staining with DAPI facilitates visualization and cell counting (**Figure 1B**). It is necessary to take pictures in a fluorescence microscope to be able to observe the DAPI staining. This means that pictures on the bright field channel will be taken in black/white, so that the "blue" staining of the SA- β gal will appear black on the pictures. Of note, not all the cells within a sample are positive for β gal. The efficiency of senescence induction is highly dependent on the stimulus- and cell type/strain used. The protocols described here yielded >50% β gal positive cells in primary fibroblasts (BJ and WI-38) in our hands.

Fewer cells incorporate EdU after induction of senescence

EdU is an analog of the nucleoside thymidine that, during active DNA synthesis, will be incorporated into the DNA [28]. The incorporation of EdU into DNA can be visualized after performing the Copper-Catalyzed Azide-Alkyne cycloaddition (CuAAC) to the EdU, reaction that cannot be performed in regular thymidine because it lacks the alkyne group [28]. In this particular protocol a Sulfo-Cy3-azide is being used. If the coupling of the azide to the alkyne has taken place, cells will display fluorescence under a Cy3 filter (**Figure 2A**). It is important to take into account that by performing the EdU incorporation assay, cells that are proliferating can be distinguished from non-proliferating cells. The non-proliferating cells can be either quiescent or senescent, meaning that the EdU incorporation assay cannot discriminate between these two types of cell cycle arrest.

Senescent Fibroblasts upregulate the CDK inhibitors p16 and p21

Senescent cells make use of inhibitors of the CDKs to stop the cell cycle [29]. Particularly p16 and p21 are often measured as markers of senescent cells [2]. Either one or both markers are normally upregulated in senescent cells, and the upregulation is often measured at the transcriptional level. It is encouraged to use both markers simultaneously since some cells do not upregulate p16 at the transcriptional level and p21 is a universal but not specific marker for cellular senescence [15,17,30]. **Figure**

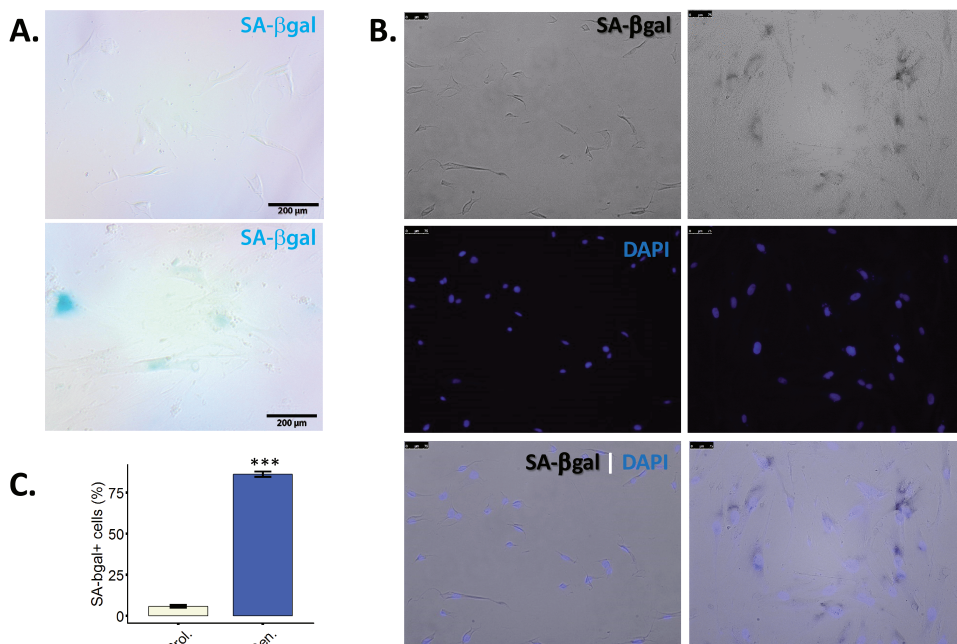


Figure 1. Enrichment of SA-βgal staining in senescent primary fibroblasts. BJ primary foreskin fibroblasts (PD 34.1) were induced to senescence by exposing them to ionizing radiation (10 Gy). Cells were stained for SA-βgal ten days after irradiation. **A.** Representative results for the SA-βgal staining in BJ primary fibroblasts either untreated (up) or exposed to ionizing radiation (down). Final magnification: 100x. **B.** Representative figure of SA-βgal co-stained with DAPI for BJ primary fibroblasts either untreated (three left panels) or exposed to ionizing radiation (three right panels). The DAPI staining (blue) helps to visualize individual cells facilitating the quantification. Pictures taken on bright-field appear in black/white. Therefore, in these particular pictures the SA-βgal staining will look like black perinuclear spots. Final magnification: 100x. **C.** Quantification of SA-βgal positive cells in proliferating (Prol., white) BJ fibroblasts versus ionizing irradiated-treated counterparts (Sen., blue). Quantification was performed by using three biological replicates. Statistical significance was determined by an unpaired two-tailed Student's t-test on delta-CT values (***) = p value < 0.01).

3 shows representative relative quantifications of p16 and p21 mRNA in fibroblasts induced to senescence. Other techniques such as immunostaining and/or western blotting to detect protein levels are also possible.

Senescent Fibroblasts display a SASP

Most senescent cells transcriptionally upregulate several genes encoding for secreted proteins, a phenomenon called SASP [6]. The SASP includes factors involved in inflammation, e.g. interleukins and chemokines, or in extracellular matrix (ECM) degradation, e.g. MMPs, but it is highly heterogeneous. Induction of SASP factors can be evaluated by measuring either mRNA expression levels via qPCR or levels of secreted protein via Enzyme-Linked Immuno Sorbent Assay (ELISA). **Figure 4** shows a representative image showing the upregulation of IL6 both at the transcriptional and secreted levels. We used IL6 only as a representation; however, it is encouraged to measure multiple members of the SASP from the suggested list on protocol 9.

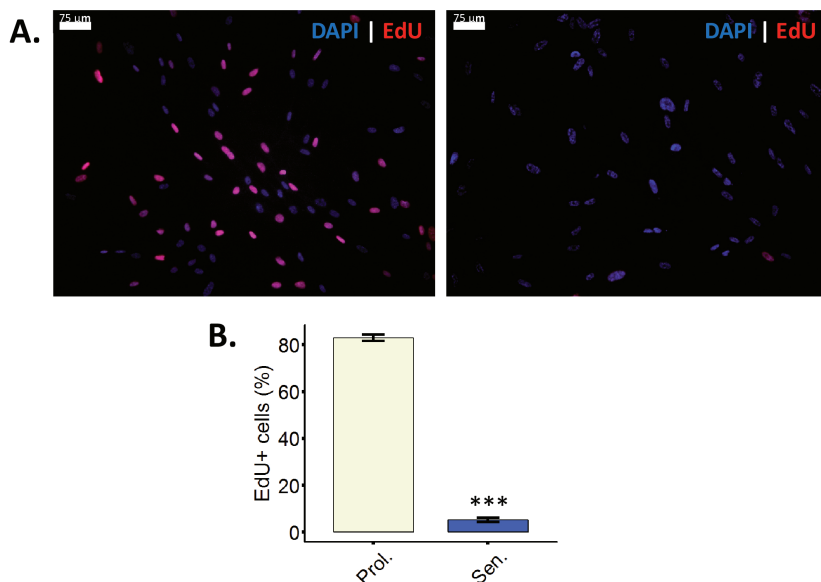


Figure 2. Fewer cells incorporate EdU after induction of senescence. **A.** Representative image of the EdU incorporation assay in proliferating WI-38 fibroblasts PD 43.86 (left) and their ionizing irradiated counterparts (right). Final magnification: 100x. **B.** Quantification of EdU positive cells in proliferating (Prol., white) BJ fibroblasts (PD 38.7) versus their irradiated counterparts (Sen., blue). Quantification was performed by using three biological replicates. Statistical significance was determined by an unpaired two-tailed Student's t-test on delta-CT values (***) = p value <0.01).

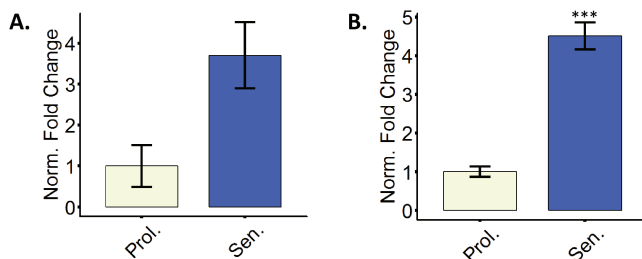


Figure 3. Senescent fibroblasts upregulate the CDK inhibitors p16 and p21. **A.** Quantification of p16 mRNA expression in proliferating (Prol., white, PD 35.3) or 5-aza-treated BJ cells (Sen., blue). **B.** Quantification of p21 mRNA expression in proliferating (Prol., white, PD 35.3) or 5-aza-treated BJ cells (Sen., blue). Quantification was performed by using three biological replicates (each with two technical replicates). Statistical significance was determined by an unpaired two-tailed Student's t-test on delta-CT values (***) = p value <0.01).

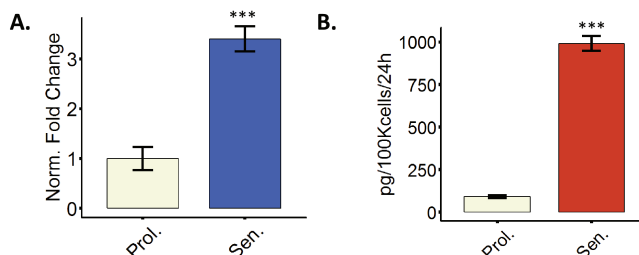


Figure 4. Senescent fibroblasts display a Secretory Phenotype (SASP). **A.** Quantification of IL6 mRNA expression in BJ fibroblasts either proliferating (Prol., white, PD 38.7) or induced to senescence by ionizing radiation (Sen., blue). **B.** Quantification of IL6 protein expression in proliferating PD 38.6 (Prol., white) or ionizing radiation-treated WI38 fibroblasts (Sen., blue). Quantification was performed by using three biological replicates. In the case of the qPCR data, each biological replicate had two technical duplicates. Statistical significance was determined by an unpaired two-tailed Student's t-test on delta-CT values (***) = p value <0.01).

Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
DMEM Media - GlutaMAX	Gibco	31966-047	
Fetal Bovine Serum	Hyclone	SV30160.03	
Penicillin-Streptomycin (P/S; 10,000 U/ml)	Lonza	DE17-602E	
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	SC-202581	
Nuclease-Free Water (not DEPC-Treated)	Ambion	AM9937	
T75 flask	Sarstedt	833911002	
Trypsin/EDTA Solution	Lonza	CC-5012	
PBS tablets	Gibco	18912-014	
1.5 ml microcentrifuge tubes	Sigma-Aldrich	T9661-1000EA	
Corning 15 mL centrifuge tubes	Sigma-Aldrich	CLS430791	
6-well plate	Sarstedt	83.3920	
24-well plate	Sarstedt	83.3922	
13mm round coverslips	Sarstedt	83.1840.002	
Steriflip	Merck Chemicals	SCGP00525	
Cesium137-source			IBL 637 Cesium-137γ-ray machine
UV radiation chamber			Opsytec, Dr. Göbel BS-02
Doxorubicin dihydrochloride	BioAustralis Fine Chemicals	BIA-D1202-1	
Hydrogen peroxide solution	Sigma-Aldrich	7722-84-1	
5-aza-2'-deoxycytidine	Sigma-Aldrich	A3656	
SAHA	Sigma-Aldrich	SML0061	
Sodium Butyrate	Sigma-Aldrich	B5887	
X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	Fisher Scientific	7240-90-6	
Citric acid monohydrate	Sigma-Aldrich	5949-29-1	
Sodium dibasic phosphate	Acros organics	7782-85-6	
Potassium ferrocyanide	Fisher Scientific	14459-95-1	
Potassium ferricyanide	Fisher Scientific	13746-66-2	
Sodium Chloride	Merck Millipore	7647-14-5	
Magnesium Chloride	Fisher Chemicals	7791-18-6	
25% glutaraldehyde	Fisher Scientific	111-30-8,7732-18-5	
16% formaldehyde (w/v)	Thermo-Fisher Scientific	28908	
EdU (5-ethynyl-2'-deoxyuridine)	Lumiprobe	10540	
Sulfo-Cyanine3 azide (Sulfo-Cy3-Azide)	Lumiprobe	D1330	
Sodium ascorbate	Sigma-Aldrich	A4034	
Copper(II) sulfate pentahydrate (Cu(II)SO ₄)	Sigma-Aldrich	209198	
Triton X-100	Acros organics	215682500	
TRIS base	Roche	11814273001	
LightCycler 480 Multiwell Plate 384, white	Roche	4729749001	
Lightcycler 480 sealing foil	Roche	4729757001	
Sensifast Probe Lo-ROX kit	Bioline	BIO-84020	
UPL Probe Library	Sigma-Aldrich	Various	
Human IL-6 DuoSet ELISA	R&D	D6050	
Bio-Rad TC20	Bio-Rad		

Counting slides	Bio-Rad	145-0017	
Dry incubator	Thermo-Fisher Scientific	Heratherm	
Dimethylformamide	Merck Millipore	1.10983	
Parafilm 'M' laboratory film	Bemis	#PM992	
Tweezers			
Needles			

Discussion

The protocols explained here were optimized for human primary fibroblasts, particularly BJ and WI-38 cells. The protocols for replicative senescence, ionizing radiation and doxorubicin, have been successfully applied to other types of fibroblasts (HCA2 and IMR90) and in other cell types (namely neonatal melanocytes and keratinocytes or iPSC-derived cardiomyocytes) in our laboratory. However, adaptations for additional cell types can be optimized by adjusting some details such as the number of seeded cells, the methods and chemicals to help cells for attaching/detaching to plastic supports and the dosage of the treatment to avoid toxicity.

Even the use of primary fibroblasts poses a number of challenges. Senescent cells are usually more difficult to detach than their proliferating counterparts, and they are often more sensitive to trypsinization or any other type of detaching method, meaning that the viability after detaching is slightly lower than the one of proliferating cells. The choice of the appropriate control for the different senescence-inducing methods is difficult. For instance, for the drug-based treatments such as doxorubicin, we suggest a short treatment with the vehicle: PBS for 24 hours in the case of the control samples for doxorubicin-treated cells followed by immediate harvesting/processing. It might be argued that cells induced to senescence go through an extended culture time after the treatment was applied (six extra days of culture for doxorubicin-treated cells) and that control cells should be cultured equal amount of time after removal of PBS. However, such a long culture would allow the cells to divide further, to become over-confluent or to require further passaging and to increase PD. Over-confluence may cause senescence markers, such as SA- β gal to appear despite cells maintaining their proliferating potential [31]. The increased PD would get them closer to their replication limit (and to replicative senescence) and make them less comparable to their doxorubicin-treated counterparts. A similar situation would apply for the other treatments. We have suggested the controls that we consider more appropriate for each case.

Most of the techniques used to induce cells into senescence seem relatively easy and straight-forward, but many factors can affect the outcome of the experiments. For instance, normal glucose concentration of conventional cell culture media for fibroblasts is 4.5g/L. However, for some cell types such as stem cells, lower concentrations of glucose extend their proliferative potential [32], while for others higher concentrations may lead to premature senescence [33]. Moreover, as senescent cells are highly metabolic and spend high amounts of energy to produce secreted factors [34], other senescence-associated phenotypes might be affected by oscillations in glucose concentrations.

Another potential variable in the cell culture medium is serum. The composition of the serum is normally not defined and varies according to the animal source and the batch. Particularly, the amount of growth factors and pro-inflammatory proteins can influence senescence [35]. We recommend that the same batch of serum is used

for the whole experiment to avoid unnecessary and confounding variability. Yet, some inevitable technical conditions such as the use of serum-free medium used for some ELISA-based protocols can reduce SASP expression.

Oxygen tension is important for the complete senescence induction. Hypoxia can inhibit geroconversion, so that cells do not proliferate but are not irreversibly arrested [36]. However, the most common problem in the experimental setup is not hypoxia but hyperoxia. Indeed, standard culture conditions often use 20% oxygen as “normoxia”, but physiological conditions for most cell types are lower. Mouse blastocysts present markers of senescence (SA- β gal and DNA damage) when cultured at 20% oxygen, unlike their *in vivo*-derived counterparts or the same cells cultured at 5% oxygen [37]. Furthermore, mouse fibroblasts cultured at more physiological conditions (3% oxygen) and then irradiated display a SASP, unlike their counterparts cultured at conventional conditions (20% oxygen) and induced to replicative senescence [38]. Here, we used 5% oxygen for all the cultures and experiments and we urge researchers to reconsider the oxygen concentrations used for the particular cell type of interest.

Finally, another factor to consider is the intrinsic heterogeneity of senescent cells. On one hand, different cell types and even cell strains display differences in senescence-associated phenotypes. For instance, some strains of fibroblasts do not upregulate p16 at the transcriptional level upon senescence induction [15–17], as it is also shown in **Figure 3A**, where despite seeing an upregulation of p16, this was not statistically significant. P16 is also controlled at the translational and post-translational level, so measuring the protein levels may demonstrate in some cases an increased activity of this CDK inhibitor. However, it may be that some cells simply rely on other CDK inhibitors like p21. We recommend measuring the transcriptional levels of both of them. The exact composition of the SASP also depends on the cell that produces its [2]. Furthermore, some cells constitutively express high levels of β -galactosidase, giving a positive result for SA- β gal staining that is not necessarily indicative of senescence [2]. In some cases, this problem might be overcome by reducing the incubation time with staining solution during the SA- β gal staining protocol. As mentioned, over-confluent cells might stain with the SA- β gal without them being senescent [31], so we urge researchers to culture cells sparsely in order to perform this staining. On the other hand, the senescence phenotype itself is not static or stable over time [39] since the composition of the SASP and the appearance of other markers of senescence are time-dependent [17,40]. Here, we have suggested the time points after each treatment in which cells are considered fully senescent and that are routinely used in our laboratory. Importantly, measuring markers at a shorter time point might render negative results due to incomplete senescence [40]. Moreover, since in most of the treatments a percentage of cells do not become senescent, using a longer time point might give enough time for the few non-senescent cells to expand and overtake the culture, reducing the expression of senescent markers. In view of the heterogeneity of senescent cells and the multiple caveats of the different markers, we highly encourage researchers to use multiple senescence markers within the same sample.

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Chapter 4

Unmasking Transcriptional Heterogeneity in Senescent Cells

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Abstract

Cellular senescence is a state of irreversibly arrested proliferation, often induced by genotoxic stress [1]. Senescent cells participate in a variety of physiological and pathological conditions, including tumor suppression [2], embryonic development [3, 4], tissue repair [5-8] and organismal aging [9]. The senescence program is variably characterized by several non-exclusive markers, including: constitutive DNA damage response (DDR) signaling; senescence-associated β -galactosidase (SA- β gal) activity; increased expression of the cyclin-dependent kinase (CDKs) inhibitors p16INK4A and p21CIP1; increased secretion of many bio-active factors (the senescence-associated secretory phenotype, or SASP); reduced expression of the nuclear lamina protein LaminB1 (LMNB1) [1]. Many senescence-associated markers result from altered transcription, but the senescent phenotype is variable and methods for clearly identifying senescent cells are lacking [10]. Here, we characterize the heterogeneity of the senescence program using numerous whole-transcriptome datasets generated by us or publicly available. We identify transcriptome signatures associated with specific senescence-inducing stresses or senescent cell types, and identify and validate genes that are commonly differentially regulated. We also show that the senescent phenotype is dynamic, changing at varying intervals after senescence induction. Identifying novel transcriptome signatures to detect any type of senescent cell or discriminate among diverse senescence programs is an attractive strategy for determining the diverse biological roles of senescent cells and developing specific drug targets.

Results and discussion

Specific senescence-associated transcriptome programs have been mainly characterized in fibroblasts. We therefore constructed a signature based on whole-transcriptome profiles of various strains of human fibroblasts subjected to different senescence-inducing stimuli. To increase statistical power, and limit biases associated with individual studies [11], we used several datasets generated at different times and in different laboratories, including ours. Only datasets generated using primary fibroblasts without genetic manipulation (with the exception of oncogenic Ras expression in the case of oncogene-induced senescence), with at least two biological replicates, and at least 50% of the cells positive for SA- β gal activity, were included in the analysis (**Data S1A**). The selected datasets covered 6 different fibroblast strains (BJ, IMR90, HFF, MRC5, WI38, HCA-2), 3 different senescence-inducing stimuli (RS=replicative senescence; OIS=oncogene-induced senescence; IRIS=ionizing radiation-induced senescence), and were generated by 5 independent laboratories (**Figure 1A**) [12-16].

Principal Component Analysis (PCA) revealed that the tissue of origin (lung or foreskin) accounted for most of the variation (data not shown). However, the second and third principal components separated the cells according to senescence status, with some influence from the study/dataset of origin (**Figure S1A**). Nonetheless, one sample within one of the datasets (RS IMR90 [14]) clustered differently from its replicates. The aberrant clustering of this sample was reported in the original study, and this sample was then removed from further analysis (**Figure S1A**).

Each sample showed transcriptional induction of at least some of the known senescence-associated genes, namely p16INK4A (CDKN2A), p21CIP1 (CDKN1A),

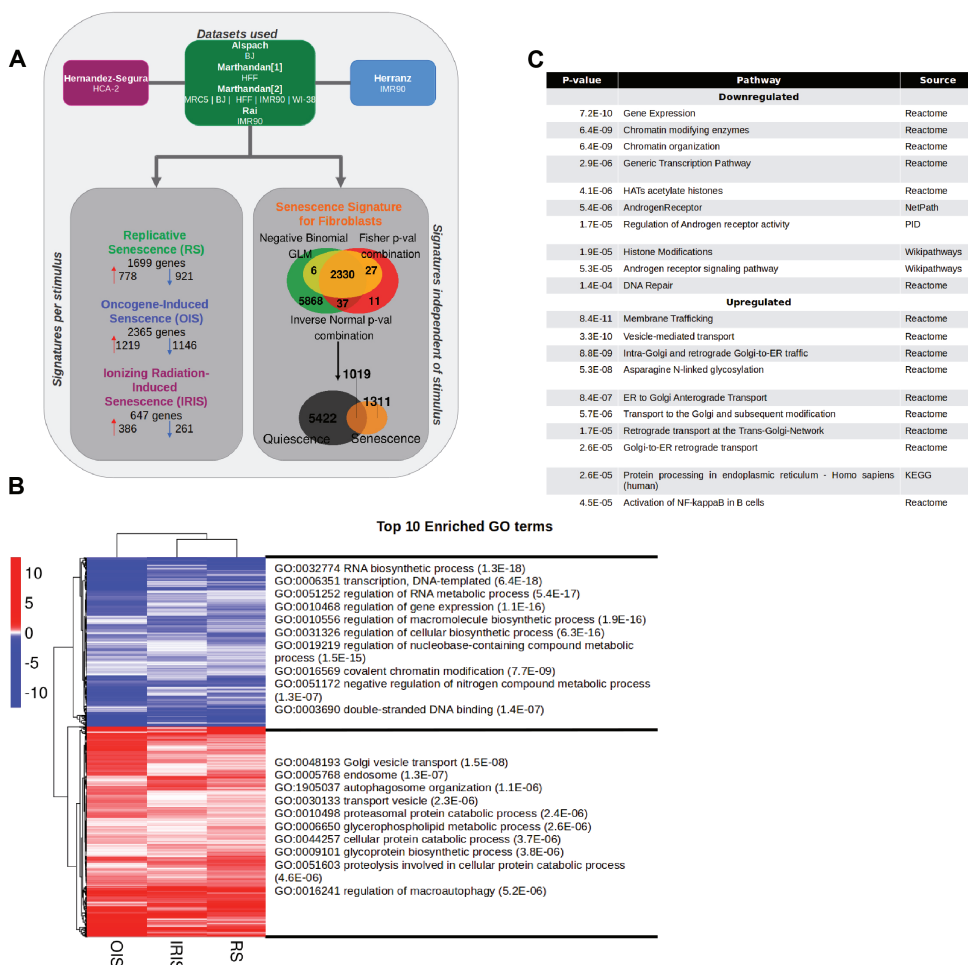


Figure 1. Meta-analysis of senescent fibroblast transcriptomics. **A.** Experimental Design. Seven RNA-seq datasets obtained from the indicated studies were used to build a stimulus-specific signature and general signature of senescent fibroblasts irrespective of the stimulus. Only genes with a p-value ≤ 0.01 , calculated by the three methods and with expression unchanged or in the opposite direction in quiescence, were included in each signature. The number of genes comprising each signature is displayed in the flower plot. **B.** Heatmap of the 1311 genes in the senescence signature of fibroblasts and the top 10 enriched GO terms. The graph shows the logarithm base 2 of the fold change for each senescence-inducing stimulus tested with respect to proliferating cells. Blue = downregulated genes; red = upregulated genes. **C.** Top 10 enriched pathways in the senescence signature of fibroblasts. The pathways enriched in genes within the senescence signature for fibroblasts (**B**) are enlisted with their corresponding p-value and source. See also **Figure S1** and **Data S1**.

SA- β gal (GLB1) and several SASP factors (**Figure S1B**). Interestingly, our analysis revealed variability due to intra- and inter-laboratory culture conditions (**Figure S1B**).

We first grouped the datasets based on the senescence stimulus (**Figure 1A**). For RS, the only group with more than one dataset, we performed a meta-analysis combining three different methods: 1) a negative binomial-generalized linear model (NB-GLM), pooling all the samples in all the datasets for one particular senescence-inducing stimulus and comparing them to proliferating counterparts. Within the model, we included a covariate that accounted for inter-laboratory and inter-strain differences (see Methods for details); 2) an analysis of each individual dataset and subsequent combination of the p-values using the Fisher method; 3) an inverse-normal p-value combination technique in which each dataset was weighted according to the number of replicates [17, 18]. We set a stringent threshold of nominal p-value ≤ 0.01 to reduce the odds of false positive results, and retained only those genes that were differentially expressed by the three different methods. For IRIS and OIS, where only one dataset for each condition was available, we used a normal differential expression analysis to select genes (adjusted p-value $p \leq 0.01$). To ensure the identification of genes associated with senescence, and not growth arrest per se, we analyzed quiescent (HCA2) fibroblasts and removed from subsequent analysis genes similarly regulated in both quiescence and each of the senescence conditions.

A number of genes were associated with a specific senescence-inducing stimulus: 1699 genes with RS, 2365 genes with OIS and 647 genes with IRIS (**Figure 1A**, **Figure S1C** and **Data S1B-D**). Of note, the stimulus-specific signature might be influenced by the fact that more than one dataset was available only for RS. However, common differentially expressed genes between the different stimuli were not affected by this variable. Thus, we performed a meta-analysis using the same three methods described for the stimulus-specific signatures, but pooled all the datasets available and compared senescent cells (regardless of the stimulus) to proliferating cells. We found 2330 genes were differentially expressed by senescent fibroblasts, regardless of the senescence inducer, and half of these genes (1311 genes) were not shared with quiescent cells (**Figure 1A**, **Data S1E**). Within the senescence-associated signature of fibroblasts, multiple genes related to transcription and RNA synthesis were downregulated, while genes involved in vesicle transport were upregulated (**Figure 1B**). Among the main gene ontology (GO) pathways [19, 20] showing altered in senescent cells, “chromatin organization”, “DNA repair”, “membrane trafficking” and “activation of NF-kappaB” were notable for their known links to senescence and aging (**Figure 1C**) [10, 21-23].

An important contributor to the heterogeneity of the senescence program is the expression of cell type-specific genes [24]. For example, upregulation of various components of the SASP has been reported to be dependent on the type of cell [25]. To identify variability due to cell type, and identify genes at the core of the senescence program, we used datasets from melanocytes, keratinocytes and astrocytes. We used inclusion criteria similar to those used for the fibroblast datasets (**Data S2A**). The datasets from melanocytes and keratinocytes were obtained in our laboratory: the cells were induced to senescence by IRIS, RNA was collected 10 days after induction, and senescence status was confirmed by SA- β gal activity and growth arrest (**Figure S2A**). The dataset from astrocytes was publicly available, and senescence was induced by oxidative stress [26] (**Figure 2A**).

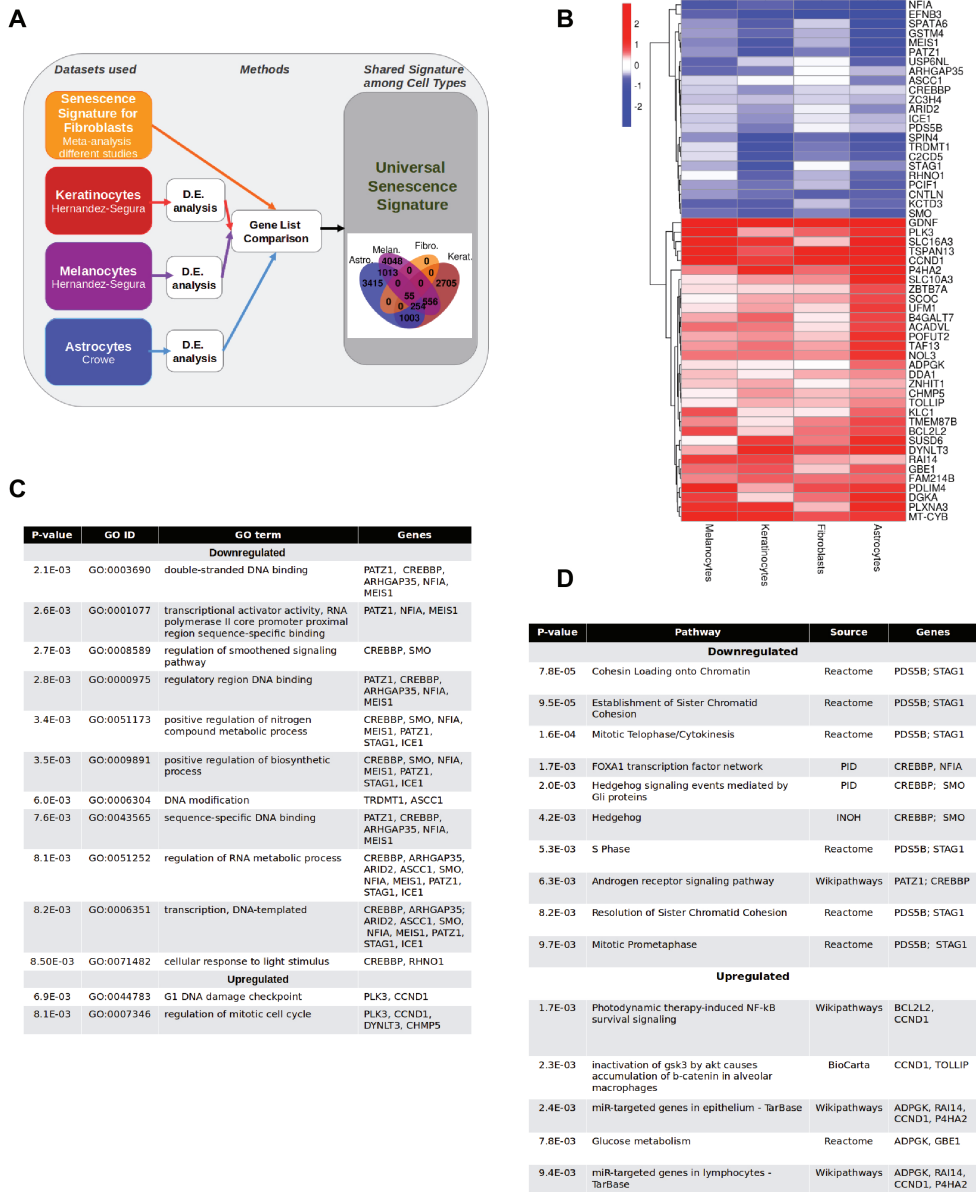


Figure 2. Characteristics of the core senescence-associated signature. **A.** Experimental Design. RNA-seq datasets obtained from the indicated studies of melanocytes, keratinocytes and astrocytes were compared to the senescence signature of fibroblasts. The intersection of genes differentially expressed (p -value ≤ 0.01) in all the datasets are shown in the flower plot. **B.** Heatmap of the 55 genes of the senescence core signature. The figure shows the logarithm base 2 of the fold change for each cell type with respect to proliferating cells. **C.** GO terms enriched in the senescence core signature. The plot shows the enriched GO terms in the up- (red) and down- (blue) regulated genes of the signature. Bars indicate the logarithm base 10 of the p -value. **D.** Pathways enriched in the core signature of senescence. The pathways enriched in genes within the core signature (**B**) are listed with their corresponding p -value and source. See also **Figure S2, S3** and **Data S2**.

To identify cell type-dependent and -independent senescence signatures, we compared the lists of differentially expressed genes in the three cell types with the senescence-specific signature derived from fibroblasts (**Figure 2A**, **Data S2B-E**). Cell type-dependent genes were enriched in different GO terms. For instance, few GO terms related to intracellular transport, RNA and/or protein expression or processing and immune system functions were associated with the different cell types (**Figure S2B**). Three of the enriched GO terms for either astrocytes or keratinocytes were associated with differentiation in their tissue of origin (namely “neurogenesis” or “neuron differentiation” and “epithelial cell differentiation”, respectively).

Importantly, upon examining the list of common differentially expressed genes, we identified a significant number of hits (55 in total, **Figure 2B** and **Data S2F**) comprising a senescence core signature. Remarkably, none of the classical senescence markers (CDKN2A, CDKN1A, LMNB1 and members of the SASP) were among these hits since they were either not differentially expressed in all the cell types or they were shared with quiescence (**Figure S2C**). Nevertheless, within the senescence core signature we found genes that had been previously linked to senescence: BCL2L2 (also known as Bcl-w), a negative regulator of apoptosis [27]; PATZ1, a transcriptional repressor whose expression inhibits senescence in endothelial cells [28]; SMO, a component of the Hedgehog pathway [29]; and CCND1, a regulatory subunit of CDK4 or CDK6, whose activity is required for the G1/S transition [28]. Moreover, a number of other genes in the signature were reported to be implicated in oncogenesis or in known senescence-associated pathways (see **Data S2G**). In terms of GO and pathways analysis, some of the downregulated genes were associated with DNA binding or regulation of transcription, while upregulated genes were mainly related to DNA damage checkpoints and mitosis (**Figure 2C-D**). Of note, most of the genes had a moderate fold change, yet 26 of them changed greater than 1.3-fold in all cell types.

From this senescence-associated core signature, we selected a number of genes for further validation using qPCR and human BJ primary fibroblasts. We induced senescence by various means, including those used in the previous analyses (ionizing radiation, replication, oxidative stress) as well as doxorubicin, a DNA-damaging chemotherapeutic agent known to cause senescence [29]) (**Figure S3A-D**). Senescence status was confirmed by SA- β gal activity (**Figure S3A**), lack of EdU incorporation (**Figure S3B**) and increased expression of p16INK4a and p21CIP1 (for RS cells, **Figure S3C**). Furthermore, to investigate potential conservation among species, we measured the expression of a subset of genes in senescent mouse cells (mouse embryonic fibroblasts (MEFs) or neonatal endothelial cells) (**Figure S3E-F**). We confirmed the differential expression of these genes under most of the conditions tested, further validating our identification of a senescence core signature. In few cases, the differential expression of individual genes was not statistically significant, suggesting that a combination of the expression level of senescence core genes might be a much stronger predictor of senescence. Moreover, we identified discrepancies between mouse and human cells for EfnB3, suggesting potential differences in senescence core signatures among species.

To validate the specificity of our senescence core signature, and test its validity in predicting senescence in diseased tissues, we analyzed additional datasets. To exclude the possibility that genes in our core signature were predictor of a general response to genotoxic stress, we interrogated published datasets that used mild forms of DNA

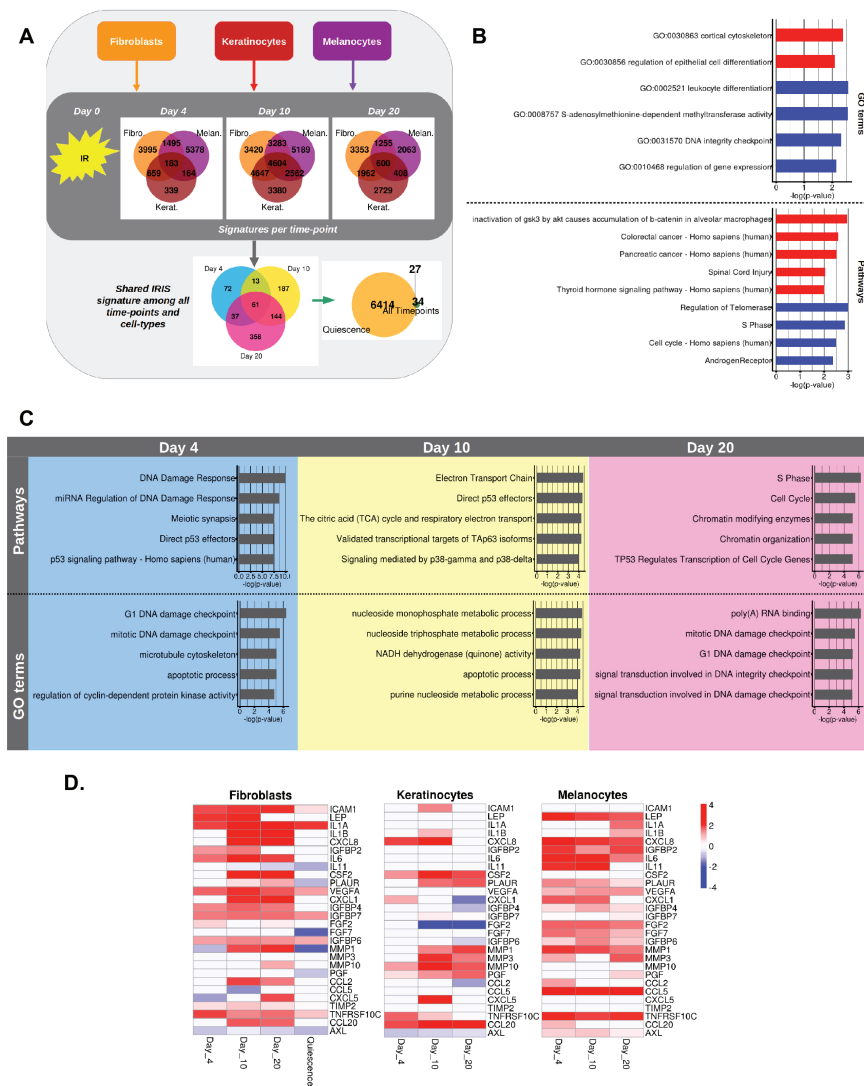


Figure 3. Temporal dynamics of the senescence transcriptome. **A.** Experimental Design. Fibroblasts (HCA-2, yellow), melanocytes (red) and keratinocytes (magenta) were exposed to ionizing radiation (IR) and RNA harvested 4, 10 or 20 days later. Transcriptomes of the different cell types and intervals after senescence induction were obtained by RNA-seq. A time-point signature with genes differentially expressed ($p\text{-value} \leq 0.01$) in all three cell types and a shared IR-induced Senescence (IRIS) signature with genes shared by all cell types and time points ($p\text{-value} \leq 0.01$) were generated. **B.** GO terms and pathways enriched in the shared IRIS signature among all time-points and cell types. The figure shows enriched GO terms in the up- (red) and down- (blue) regulated genes of the signature. Bars indicate the logarithm base 10 of the p-value. **C.** Top 5 GO terms and pathways enriched at each time point. The figure shows the enriched GO terms and pathways for days 4, 10 and 20. Bars indicate the logarithm base 10 of the p-value. **D.** Heatmap showing the dynamics of genes encoding SASP factors for each cell type. Known SASP factors that were significantly differentially expressed during at least one time point in each cell type are shown. The heatmap shows the logarithm base 2 of the fold change for each time post-irradiation with respect to proliferating cells. Quiescence was measured only on fibroblasts. The violet arrows highlight MMP1, the only SASP factor commonly regulated at day 10 and 20 in all cell types. See **Figure S4** and **Data S3**.

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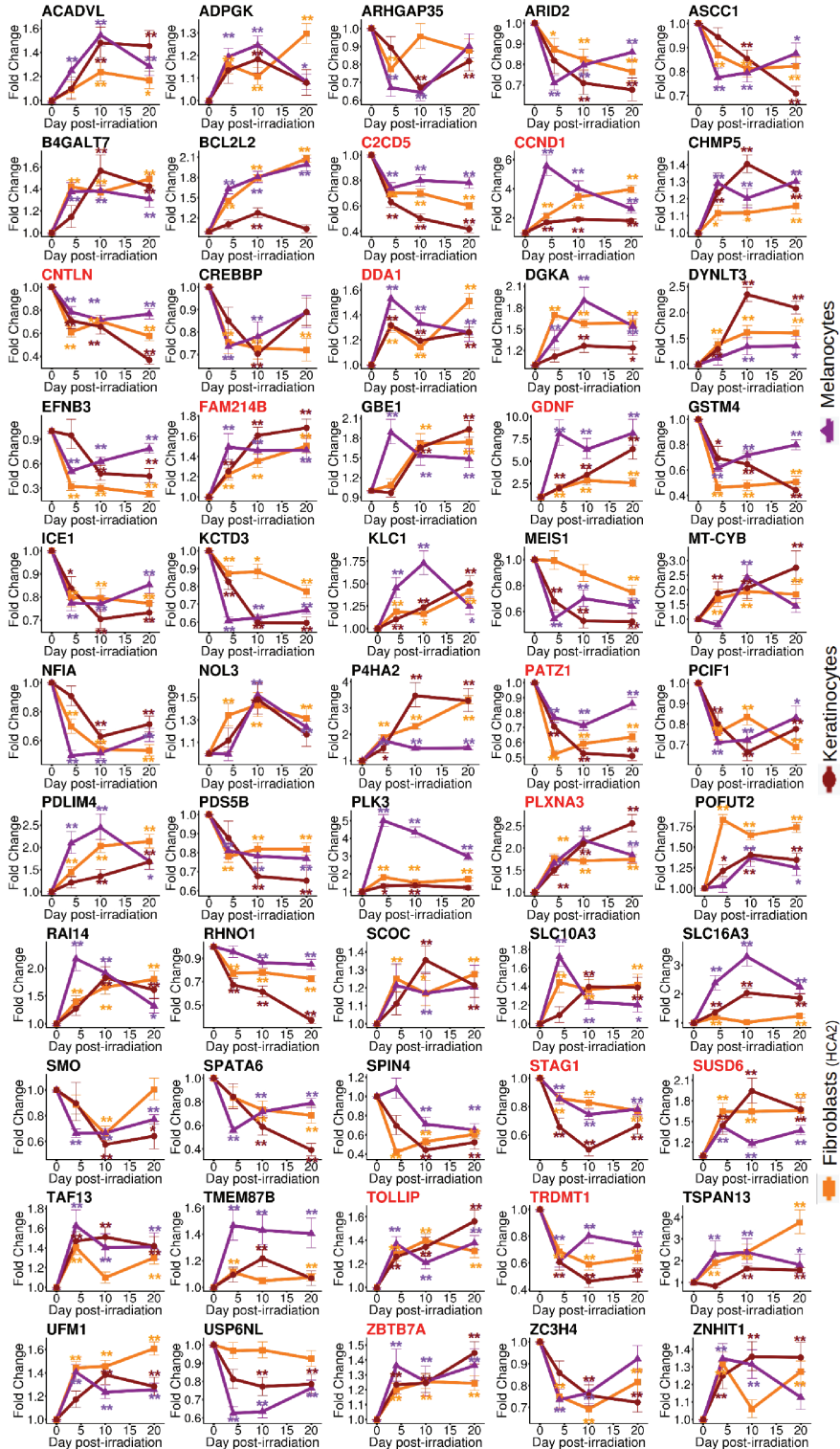
damage ([30]; GEO accession: GSE80207). Only one gene in our signature (PLK3) was reported differentially expressed in a radiosensitive lymphoblast line 4 hours after exposure to 2 Gy ionizing radiation ($FDR \leq 0.05$), a dose that causes damage without pervasive permanent cell cycle arrest. Importantly, this gene was no longer differentially expressed 24 hours or 14 days after the radiation or at any time after radiation in a radioresistant lymphoblast line (data not shown).

To understand whether genes present in the core signature were differentially regulated in diseased and senescence-enriched tissues, we used a RNAseq dataset from lung tissues of patients with idiopathic pulmonary fibrosis, in which known senescence markers were detected [31]. Strikingly, ten genes in our core signature (DGKA, GBE1, GDNF, KCTD3, MEIS1, PDLIM4, RAI4, SPIN4, TAF13 and TRDMT1) were also present in this dataset (adjusted p -val ≤ 0.01 , data not shown). These findings suggest that even in whole tissues, where the number of senescent cells is small (most aged or diseased tissues contained 1-3% senescent cells [32]), some of the genes of our core signature can be identified as differentially regulated.

The senescence phenotype, including the transcriptome, is highly dynamic meaning that not all the common senescence markers appear or persist simultaneously [33, 34]. However, most studies analyze senescent phenotypes 7-10 days after applying the senescence inducers [35, 36]. To explore the temporal dynamics of senescence-associated gene expression, we generated RNAseq datasets using fibroblasts (HCA-2), melanocytes and keratinocytes (6 biological replicates each) at 4 (early-), 10 (intermediate-) and 20 (late-) times after ionizing radiation (**Figure 3A, Data S3A-C**). We identified 61 genes that were shared among all cell types and time points, 34 of which were not shared with quiescent cells (**Figure S4A, Data S3D**). GO annotations and pathways analysis revealed the differential regulation of genes involved in cancer and cell cycle progression (**Figure 3B**).

We then obtained transcriptomic signatures that were specific for each time point to determine the dynamics of the response to a senescence-inducing stimulus (**Figure 3C, Data S3A-C**). Early senescence was characterized by DNA damage response and p53 signaling, perhaps reflecting the first response to the damage caused by the radiation. Intermediate senescence was characterized by metabolic changes (the citric acid cycle and respiratory electron transport), p53-associated pathways, and signaling mediated by p38-gamma and p38-delta, two isoforms of p38-MAPK known to have an important role in senescence and the SASP [37]. Notably, only in late senescence were cell-cycle arrest and chromatin remodeling among the top differentially expressed pathways, surpassing other pathways in importance (**Figure 3C**). Interestingly, genes encoding SASP factors showed significant time point- and cell type-dependent heterogeneity (**Figure 3D**). This variability highlights the importance of time and cellular identity in determining the SASP, and reflects the complexity of its regulation and biological

Figure 4 (next page). Dynamic changes in expression of genes in the core senescence signature. Each panel shows one of the 55 genes in the core signature at the indicated points before and after irradiation. All genes show a dynamic temporal behavior at the time points tested: day 0 (proliferation), day 4, day 10 and day 20 after irradiation. Notably, all genes show a similar trend in the three cell types tested: fibroblasts (yellow), melanocytes (red) and keratinocytes (magenta). Genes in red correspond to those that reached significance (p -value ≤ 0.01) at all time points tested. $N=6$. * $p \leq 0.05$, ** $p \leq 0.01$. See also **Figure S4** and **Data S3**.



4

functions [1].

Finally, we followed over time the 55 genes that comprised the senescence core signature (**Figure 4** and **Figure 2B**). While we found differential expression of all the genes at day 10 for melanocytes and keratinocytes, other time points showed high variability. In the case of fibroblasts, the lack of significance of some genes at day 10 is likely due to the lack of power from using a single dataset since these genes reached statistical significance only when multiple datasets were pooled. We also validated a subset of these genes in primary human HCA-2 fibroblasts, and confirmed differential expression compared to proliferating and quiescent cells (**Figure S4B-C**). Nonetheless, among the 55 genes of the senescence core signature, 13 genes were differentially regulated at every time point and in every cell type (**Figure 4** – genes in red).

Many factors can influence the senescence program and the biological functions of senescent cells. Here, we investigated the senescence phenotypes related to 3 main variables: the stress signal, the type of cell and the time after senescence induction. We found 1311 genes uniquely differentially regulated in senescent fibroblasts, and not quiescent fibroblasts. 55 genes were also shared among four different cell types induced to senesce by four different stimuli. The temporal dynamics of these genes exposed a new layer of complexity, revealing that the senescence signature found is highly time-dependent. Nevertheless, we were able to identify 13 genes as differentially regulated in all the conditions considered, including time.

Among the known senescence-associated genes, we identified PATZ1, a transcriptional repressor that inhibits senescence [28] and CCND1, a regulatory subunit of CDK4 or CDK6 [28]. It is noteworthy that the common senescence markers p16INK4A, p21CIP1 and LMNB1 were not within the core signature, supporting the idea that known senescence markers lack universality for different cell types and inducers of senescence [10]. Indeed, one of the studies used here [14] showed that p16INK4A mRNA levels are not always significantly changed in senescence in some fibroblast strains. Although RNA levels do not always reflect protein levels, p16INK4A expression is often used as a senescence marker. However, it is possible that samples collected to perform the RNAseq experiments might have been in a pre-p16INK4A engagement phase, as p16INK4A is often induced late after senescence induction [38]. By contrast, p21CIP1 was among the genes differentially expressed by all senescent cell types and in response to all stimuli, although it was also slightly upregulated in quiescence, concordant with its known involvement in both types of cell cycle arrest [39]. LaminB1 was strongly downregulated in all cell types with the exception of melanocytes, but was also somewhat downregulated at quiescence.

Finally, we showed that temporal dynamics strongly influence the detection of differentially expressed genes. In addition to the expression of the genes in our core signature, we included genes encoding known SASP factors (**Figure 3D**), further illustrating the heterogeneity among cell types and time points after senescence induction. These findings emphasize the need to include time as a variable when studying senescence. It is clear though that there is as yet no universal marker of senescence. Others have proposed the use of multiple markers [10], and we propose here the use of core transcriptome signatures such as we provide here. These signatures can help identify senescent cells and discriminate among different senescence programs. The number of genes to be tested to achieve precision still needs to be determined,

particularly in vivo. However, our data clearly highlight the heterogeneity of senescent cells in culture, anticipating that these challenges might be amplified in in vivo, where less is known about the stimuli and cell-types associated with senescence.

Author contributions

Conceptualization, AHS, JC and MD; Methodology, AHS, VG and MD; Formal analysis, AHS, TdJ, SM, VG and MD; Investigation, AHS, SM and MD; Writing, original draft: AHS and MD; Writing, Review and Editing, AHS, VG, JC and MD; Supervision, VG, JC and MD; Funding acquisition, JC and MD.

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Annex 1. Methods

Experimental Model and Subject Details

Cell strains and culture. Human foreskin fibroblasts HCA2 (male) were obtained from the laboratory of O. Pereira-Smith (University of Texas Health Science Center, San Antonio); human foreskin fibroblasts BJ were purchased from ATCC (Cat: CRL-2522); Human neonatal foreskin epidermal melanocytes and keratinocytes (male) were purchased from ATCC (Cat: PCS-200-012 and PCS-200-010, respectively). HCA2 cells were not re-authenticated by the laboratory, but regularly monitored for mycoplasma contaminations (once/2 weeks). Commercial cells were authenticated by ATCC. Mouse Embryonic Fibroblasts (MEFs – from unknown gender due to developmental stage) were produced from 13.5 day embryos as previously described [40]; mouse primary skin microvascular endothelial cells (gender not provided by the source) were purchased from Cellbiologics (Cat: C57-6064). All cells were cultured in 5% oxygen and 37C for at least 4 Population Doublings (PD) prior to use and tested regularly for mycoplasma infection. Fibroblasts were cultured in DMEM (Thermo Fisher Scientific) enriched with 10% fetal bovine serum (FBS, GE Healthcare Life Sciences) and 1% penicillin/streptomycin (Lonza). Endothelial cells were grown in an endothelial cell growth media (ATCC).

Method details

Technical and biological replications are described in the individual methods. Randomization and blinding of most experiments were not possible, but otherwise described. Statistical methods of computation are described in specific paragraphs. Inclusion and exclusion criteria for RNAseq datasets are provided in **Data S1A**.

Sample preparation. For each condition, 3 biological replicates were generated.

Quiescence was induced by culturing the cells for 48 hours in DMEM supplemented with 0.2% FBS.

For ionizing radiation-induced senescence (IRIS), cells were subjected to a 10 Gy dose of γ -radiation using a ¹³⁷Cesium source and medium was refreshed every 2

days. Cells were harvested at day 10 after irradiation for most of the experiments and validations. For the time series, cells were harvested at day 4, 10 and 20 after irradiation.

For replicative senescence (RS), cells were propagated in culture for ~4 months (re-cultured at 30-40% density every time they reached 70-80% confluency) until they stopped growing (~PD 65 for BJ cells).

For oxidative stress-induced senescence (OSIS), cells were treated with 200uM of hydrogen peroxide (Sigma Aldrich) for 2 hours, followed by drug removal and culturing in fresh DMEM supplemented with 10% FBS. Treatment was repeated at day 0, 3 and 6, with medium refreshed every 2 days in between, and cells harvested on day 10 after the first treatment.

Doxorubicin (Tebu-bio) was used in a concentration of 250 nM for 24 hours. The medium was then replaced by normal DMEM supplemented with 10% FBS and refreshed every 2 days. Cells were harvested on day 7 after treatment.

Proliferating controls for each condition were generated stimulating cells with the corresponding vehicles and/or considering the same PD of the treated samples. When only one control for multiple conditions is shown, it represents the average of controls for each condition.

SA-βgal assay. Cells were plated in a 24-well plate, fixed in a mixture of gluteraldehyde and formaldehyde (2%/2%) for 10-15 minutes and stained overnight with an X-Gal solution using a commercial kit (Biovision). Cells were counter-stained with a 1μg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, D9542) solution for 20 min. Every biological replicate was stained in duplicate, and counting was made in blind.

EdU staining. Cells were cultured for 24 hours in the presence of EdU, and fixed and stain using a commercial kit (Click-iT EdU Alexa Fluor 488 Imaging kit; Thermo Fisher Scientific). Every biological replicate was stained in duplicate, and counting was made in blind.

Real Time-PCR. Total RNA was prepared using the Isolate II Rna Mini Kit (Bioline). 255 – 500 ng of RNA was reverse transcribed into cDNA using a kit (Applied Biosystems). qRT-PCR reactions were performed as described [1] using the Universal Probe Library system (Roche) and a SENSIFast Probe kit (Bioline). Expression of tubulin was used to normalize the expression of CT values. List of primers used is provided as Table S1. Every biological replicate was analyzed in duplicate.

Public Datasets. A summary of the selection of the datasets and the samples used can be found in **Data S1A**. The raw data for the different public datasets used was collected from the “GEO repository” (<https://www.ncbi.nlm.nih.gov/geo/>). Five public datasets studying the transcriptome of senescent fibroblasts were included: 1) Alspach *et al* (ID code: GSE56293) used RS in BJ cells as a model to study SASP induction. 2) Herranz *et al* (ID code: GSE61130) studied the control of SASP factors in an OIS (induced by Ras) model in IMR90 cells. 3) Marthandan *et al* [2] (ID code: GSE64553) used five different strains of fibroblasts (BJ, WI-38, IMR90, HFF and MRC-5 cells) to study RS. 4) Marthandan *et al* [1] (ID code: GSE63577) used MRC-5 and HFF cells to study the effect of rotenone in different PD. Only the first (proliferation) and last time point (RS) for HFF cells were used. 5) Rai *et al* (ID code: GSE53356), used IMR90 cells to study the chromatin landscape of RS. One public dataset produced by Crowe *et al* (ID code:

GSE58910) studying OSIS in astrocytes was used for the core signature of senescence shared by different cell types. The ID code for this dataset is: GSE58910.

RNAseq. Cells (6 biological replicates per condition) were prepared for RNA extraction via an RNAeasy mini kit (Qiagen). Samples were treated with Qiasol lysis buffer and extracted for total RNA on a Qiacube robot per the manufacturer's instructions (Invitrogen). The extracted RNA was quantitated using a NanoDrop (higher than 1 microgram) and RNA quality was measured via BioAnalyzer chip (Agilent) (RIN of 8 or greater). Purified RNA samples were then sent to the University of Minnesota BioMedical Genomics Center for Illumina HiSeq RNA sequencing, where RNA-Seq library preparation was carried out using Illumina's Truseq RNA Sample Preparation kit (Cat. # RS-122-2001 or RS-122-2002) according to the manufacturer's protocols. Briefly, RNA was oligo-dT purified using oligo-dT coated beads and then reverse transcribed into cDNA. The cDNA is fragmented, blunt-ended, and indexed (barcoded) adaptors are ligated to the ends of the fragments that are then amplified using 15 cycles of PCR. The final library size distribution was validated using capillary electrophoresis and quantified using fluorimetry (PicoGreen) and via qPCR. Indexed libraries are then normalized, pooled and size selected to 320bp +/- 5% using Caliper's XT instrument. Samples were then paired-sequenced on the Illumina HiSeq 2000 System using Illumina's HiSeq® PE Cluster Kit v4 – cBot™ (PE-401-4001) HiSeq® SBS Kit v4 (50 cycles) (FC-401-4002). For the primary analysis and de-multiplexing, base call (.bcl) files for each cycle of sequencing are generated by Illumina Real Time Analysis (RTA) software. Primary analysis and de-multiplexing were performed using Illumina's CASAVA software 1.8.2. Average Quality scores for the completed run across all samples was >30, with an average number of reads for each pooled sample greater than 10 million reads.

Quality Control and Alignment of Sequencing Datasets. Raw data of the public datasets was downloaded as fastq files using the SRA Toolkit 2.6.2. Quality control of all samples, including our own, was performed using the FastQC software v0.11.5 and the low quality reads (Average Quality: <20) were discarded. End-trimming was performed when necessary by using the tool Trimmomatic 0.36. Samples were aligned to the GRCh38 genome using STAR-2.5.1b aligner and a count table was directly obtained with Star. Only genes annotated as protein-coding were included in the analysis.

Meta-Analysis of Fibroblasts. The heterogeneity of the data was evaluated with a Principal Component Analysis (PCA)-plot of the log-transformed normalized counts for the protein-coding genes, evaluating whether they clustered with similar samples in the same dataset.

For the meta-analysis (both for RS and for the senescence signature for fibroblasts), we used three different methods: negative-binomial generalized linear model (GLM), Fisher p-value combination and Inverse Normal p-value combination. In the case of the senescence signature for fibroblasts, we included all the samples in one unique meta-analysis. The first approach for the meta-analysis used the R-package DESeq2 for differential expression analysis. We included two variables in the model: a) Condition: senescence versus proliferation as the main variable of the model; b) Batch: we created this covariate to account for the differences in cell strain and the study they belonged to. In this variable, an identifier was assigned to each set of samples that belonged to the same dataset and same cell strain. Thus, every dataset included as many identifiers

as number of cell strains used. The other two approaches were done by analyzing each dataset individually using DESeq2 to later combine the p-values of the results by using the R-package MetaRNAseq. Therefore, the second approach used a Fisher- and the third one an Inverse Normal-p-value combination. Genes with an adjusted p-value ≤ 0.01 in the negative-binomial GLM and a combined p-value ≤ 0.01 in the other two methods were included in the corresponding signature.

Genes that were also differentially regulated in the quiescence samples (adjusted p-value ≤ 0.01 and sign of the fold change in the same direction than senescence) were removed from the list of possible senescence markers after each meta-analysis was finished.

Enriched pathways and Gene Ontology (G.O.) terms in the differentially expressed genes within the “Fibroblast Senescence signature” were evaluated by using the online tool “Over-representation analysis” of the Consensus Path DB-human (<http://cpdb.molgen.mpg.de/>) [19, 20].

Universal Senescence Signature shared in different Cell Types. Differential Expression analysis was also performed with DESeq2 for each individual dataset separately and the gene lists of differentially expressed genes were compared to the senescence signature of fibroblasts, without combining their p-values. As before, only genes with a p-value ≤ 0.01 in every dataset and within the signature of fibroblasts were included in the core senescence signature.

Quantification and statistical analysis

SA- β gal assay. Images were acquired at 100X magnification, and the number of cells counted by the software ImageJ (www.rsbweb.nih.gov/ij/). The number of positive cells was counted manually in blind.

EdU staining. Images were acquired at 400X magnification, quantified using ImageJ (www.rsbweb.nih.gov/ij/). In all cases, both for SA- β gal assay and for EdU staining, samples were done in triplicates and at least 100 cells were counted in each replicate (in blind) and corresponding barplots were generated, where error bars represent the Standard Error of the Mean (SEM).

Real Time-PCR. Tubulin was used for normalization of the CT values. All samples were run with a technical replicate and in 2-3 biological replicates. An unpaired two-tailed Student’s t-test was used to determine statistical significance based on delta-CT values. P values of .05 or less were considered statistically significant.

Data and software availability

Self-generated RNAseq data: RNAseq data has been deposited in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under the ID code E-MTAB-5403).

Plots. All plots were made in R using the following R-packages: “pheatmap”, “ggplot2”, “ggfortify”, “RColorBrewer” and “VennDiagram”.

Annex 2. Supplementary Material

All supplementary datasets can be found in the published online version of this article (<http://dx.doi.org/10.1016/j.cub.2017.07.033>).

For supplementary figures see next pages.

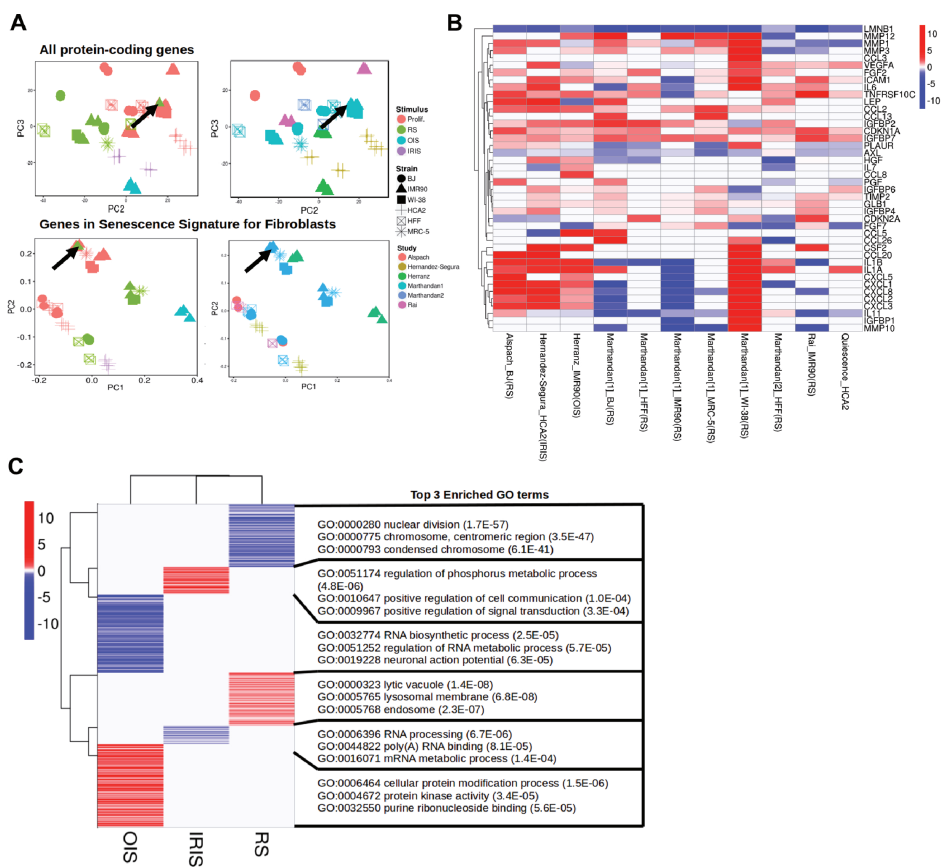


Figure S1. Transcriptional signatures of senescent fibroblasts. **A.** Principal Component Analysis (PCA) of all samples in the selected datasets. The upper panels displays the principal component 2 vs 3 for all the protein-coding genes. The panel on the left upper corner shows each sample colored according to their proliferating or senescence status. The panel on the right upper corner shows each sample colored according to the dataset that they derive from. Samples for each cell type in the same dataset clustered together, with a separation between senescent and proliferating cells from the same dataset. The black arrow shows the only sample (Replicative Senescence in IMR90 cells) that clustered incorrectly. The lower panels display only the genes that were within the Signature of Senescence in Fibroblasts (1311 genes) where it is also evidenced that the same sample clustered differently than its counterparts from the same and other datasets. Based on this evidence, it was decided to remove that sample from further analysis. **B.** Heatmap of known senescence markers in all the datasets included in the meta-analysis. The figure shows the logarithm base 2 of the fold change for senescent cells versus proliferating cells of senescence markers: CDKN1A (p21), CDKN2A (p16), GLB1 (beta-gal) and known members of the SASP. Samples are named according to the name of the first author of the dataset, followed by an underscore and the strain of fibroblast depicted in each column. The stimulus used in each dataset is in parentheses: Replicative Senescence (RS), Ionizing Radiation-Induced Senescence (IRIS) and Oncogene-Induced Senescence (OIS). **C.** Heatmap of genes that were differentially expressed exclusively by one of the stimuli tested and the corresponding GO terms. The figure shows the logarithm base 2 of the fold change for RS, IRIS and OIS versus proliferating cells. The right side of the panel shows the top 3 enriched GO terms for each stimulus and either up- (red) or down- (blue) regulated genes.

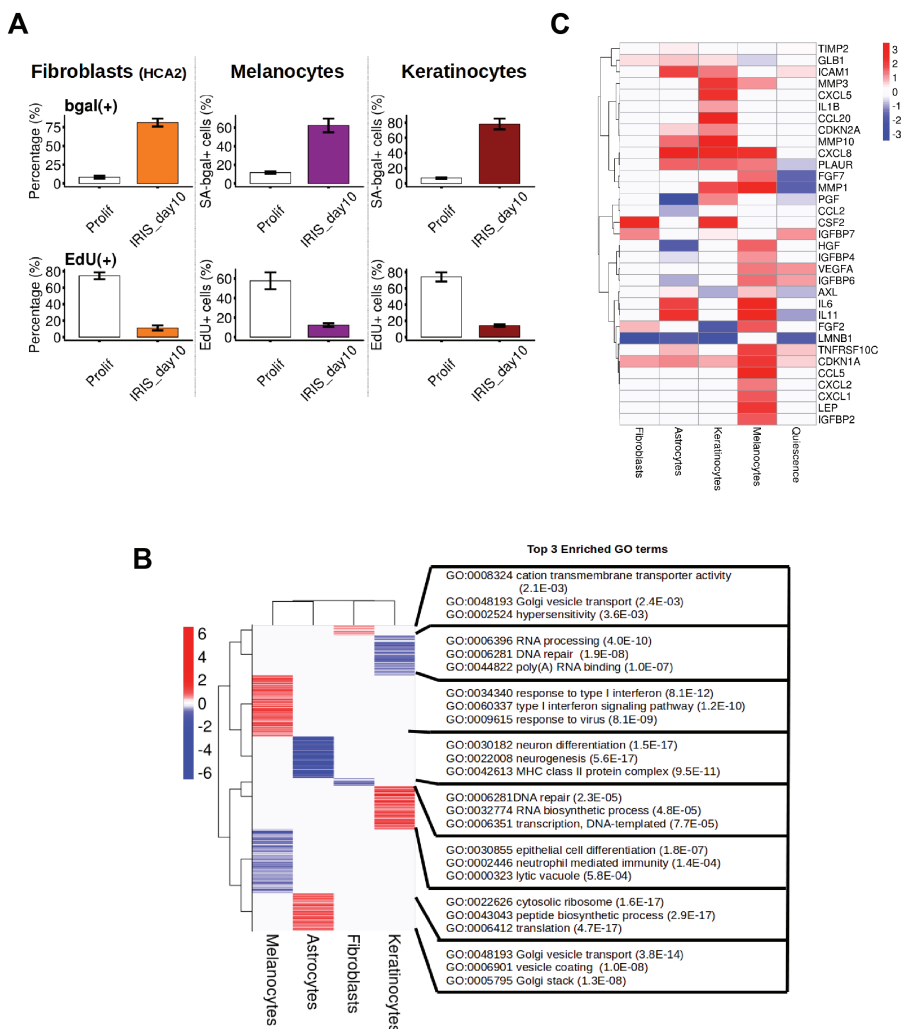


Figure S2. Core senescence-associated transcriptomic signatures. **A.** Senescence-induction in the three cell types used by our laboratory was confirmed by senescence-associated b-galactosidase (SA-bgal) activity and incorporation of EdU into DNA of proliferating cells. The percentage of positive cells for senescent fibroblasts (yellow), melanocytes (magenta) and keratinocytes (red) and their proliferating counterparts (white) are shown, demonstrating increased SA-Bgal activity and decreased EdU incorporation (proliferation) in senescent cells (10 days post-irradiation). **B.** Heatmap of genes that were differentially expressed exclusively in one cell type and the corresponding GO terms. The figure shows the logarithm base 2 of the fold change for senescent fibroblasts, melanocytes, keratinocytes and astrocytes versus their proliferating counterparts. The right side of the panel shows the top 3 enriched GO terms for each stimulus and either up- (red) or down- (blue) regulated genes. **C.** Heatmap of known senescence markers in the different Senescent cell types tested and in the Quiescence dataset. The figure shows the logarithm base 2 of the fold change for senescent cells versus proliferating cells of senescence markers: CDKN1A (p21), CDKN2A (p16), GLB1 (SA-bgal) and known members of the SASP. The Fibroblasts refers to the Differentially Expressed Genes in Senescent Fibroblasts product of the meta-analysis (before extracting genes similarly regulated in Quiescence). Melanocytes and Keratinocytes were induced to Senescence with IRIS and Astrocytes with OSIS. Quiescence refers to the sample of HCA2 fibroblasts that was induced to quiescence by serum starvation.

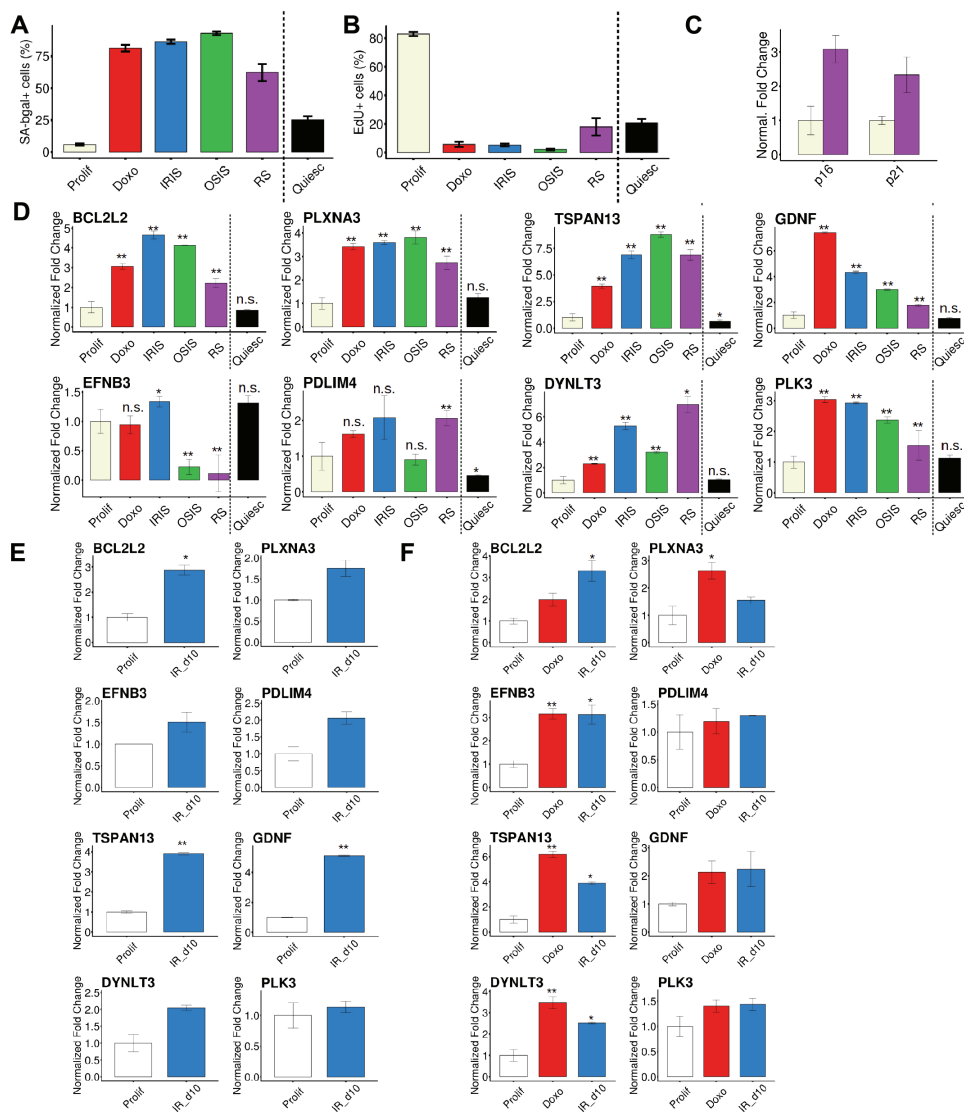


Figure S3. Validation of the core senescence signature. **A-C.** Different senescence-inducing stimuli were applied to BJ cells: doxorubicin (red), IRIS (blue), OSIS (green) and RS (violet). Signatures were compared to proliferating (white) and quiescent (black) cells. **A.** Percentage of SA-βgal⁺ cells in proliferating and senescent populations. **B.** Percentage of EdU⁺ cells in proliferating and senescent populations. **C.** p16 and p21 levels were measured by Real Time-PCR during RS, and tubulin used to normalize. **D.** Eight genes in the core signature of senescence were validated by Real Time-PCR: BCL2L2, PLXNA3, EFNB3, PDLIM4, TSPAN13, GDNF, DYNLT3 and PLK3. The expression of tubulin was used to normalize the fold changes. **E-F.** The above eight genes in the core signature of senescence that were validated in BJ cells (human) were measured in mouse cells. All the genes tested (BCL2L2, PLXNA3, PDLIM4, TSPAN13, GDNF, DYNLT3 and PLK3) followed the same trend, with the exception of EFNB3, which showed an opposite trend. The expression of tubulin was used to normalize the fold changes. **E.** Validation of the eight core senescence signature genes in mouse endothelial cells. **F.** Validation of the eight core senescence signature genes in mouse embryonic fibroblasts (MEFs). All samples included two or three biological and two technical replicates. Statistical significance was determined by an unpaired two-tailed Student's t-test on delta-CT values (* = $p < 0.05$, ** = $p < 0.01$ and n.s.=not significant).

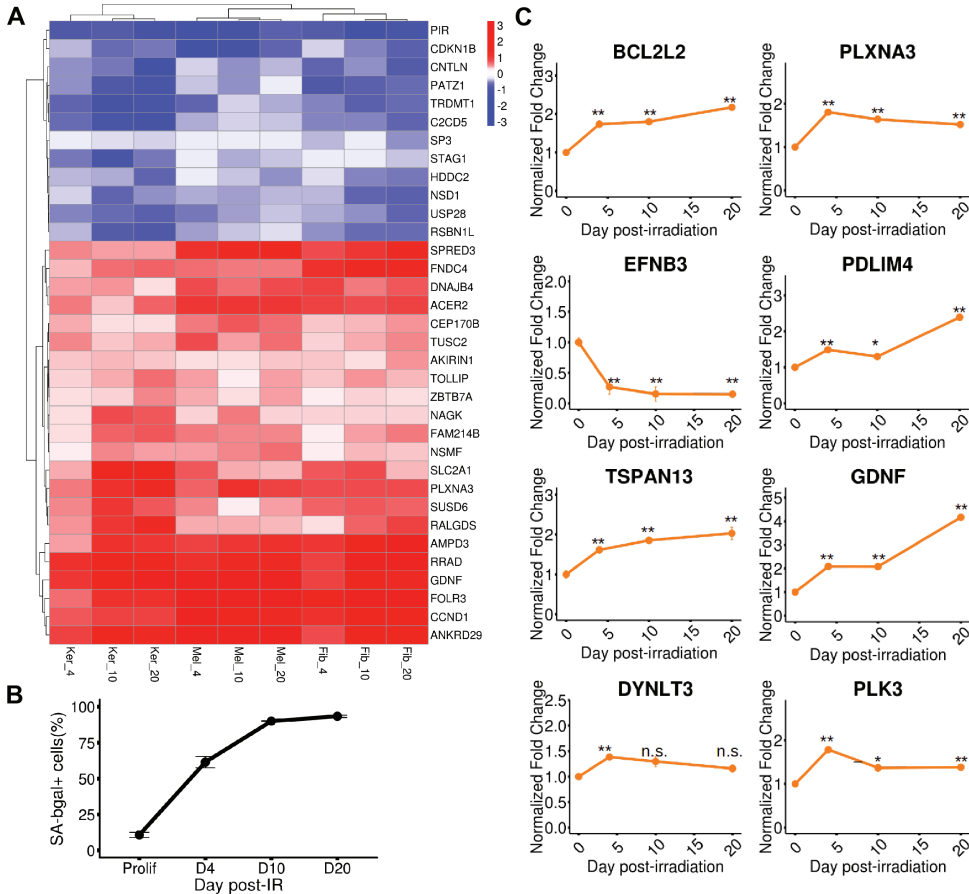


Figure S4. Temporal dynamics of the senescence transcriptome. **A.** Heatmap of the genes comprising the shared IRIS signature among all time-points and cell types. The heatmap shows the logarithm base 2 of the fold change for each time point (days 4, 10 and 20 post-irradiation) and for each cell type (Fib=fibroblasts, Mel=melanocytes and Ker=keratinocytes) with respect to their proliferating counterparts. **B.** Percentage of SA- β gal+ BJ cells at day 0 (proliferation) and days 4, 10 and 20 post-irradiation demonstrating an increase in SA- β gal activity upon irradiation. **C.** The eight genes in the core signature of senescence that were validated by Real Time-PCR in BJ cells were confirmed in HCA-2 cells. The temporal dynamics of the genes are demonstrated by the expression trends and lack of statistical significance at some of the time points. The expression of tubulin was used to normalize the fold changes. All samples included three biological and two technical replicates. Statistical significance was determined by an unpaired two-tailed Student's t-test on delta-CT values (* = p < 0.05, ** = p < 0.01 and n.s.=not significant).



Chapter 5

Identification of Stable Senescence-Associated Reference Genes

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Abstract

Cellular senescence is a state of permanent cell cycle arrest deriving in response to damaging stimuli. Many hallmarks associated to senescent cells are measured by quantitative real time PCR (qPCR). However, interpretation of qPCR data requires the selection of stable reference genes for normalization, a step that is often overlooked. Here, we perform a systematic review to understand normalization strategies entailed in experiments involving senescent cells. We find that, in violation of the Minimum Information for publication of qPCR Experiments (MIQE) guidelines, most reports use only one reference gene to normalize qPCR data, and that stability of the reference genes was either not tested or not reported. We use public RNAseq datasets to identify new and more stable reference genes -using the Shapiro-Wilk normality test and the coefficient of variation per gene- to be used in experiments that include senescent fibroblasts as model. We compare the new reference gene candidates with commonly used ones by using both RNAseq and qPCR data. Finally, we give suggestions on the best reference genes that can be used universally or in a strain-dependent manner. This study intends to raise awareness of the instability of classical reference genes in senescent cells, and to serve as a first attempt to define guidelines for the selection of more reliable normalization methods.

Results and Discussion

Cellular senescence is a state of permanent cell cycle arrest activated by various damaging stimuli [1]. Senescent cells develop several morphological and functional changes, from enlarged and misshaped cell body to secretion of various bioactive molecules – the Senescence-Associated Secretory Phenotype (SASP). However, studies from many research groups, including ours, have highlighted that the senescence program is complex and heterogeneous [2–4]. Most, if not all, senescence-associated markers are not senescence-specific and often the classification of a cell as senescent is oversimplified. One of the most powerful techniques to monitor several senescence-associated traits is quantitative real-time PCR (qPCR). qPCR is often used to measure the expression of senescence-associated growth arrest markers, such as the Cyclin-Dependent kinase inhibitors p16 and p21, of various SASP factors and of other effectors of morphological alterations, for example the down-regulation of the nuclear lamina protein LMNB1 [5,6]. qPCR is fast, accurate, relatively easy to perform, inexpensive and allows to measure multiple markers simultaneously. Interpretation of qPCR data heavily relies on the use of a normalization factor which is often calculated based on the expression of a reference gene- a gene whose levels remain unchanged among the different conditions analyzed [7]. The MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) also recommend to use at least two reference genes in every qPCR experiment [8,9]. In contrast, the common practice is to use a single housekeeping gene –a gene that covers an essential cellular function [9,10], despite housekeeping genes being not always stable [11]. For example, GAPDH, a common housekeeping gene used for qPCR normalization, is unstable in many conditions and cell types [11,12]. Particularly in the senescence field, recent experiments of single-cell qPCR –a variation of the qPCR that does not rely on the use of reference genes for normalization– reported changes in GAPDH expression in senescent versus proliferating cells [4].

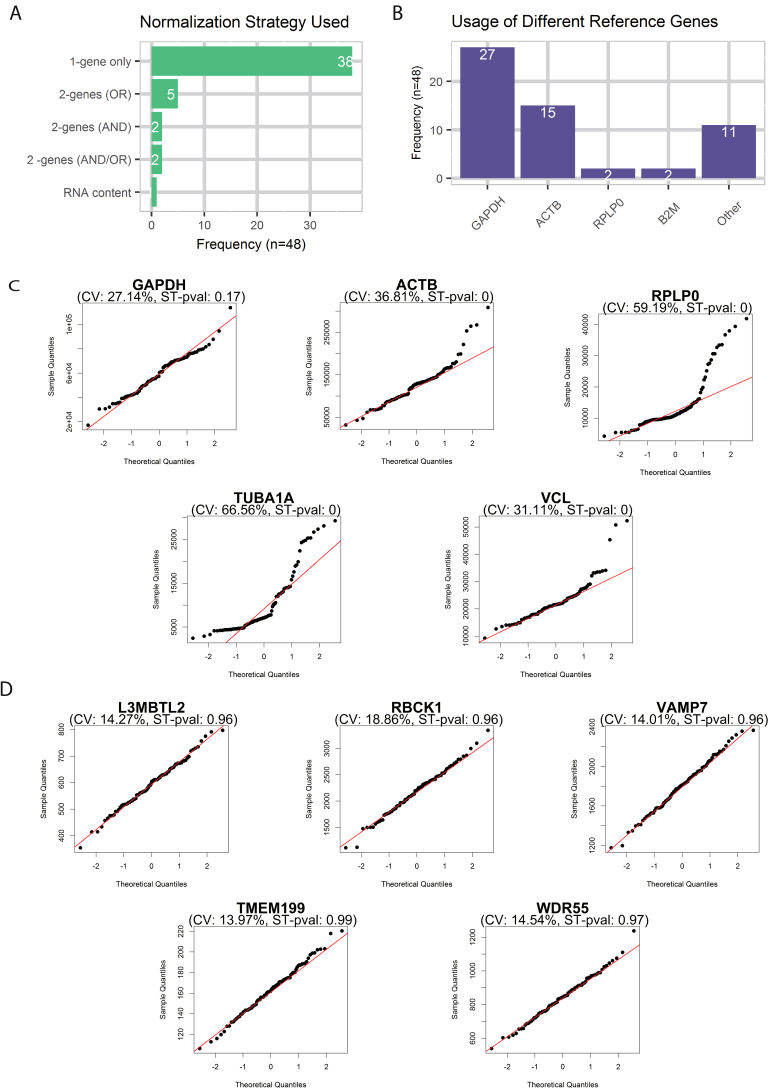


Figure 1. The Reference Genes for qPCR Experiments used in the Senescence Field are not always stable. **A-B.** Results for a systematic review on articles using qPCR data in senescent fibroblasts to evaluate the usage of reference genes in the field. **A.** Barplot showing the method of choice to normalize qPCR data in the senescence field. 1-gene only= only one reference gene used to normalize data, 2-genes (OR)= two different reference genes used one at a time for different experiments, 2-genes (AND) = two reference genes used together to calculate a normalization factor, 2-genes (AND/OR) = two reference genes used either one at a time or together in different experiments, RNA content = RNA content per sample used to normalize qPCR data (n=48 articles). **B.** Barplot showing the reference genes used in the senescence field (n=48 articles). The usage of a gene was counted regardless if it was used alone or in combination with another reference gene. **C.** Quantile-Quantile plots for the expression of five reference genes commonly used in the Senescence Field to normalize qPCR data of Senescent Fibroblasts as evaluated by public RNAseq datasets. 99 samples from ten different datasets were used to build the plots. The calculated CV and the p-value for the Shapiro-Wilk normality test (ST-pval). **D.** Quantile-Quantile plots for the top five reference gene candidates picked having the highest ST-pval and a CV lower than 20. RNAseq data for different fibroblast strains was used in combination for C and D.

In order to compile a list of the most common reference genes used to normalize qPCR in experiments involving senescent cells, we performed a systematic review of articles published in 2017 and 2018 which included senescent fibroblasts – arguably the most widely used cell type to model senescence in culture. Articles performing qPCR using microRNAs as a target were excluded since the normalization methods are still debated and are not comparable to other targets [13]. Our search (a description of it is provided in “Experimental Procedures”) yielded 105 results from which 48 were included after examination for availability of the required information and suitability according to inclusion/exclusion criteria (**Table S1**). Only one article used RNA content to normalize the qPCR data, while all the others made use of reference genes. Despite the recommendation in the MIQE guidelines, the majority of articles (38/48 studies) used only one reference gene, while only two articles used two genes to normalize their qPCR data (**Figure 1A**). Remarkably, the remaining seven articles used different reference genes for different experiments within the same article or one reference gene for some experiments and two reference genes for some others. In these cases, the reasoning to use different normalization strategies in different experiments was not clear.

We also evaluated the frequency of specific reference genes. GAPDH was the most commonly used gene (27/48 studies) either alone or in combination with other reference genes. ACTB was the second most used reference gene (15/48 studies), followed by RPLP0 (2/48 studies) and B2M (2/48 times). Other genes (TBP, Rps29, GUSB, G6PD, Polr2a, HPRT, TFRC, SMARCA1, TUBA1A, Rps13) were used in only one study each (**Figure 1B**). Of note, all the articles used a gene with a housekeeping function and none of them made clear whether the stability of the reference genes was tested beforehand.

A major issue is that several housekeeping functions, such as metabolism, cell structure and protein synthesis, are altered in senescent cells [3], and housekeeping genes might be differentially expressed in senescent samples [11,14][11,14]. To determine the stability of the most common reference genes used in experiments involving senescent cells, we analyzed ten public RNAseq [3,15–23]. These datasets used different types of fibroblasts (foreskin fibroblasts: BJ, HFF and HCA2 and lung fibroblasts: IMR90, MRC5 and WI38), and included proliferating, quiescent and different types of pre- or fully senescent cells (induced by replicative senescence, oncogene-induced senescence and ionizing radiation-induced senescence) (**Table S2**). We evaluated the stability of five commonly used reference genes: GAPDH, ACTB and RPLP0, which were the top three reference genes identified in our systematic review analysis (**Figure 1**); TUBA1A, which our laboratory uses as reference; and VCL, often used as reference in protein expression experiments, namely, Western blots. Following a similar strategy used by [24], we evaluated the stability of each gene using these two criteria: 1) We assumed that the expression of reference genes should be stable in every sample independently of the condition. Therefore, the expression of a reference gene in all samples should follow a Gaussian distribution, which can be tested using a Shapiro-Wilk normality test; 2) The variation in expression among different samples, defined as Coefficient of Variation (CV), should be small for a reference gene. Following the indications provided by [24], we considered that a stable and reliable reference gene should have a p-value higher than 0.6 for the Shapiro-Wilk normality test and a CV lower than 20. Intriguingly, none of

the common five reference genes passed the threshold (**Figure 1C**). We then expanded the analysis to every protein-coding gene present in the pool of RNAseq datasets that we had collected. In this way, the reference gene candidates could be suitable for any of the cell strains and conditions contained in the datasets tested, avoiding the need to adapt several reference genes for routine studies that engage different senescence models. We identified 65 out of the 13,968 sequenced genes that met the criteria and we selected the top five: L3MBTL2, RBCK1, TMEM199, VAMP7 and WDR55 (**Table S3**). (**Figure 1D**). The absolute expression levels of the five selected candidates were lower than common housekeeping genes, but high enough to be easily detected by qPCR (**Figure S1**). Moreover, there is no reason why genes that are expressed at a mid-level would perform any worse than highly expressed genes in qPCR experiments [11].

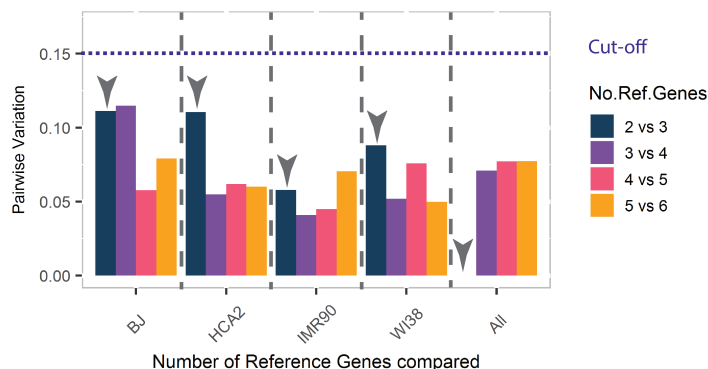
Two analytical methods, namely geNorm and NormFinder, are commonly used for identification/validation of reference genes [25,26]. GeNorm uses the mean pairwise variation for a given reference gene candidate compared to the other candidates (M-value) and excludes the least stable gene before repeating the analysis until only two (the most stable) genes are left. NormFinder uses a mathematical model of gene expression that measures the intra- and inter-group variation of the candidate reference genes, giving a “stability value” as a result. In both cases, a lower M-value and a lower stability value define the best reference gene. Both methods have pitfalls: geNorm is sensitive to gene co-regulation, so two co-regulated genes would maintain their pairwise variation despite not being stable. Indeed, some genes (mainly the commonly used ones) may be co-regulated albeit evidence is not strong (**Figure S2**). NormFinder requires a bigger sample size per condition or treatment and, unlike geNorm, it does not provide a systematic way to choose the optimal number of reference genes required for a given experiment [27]. As both methods would be biased if used alone, we validated the stability of the candidate reference genes in qPCR experiments by combining them.

We generated 99 samples that included different strains of fibroblasts (BJ: 27 samples, HCA2: 27 samples, IMR90: 18 samples and WI38: 27 samples) and different methods of senescence induction (doxorubicin, inhibition of different histone deacetylases, ionizing radiation, replicative senescence, inhibition of DNA methylation) (summarized in **Table S4**).

We measured the expression of ten reference gene candidates, the five commonly used (GAPDH, ACTB, RPLP0, TUBA1A, VCL) and the novel five previously identified (L3MBTL2, RBCK1, VAMP7, TMEM199, WDR55). We used geNorm and NormFinder to rank them according to their stability in each of the four cell types tested and in the combination of all of them together (**Figure S3**). Then, we built an overall ranking by averaging the information derived from the two methods (**Figure 2B**). For instance, if a gene scored 2 in geNorm and 4 in NormFinder, the overall rank would be 3. If two or more genes had the same overall ranking, the tie was solved by choosing the one with the lowest standard deviation for the overall ranking. This was done in order to avoid giving more weight to one of the reference gene selection methods. Overall, TMEM199 showed the highest stability and reliability among the tested reference genes (**Figure 2B**).

Finally, the MIQE guidelines suggest the use of at least two reference genes for every qPCR experiment and to test whether more than two are necessary. geNorm allows this evaluation by calculating the normalization factor (geometric mean of the

A



B

Rank	BJ		HCA2		IMR90		WI38		All cell strains	
	Gene	GN NF	Gene	GN NF	Gene	GN NF	Gene	GN NF	Gene	GN NF
1	TMEM199	3 2	L3MBTL2	2 2	L3MBTL2	3 2	TMEM199	1 2	TMEM199	2 1
2	VAMP7	1 4	TUBA1A	1 3	VAMP7	2 3	RPLP0	3 3	ACTB	4 2
3	L3MBTL2	2 5	TMEM199	3 4	RPLP0	1 4	VAMP7	2 4	RPLP0	2 4
4	WDR55	6 3	VAMP7	5 2	ACTB	5 2	WDR55	5 2	WDR55	5 3
5	RPLP0	7 2	ACTB	6 5	TMEM199	4 5	RBCK1	3 6	VAMP7	3 5
6	ACTB	5 6	GAPDH	7 6	WDR55	6 6	ACTB	5 5	RBCK1	6 6
7	GAPDH	4 7	RBCK1	4 9	VCL	7 7	TUBA1A	7 7	VCL	7 7
8	RBCK1	9 8	RPLP0	8 8	TUBA1A	8 8	VCL	8 8	L3MBTL2	8 8
9	VCL	8 9	WDR55	9 7	GAPDH	9 9	GAPDH	9 9	GAPDH	9 9
10	TUBA1A	10 10	VCL	10 10	RBCK1	10 10	L3MBTL2	10 10	TUBA1A	10 10

Figure 2. New Reference Genes Candidates to normalize qPCR data in Senescence Experiments. **A.** The stability of the best reference gene candidates was tested using qPCR data and the algorithms proposed by geNorm and NormFinder. The normalization factor (geometric mean) using two, three, four, five or six top reference genes were calculated for each cell type and for all cell types in combination (All). The performance of the different normalization factors was evaluated using geNorm. A difference in pairwise variation lower than 0.15 was used as a cut-off as recommended by [25]. In all cases two reference genes were sufficient for the calculation of the normalization factor. **B.** Final ranking of the ten reference gene candidates tested by qPCR with both, geNorm (GN) and NormFinder (NF). Genes in orange mark the top two genes that were sufficient for the calculation of an adequate normalization factor.

expression of reference genes) combining the best two reference gene candidates and comparing it to the normalization factor using three, four or more candidates. The pairwise variation of the different normalization factors is calculated and a decision is taken on whether adding an extra gene would improve the analysis. In the original paper it was proposed that if the use of an extra reference gene would decrease the pairwise variation more than 0.15 units, it would be necessary to include it in the normalization method. Following this protocol, we compared the performance of the normalization factor using two, three, four, five or six reference genes (see **Figure 2A**). In all cases, the use of three genes did not significantly decreased the pairwise variation, so only the top two reference genes are necessary to normalize the qPCR data for the four cell types and conditions tested. This report and particularly the list shown on **Figure 2B** can be used as a guidance for the selection of candidate genes in experiments involving senescent fibroblasts.

Some of the commonly used housekeeping genes that were not stable in the RNAseq data, ranked well in the qPCR data. These discrepancies might reflect the fact that the RNAseq analysis was used combining all the cell types together, so that stability in particular cell types is not tested. Moreover, the induction of senescence was not performed in the same way in both datasets. Another source of discrepancy might be the different transcript variants. Indeed, all the genes tested encode for multiple transcript variants which are all included in the RNAseq analysis. In contrast, the qPCR assays detect only a selection of those variants (see **Table S5**). In any case, our predicted candidates ranked generally better than the common reference genes.

With this report we do not aim at criticizing experiments from other laboratories, but rather to raise awareness and encourage improvement. First, we cannot consider ourselves blameless because we used non-tested and unstable genes as reference in previous studies, failing to critically address the problem of data normalization. Second, the conclusions stated in the articles used for the systematic review would probably hold, since in most cases different techniques were used to validate the findings. However, we believe that reproducibility of results would be improved if the description of the qPCR experiments would receive more attention.

We encourage choosing appropriate genes for every experiment tested, but the candidates suggested in **Table S3** and **Figure 2B** set a starting point for genes to test. It is important that the field makes a shift towards better laboratory practices, particularly in times in which reproducibility of reports has been questioned [28–31].

Acknowledgements

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Author contributions

Alejandra Hernandez-Segura prepared most samples used, performed all the qPCR experiments and the selection of reference genes both by RNAseq and qPCR. Richard Rubingh performed part of the selection of reference genes by RNAseq. Marco Demaria led and supervised the project and the manuscript writing. Marco Demaria and Alejandra Hernandez-Segura formulated the idea and wrote the manuscript.

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Annex 1. Experimental Procedures

Systematic review of reference genes used for studies including senescent cells

We scouted for original scientific articles that used qPCR to measure gene expression in senescent versus proliferating/cancer/quiescent human fibroblasts. The search was done in PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>) including only articles written in English between the beginning of 2017 until the moment of performing the systematic review (10/08/2018). The exact searching method used was: “senescence”[Title/Abstract] AND (“real-time polymerase chain reaction”[MeSH Terms] OR (“real-time”[All Fields] AND “polymerase”[All Fields] AND “chain”[All Fields] AND “reaction”[All Fields]) OR “real-time polymerase chain reaction”[All Fields] OR “qpcr”[All Fields]) OR (“gene expression”[MeSH Terms] OR (“gene”[All Fields] AND “expression”[All Fields]) OR “gene expression”[All Fields]) OR transcript[All Fields] AND (“humans”[MeSH Terms] OR “humans”[All Fields] OR “human”[All Fields]) AND (“fibroblasts”[MeSH Terms] OR “fibroblasts”[All Fields] AND (Journal Article[ptyp] AND (“2017/01/01”[PDAT] : “2018/08/10”[PDAT]) AND English[lang])). The search rendered 105 results that were further evaluated to ensure that they included bulk (no single-cell) qPCR experiments on human fibroblasts, qPCR of mRNAs or lncRNAs (no miRNAs) and that they included sufficient information about the normalization method of the qPCR data on the article or supplementary material. There was no restriction for the type of fibroblast used. Ten articles were excluded because we could not get access to the full-text. In total, we included 48 original articles. In each of them, we extracted the name of the gene(s) used to normalize the qPCR data or any other method used (if applicable).

Acquisition, quality control and alignment of public RNAseq datasets

Ten public datasets were collected from public repositories. The datasets can be found in the Gene Expression Omnibus under the following accession numbers: GSE77675, GSE56293G, GSE78138, GSE70668, GSE55949, GSE61130, GSE63577, GSE64553 and GSE53356 or in ArrayExpress database: E-MTAB-5403. Every dataset consisted on a set of senescent and proliferating fibroblasts (lung fibroblasts: WI-38, IMR90 or MRC-5 or foreskin fibroblasts: BJ,HFF or HCA-2), inducing senescence by different methods. A summary of the type of senescence, number of replicates and cell strain used in the generation of each dataset can be found on Table S2. A total of 99 samples were included in the analysis.

Raw data of the public datasets was downloaded as fastq files using the SRA Toolkit 2.6.2. Quality control of all samples was performed using the FastQC software v0.11.5 and the low quality reads (Average Quality: < 20) were discarded. End- trimming was performed when necessary by using the tool Trimmomatic 0.36. Samples were aligned to the GRCh38 genome using STAR-2.5.1b aligner and a count table was directly obtained with Star. Only genes annotated as protein-coding and showing more than 90 reads (while adding up the expression in all samples) were included in the analysis.

Selection of reference genes based on RNAseq data

Data was normalized to account for different sequencing depth in the different

samples by calculating size factors using the DESeq2 software [1]. The p-value of the Shapiro-Wilk normality test and the CV of each gene was calculated and used to evaluate the suitability of a gene to be used as a reference gene, as reported before [2]. Genes that had a p-value higher than 0.6 and a CV lower than 20 were considered as reference gene candidates (see Table S3).

Cell culture and senescence/quiescence induction

Human foreskin fibroblasts HCA2 (male) were obtained from the laboratory of O. Pereira-Smith (University of Texas Health Science Center, San Antonio); human foreskin fibroblasts BJ were purchased from ATCC (Manassas, Virginia, USA, Cat: CRL-2522); human lung fibroblasts MRC-5 and human lung fibroblasts WI-38 were obtained from the laboratory of Judy Campisi (Buck Institute for Research on Aging, San Francisco). Each cell strain used was regularly monitored for mycoplasma contaminations (once/2 weeks). All cells were cultured in 5% oxygen and 37C for at least 3 Population Doublings (PD) prior to use and tested regularly for mycoplasma infection. Fibroblasts were cultured in DMEM (Thermo Fisher Scientific, Waltham, Massachusetts, USA, Cat: 31966-047) enriched with 10% fetal bovine serum (FBS, GE Healthcare Life Sciences, Chicago, Illinois, USA, Cat: 758092, Origin: South America, Batch: 41213-C05) and 1% penicillin/ streptomycin (Lonza, Switzerland, Cat: LO DE17-602E).

Quiescence was induced by culturing the cells for 48 hours in DMEM supplemented with 0.2% FBS.

Senescence was induced by different methods following standardized protocols [3]. In brief, ionizing radiation-induced senescence, cells were subjected to a 10Gy dose of γ -radiation using a 137-Cesium source and medium was refreshed every 2 days. Cells were harvested at day 4, 10 or 20, as stated in Table S4. after irradiation for most of the experiments and validations.

For replicative senescence, cells were propagated in culture for ~3 months (re-cultured at 30%–40% density every time they reached 70%–80% confluence) until they slowed down their growth significantly (~PD 55 for BJ cells, with one population doubling every two weeks).

Doxorubicin (Tebu-bio, Netherlands, Cat: BIA-D1202-1) was used in a concentration of 250 nM in PBS for 24 hr. The medium was then replaced by normal DMEM supplemented with 10% FBS and refreshed every 2 days.

For epigenetic-induced senescence cells were treated with either 10 μ M of 5-aza-2'-deoxycytidine (Sigma-Aldrich, St. Louis Missouri, USA, Cat: A3656), 1 μ M of suberoylanilide hydroxamic acid (Sigma-Aldrich, St. Louis Missouri, USA, Cat. SML0061), 1 μ M of RGF966 or 1 μ M of entinostat, all of them using DMSO as solvent. In every case, cells were treated for 3 days changing with fresh DMEM supplemented with 10% FBS medium + drug every 24 hours. Then they were cultured for 3 extra days with normal medium DMEM supplemented with 10% FBS.

Proliferating controls for each condition were generated stimulating cells with the corresponding vehicles and using cells of the same PD than the treated samples. In all cases (with the exception of the replicative senescence), cells were between PD 35-40.

Senescence-associated β -galactosidase assay

Cells were plated in a 24-well plate, fixed in a mixture of glutaraldehyde and formaldehyde (2%/2%) for 10-15 min and stained overnight with an X-Gal solution as described in [3]. Cells were counter-stained with a 1 mg/ml 40,6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis Missouri, USA, D9542) solution for 20 min. Images were acquired at 100X magnification, and the number of cells counted by the software ImageJ (<http://www.rsweb.nih.gov/ij/>). The number of positive cells was counted manually in blind and the percentage of positive cells was calculated (data not shown).

RNA extraction and cDNA synthesis

Once the full treatment (if applicable) was performed, cells were collected in 350 μ l of RNA Lysis buffer RLY (Bioline, UK, BIO-52079) and 3.5 μ l of β -mercaptoethanol (Sigma-Aldrich, St. Louis Missouri, USA, M6250) using a cell scraper. Samples were frozen no more than 1 month at -80C before being processed. Total RNA was prepared using the Isolate II RNA Mini Kit (Bioline, UK, BIO-52073) following manufacturer's instructions. 150–500 ng of RNA was reverse transcribed into cDNA using a kit (Applied Biosystems, Foster City, California, USA, Cat: 4368813) in a 20 μ l reaction and incubating 10 min at 25C, 120min at 37C and 5 minutes at 85C. cDNA was diluted 6 times for a total volume of 120 μ l before using it for qPCR.

qPCR

qPCR reactions were performed using the Universal Probe Library system (Roche, Switzerland, Cat: 04683633001) and a SENSIFast Probe kit (Bioline, UK, Cat: BIO-76001) in a 10 μ l reaction from which 2.5 μ l corresponded to the diluted cDNA. Reaction was run in a Roche LightCycler 480 with the following program: 1) 7 min at 95C, 2) 40 cycles of 5 sec at 95c and 30sec at 60C and 3) 1 min at 37C. Each sample was run in duplicate.

All the primers were designed using the online software from Universal Probe Library Design Center (https://lifescience.roche.com/en_nl/brands/universal-probe-library.html). The primer sequences can be found in Table S5 and their respective experimental PCR efficiency and the r^2 linearity of their standard curves in Table S6. Except for RBCK1 (87%), all the other primers had an efficiency of 95% or higher.

qPCR data analysis

The analysis to evaluate the suitability of a gene as a reference gene according to qPCR data was performed using the algorithms described for geNorm and NormFinder. In brief, the average C_q value from qPCR technical replicates was calculated. Measurements that were under the limit of detection were arbitrarily set to C_q=36 (limit of detection +1). The intra- and inter-group variation in the proliferating and senescent samples was calculated using an R function provided by NormFinder (<https://moma.dk/files/r.NormOldStab5.txt>) [4].

For the geNorm analysis, the lowest C_q value for each gene was subtracted from all the other measurements. Then, the control stability measure M was calculated in R for each gene, followed by a stepwise exclusion of the least stable gene so that a list

was constructed ranking the candidate reference genes according to their stability. The need of two or more reference genes was also calculated. The exact algorithms were described in [5].

Co-regulation data used on Figure S2 was downloaded from <https://string-db.org> (version 10.5).

Software used for analysis and plots

All plots were made either in Excel 2016 or in R version 3.5.1 using the packages “ggplot2” and “DESeq2”.

Annex 2. Supplementary Information

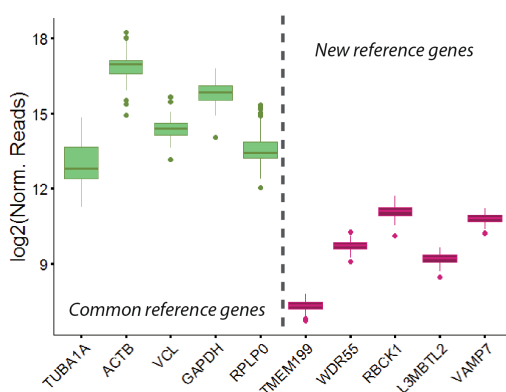


Figure S1: Average expression of common and new reference gene candidates. The logarithm of the normalized expression (normalized read counts using size factors as calculated in [1]) in the five commonly used (green) and the five newly proposed (dark pink) reference genes is shown here.

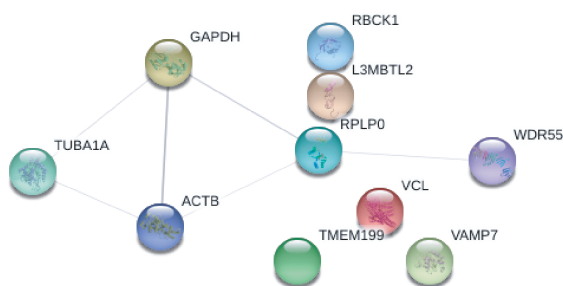


Figure S2. Co-regulation of Reference Gene Candidates. The co-regulation of the ten reference gene candidates was inquired using the online software STRING-db. Each node represents a protein and the connectors represent protein-protein associations (i.e. proteins contributing to shared function and not necessarily physically binding). The thickness of the connector represents the confidence of the association: low (0.150), medium (0.400), high (0.700) and highest (0.900). Only the association between ACTB and GAPDH was medium, all the others showed low confidence. Downloaded from <https://string-db.org/> on 30/08/2018.

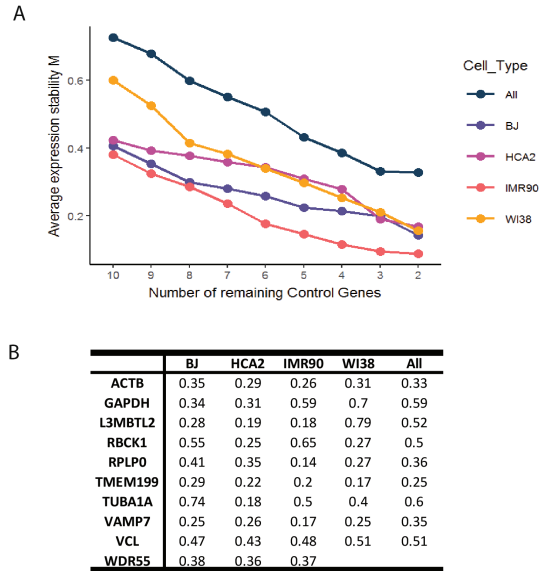


Figure S3: M-values and Stability values as calculated by geNorm and NormFinder, respectively. The results of the analysis for each reference gene candidate tested for every fibroblast strain. A. Average expression stability (M-value) after the stepwise exclusion of the least stable reference gene candidate for each fibroblast strain. B. Stability values for each reference gene candidate studied as calculated by NormFinder.

Supplementary tables are available on the online version of the article: <https://onlinelibrary.wiley.com/doi/full/10.1111/acel.12911>



Chapter 6

Heterogeneity of the Senescence-Associated Programs: an Update

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Introduction

Cellular senescence is a state of stable cell cycle arrest often occurring in response to damaging stimuli [1–3]. *In vitro*, these stimuli can be: continuous culture (replicative senescence, or RS), ionizing radiation (ionizing radiation-induced senescence, or IRIS), genotoxic drugs (therapy-induced senescence, or TIS), oncogenes (oncogene-induced senescence, or OIS), oxidative agents (oxidative stress-induced senescence, or OSIS), among others [2,3]. Organisms are also exposed to similar stimuli throughout their lives, with a consequent steady accumulation of senescent cells with aging [4]. The extent to which each stimulus contributes to cellular senescence *in vivo* is not known and probably it differs among individuals. What is clear is that cellular senescence is a stress response that prevents the division and spread of damaged cells, making it a potent anti-tumor mechanism [3]. Moreover, cellular senescence is also involved in development and wound healing [5–7]. However, multiple studies have evidenced the role of cellular senescence in aging, age-related diseases and, paradoxically, cancer [8–12]. This negative side of the senescent program is attributed to the limited regeneration potential of a senescent cell population and to the senescence-associated secretory phenotype (SASP) that promotes inflammation and changes in the micro-environment that ultimately promote cancer and inflammaging [13,14]. Thus, cellular senescence has gained attention as a target for the prevention of aging and age-related diseases [4,15,16].

Besides the stable cell cycle arrest and the SASP, cellular senescence is characterized by other features: increased lysosomal content (often reported as high activity of the Senescence-Associated β -galactosidase, or SA- β gal), DNA damage, mitochondrial dysfunction, loss of nuclear lamina proteins, chromatin rearrangements, changes in metabolism and in the composition of the plasma membrane, endoplasmic reticulum stress and apoptosis resistance [17]. However, as seen in chapter IV, the senescent phenotype is heterogeneous and different stimuli and cell types do not engage the same molecular players [18]. Indeed, even p16 –a CDK4/6 inhibitor known as a key driver of senescence-associated growth arrest [19]– is not upregulated at the transcriptional level in every senescent sample [18,20]. The heterogeneity of cellular senescence hinders the discovery of a reliable, specific and universal marker of senescence [2]. In cell culture, the current markers are often sufficient to classify a sample as senescent or non-senescent. *In vivo*, however, the complexity of tissues and the scarce information about the particular source or stimulus causing cells to enter a senescent state reclaim the need to identify novel, reliable and unequivocal senescence-associated markers. In view of this problem, the study of heterogeneity of different senescent programs is indispensable.

Few groups focus their topic of research on this crucial question [20–24]. To our knowledge, our recent report is the first one systematically studying the heterogeneity of cellular senescence in a great diversity of programs in primary cells with a tool as powerful as RNA-sequencing [18]. Here, we update the study of the heterogeneity of the senescence-associated programs induced by different stimuli by analyzing whole pathways or biological processes that could be commonly regulated even when differential regulation of individual genes is diverse. Moreover, besides RS, OIS and IRIS as models as in the last report, we also include three more datasets in which BJ foreskin fibroblasts were induced to senescence by three different TIS stimuli that were not studied before: Doxorubicin, Palbociclib and Abemaciclib.

Furthermore, in line with a recent report [25], we show that there is a large amount of cell-to-cell variability within seemingly homogeneous senescent cell populations.

Results and Discussion

In Chapter IV, we had obtained ten public or self-generated RNA-sequencing datasets in which primary fibroblasts (BJ, HCA2, HFF, IMR90, MRC5 and WI38) were induced to senescence by three different stimuli: RS, IRIS and OIS [18]. We had performed a meta-analysis to look for differentially expressed genes that were conserved in different senescent programs. Here, we update the meta-analysis by adding three more datasets in which BJ foreskin fibroblasts were induced to senescence using Doxorubicin, Palbociclib or Abemaciclib (**Figure S1A**). Doxorubicin intercalates in the DNA and disrupts the topoisomerase-II-mediated DNA repair, therefore inducing double-strand breaks that, if unreparable, lead to senescence [26]. Moreover, doxorubicin also generates free radicals which further damage the DNA and other organelles. On the other hand, Abemaciclib and Palbociclib, are two p16 mimickers that inhibit the kinases CDK4/CDK6, therefore preventing the phosphorylation of Rb and the G1/S transition [27]. This leads to a stable cell cycle arrest in G1 phase. Interestingly, Abemaciclib and Palbociclib do not induce DNA damage [28] (Wang *et al*, in progress) and yet they share many of the same features with other senescence programs, which gave us the opportunity to study an uncommon type of cellular senescence.

We found 435 genes that constituted the signature of cellular senescence in fibroblasts and that were not differentially expressed in quiescent cells (**Table S1**). Genes in the signature could differentiate control cells (proliferating untreated or proliferating + vehicle) from senescent ones (**Figure 1A**). However, from our previous report [18] and follow-up studies, we knew that these potential senescence markers have two downsides: 1) the fold changes are often small so that it is still difficult to use them to discriminate senescent from non-senescent cells and 2) these genes are often not expressed in all cell types. From our new signature of senescence in fibroblasts, 99 genes had an average fold change of \geq two (**Figure S1B**) and only four of them (TSPAN13, NFIA, EFN3 and CCND1) were also differentially expressed in astrocytes, keratinocytes and melanocytes (**Figure S1B** marked in green or orange). Even within our current dataset, which included six types of fibroblasts derived from two different tissues, foreskin (BJ, HFF and HCA2) and lung (IMR90, MRC5 and WI38), we realized that the tissue of origin accounted for a big part of the heterogeneity (**Figure 1A** and **Figure S1A**). In any case, the phenotype of the different populations of senescent cells was quite similar: stable cell cycle arrest, enlarged cell body in culture, irregular shape and high percentage of SA- β gal positive cells. Thus, we decided to investigate whether different senescent programs were actually converging into the same pathways, processes and cellular functions, despite engaging different genes.

Figure 1B shows the pathways of the Kyoto Encyclopedia of Genes and Genomes (KEGG) that were most commonly differentially regulated in senescent versus proliferating cells in at least 9/13 datasets studied ($p\text{-val} \leq 0.01$ in each dataset). Not surprisingly, “cellular senescence” and “cell cycle” were among the main pathways found. Interestingly, all datasets relied on the “cytokine-cytokine receptor pathway”, with the sole exception of Abemaciclib. Findings from our laboratory demonstrate that Abemaciclib induces a milder SASP (Wang *et al*, in progress), but their inability to

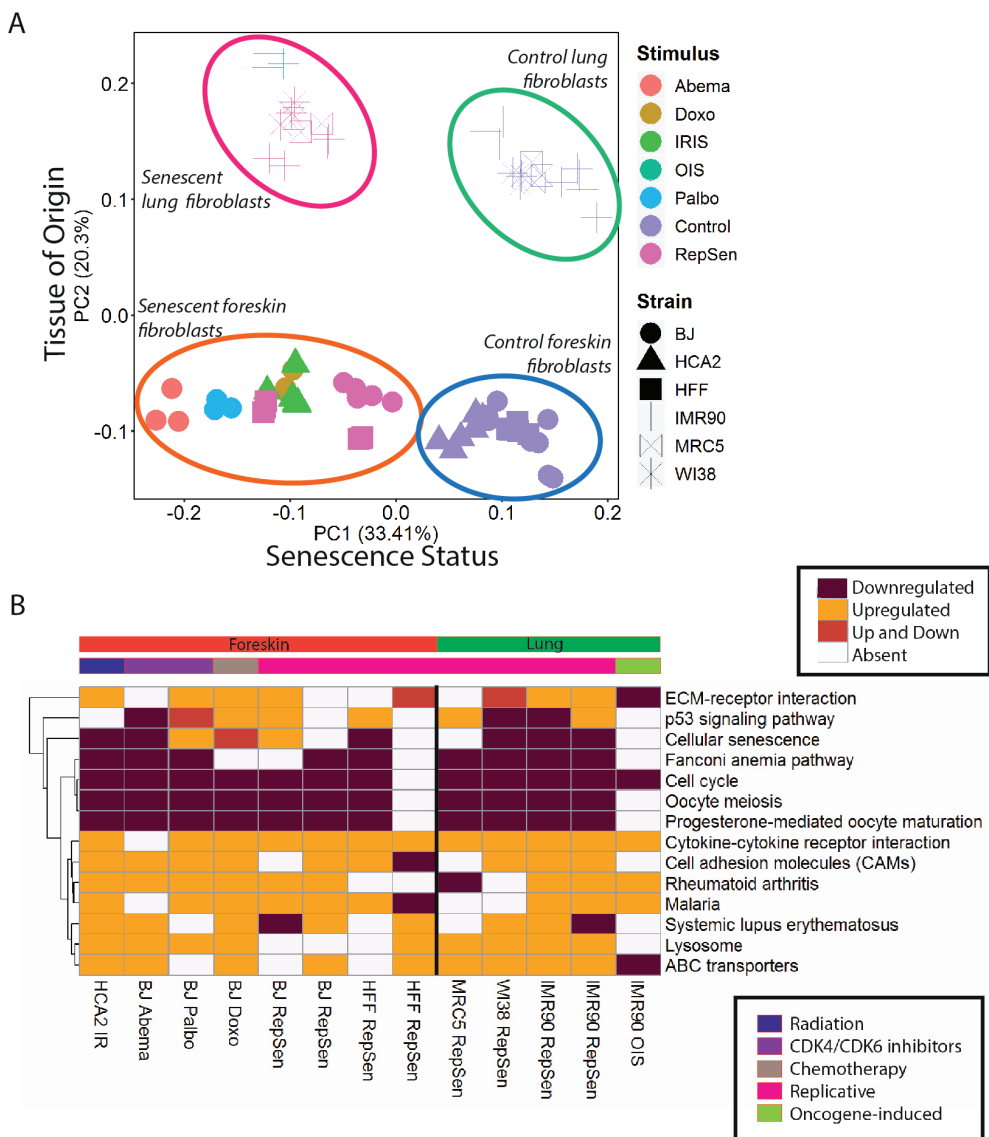


Figure 1. Heterogeneity of Different Senescence-associated Programs in Primary Fibroblasts. **A.** Principal Component Analysis of different samples of lung (IMR90, MRC5 and WI38) or foreskin (BJ, HFF, HCA2) primary fibroblasts induced to senescence by different stimuli. The log-transformed normalized pseudo-counts of the 435 genes that constituted the Signature of Senescence in Fibroblasts were used to do the Principal Component Analysis. Four different clusters were distinguishable corresponding to Control Lung Fibroblasts, Senescent Lung Fibroblasts, Control Foreskin Fibroblasts and Senescent Foreskin Fibroblasts. **B.** Heatmap showing enrichment of KEGG pathways in different fibroblast samples induced to senescence by different stimuli. In every case the colors mark whether every KEGG pathway was enriched in up-regulated genes (yellow), down-regulated genes (purple) or in both (pink) ($p \leq 0.01$). IR: Ionizing Radiation; Abema: Abemaciclib; Palbo: Palbociclib; Doxo: Doxorubicin; Repsen: Replicative Senescence; OIS: Oncogene-Induced Senescence.

increase the expression of cytokine receptors may mean they are also less responsive to autocrine and paracrine signaling. Also, the “p53 signaling pathway” is in the top list of pathways used in cellular senescence, although in some cases this pathway was enriched in down- while in others up-regulated genes. In the context of cellular senescence, p53 acts in response to DNA damage and induces p21, a CDK inhibitor that is also important for the cell cycle arrest [29,30]. However, p53 can additionally lead to metabolic changes and resistance to apoptosis, which are also features of senescent cells [31–33]. The DNA damage-independent activation of the p53 signaling pathway observed in abemaciclib and palbociclib-induced senescence could be due to the metabolic reprogramming. Thus, different senescent programs can engage p53 for regulating various features of the senescence phenotype pointing at it as a key node in which different pathways converge and others derive. However, p53 itself is mainly regulated post-translationally through many different modifications and is influenced by multiple proteins [34], therefore using p53 as an unequivocal marker of senescence is difficult. The “Lysosome” pathway was also enriched in up-regulated genes, which is in line with the high lysosomal content observed in cellular senescence [17,35]. It is known that high lysosomal content derives partly from old lysosomes that are not being removed from the senescent cells, causing them to accumulate molecules like lipofuscin [36,37]. Nevertheless, we found that many lysosomal enzymes and proteins were upregulated in the different samples (**Table S2**), suggesting that lysosomes are being actively generated, which is in line with one previous report [38].

Figure S2A shows a similar analysis than **Figure 1A**, but using Reactome pathways. Of note, “extracellular matrix organization” was enriched in up-regulated genes in every dataset analyzed. Also, genes involved in “collagen formation” were upregulated in most datasets in foreskin fibroblasts but not so in lung fibroblasts. This is of interest in view of the reported involvement of cellular senescence in Idiopathic Pulmonary Fibrosis [39]. Interestingly, the SASP pathway was not consistently regulated in all datasets and so the MAPK1/MAPK3 signaling pathways, which is one of the regulators of the SASP [40]. This may reflect the different SASP profiles displayed by different cell types and senescence-inducing stimuli [18,41]. Finally, the Regulation of Insulin-like Growth Factor transport and uptake by Insulin-like Growth Factor was enriched in up-regulated genes in 12/13 datasets, further supporting its proposed role in induction of cellular senescence [42].

Figure S2B also shows a heatmap of the GO terms that are enriched in the different senescence-associated datasets. These terms include Cellular Compartments, Biological Processes and Molecular Functions. As expected, many GO terms involved in cell division and cell cycle were enriched in down-regulated genes. Interestingly, few GO terms related to development were enriched in both, up- and down-regulated genes. Finally, many GO terms involved in plasma membrane or in secretion by cell or extracellular space were enriched in up-regulated genes, probably reflecting the well-known changes in plasma membrane and the SASP [43–45]. However, so far there is not universal surface marker of senescence and neither a universal SASP factor.

We mentioned before that TSPAN13 was among the four genes that were differentially expressed in all the fibroblast samples tested, in keratinocytes, melanocytes and astrocytes and that had an average fold change higher than two. Interestingly TSPAN13 or Tetraspanin 13 is a cell surface protein that is largely unstudied. Few reports list it as

a potential biomarker for different types of cancer [46,47] and one mentions its action as a tumor suppressor gene, mainly by promoting apoptosis [48]. We decided to test its potential as a novel senescence biomarker. For that purpose, we measured the protein expression of TSPAN13 on the surface of BJ fibroblasts induced to senescence by ionizing radiation that had been previously validated by SA- β gal staining (**Figure S3A**).

We used an antibody against TSPAN13 and analyzed the cells by fluorescent-automated cell sorting (FACS). Interestingly, while sorting the cells we realized that the size of senescent fibroblasts was very heterogeneous compared to proliferating counterparts (**Figure 2A**). The great majority of untreated fibroblasts formed a defined population with similar cell size. However, this population almost disappeared in irradiated fibroblasts and instead we saw an increase in large but heterogeneous cells without any defined population. The ratio of small/large cells increased 17 times in senescent fibroblasts. Although the enlarged cell body is a well-known hallmark of senescence [3,17], this feature has been mainly observed in culture, where it is difficult to determine whether the larger size is a consequence of cell flattening or a true bigger size. The large and irregular shape of senescent cells in suspension required optimization and the use of large nozzle size (see Materials and Methods).

Notably, the expression of TSPAN13 was indeed upregulated in senescent fibroblasts but it was also very heterogeneous (**Figure 2B**). The percentage of TSPAN13+ cells went from less than 5% in proliferating cells to more than 35% in senescent cells, but still it was not a universal marker at the protein level. However, our original senescence signature had been constructed with RNA-sequencing data and it could still be that most senescent cells upregulate TSPAN13 at the transcriptional level and only the translational and post-translational regulation make the difference in protein expression. We decided to measure TSPAN13 transcriptional expression in single-cells by single-cell qPCR. Although again we could confirm the upregulation of TSPAN13, this gene was also expressed in a heterogeneous manner in senescent cells (**Figure 2C**). This is in line with a recent report that showed that the expression of some genes shows more variability in senescent than in quiescent cells [25]. Like them, we could also demonstrate the heterogeneity of many other genes associated to different hallmarks of senescence such as proliferation and cell cycle (MKI67, RB1, CDK4), the SASP (IL6, MMP1, FGF2), chromatin and nuclear rearrangements (LMNB1, PCNA), metabolism (GAPDH), endoplasmic reticulum stress (BiP) and changes in the plasma membrane (Cav1) (**Figure S3C**). Perhaps that heterogeneity hindered the reaching of statistical significance in many cases. Still, the expression of these genes could partially differentiate proliferating from senescent cells (**Figure 2D**) and we foresee that if more genes were included we would be able to define sub-populations of senescent cells.

Discussion

In this report we updated and extended the study of gene expression heterogeneity in senescent cells, once more emphasizing that few genes are widely expressed in multiple senescence-associated programs. Here we focused in the study of TSPAN13 due to its potential as a surface marker, however, the other three genes that show a broad applicability can be of interest not only as markers but also as functional players in senescence. For instance, NFIA or Nuclear Factor IA, which is downregulated in our signature, has been reported as promoter of glioblastoma aggressiveness via negative

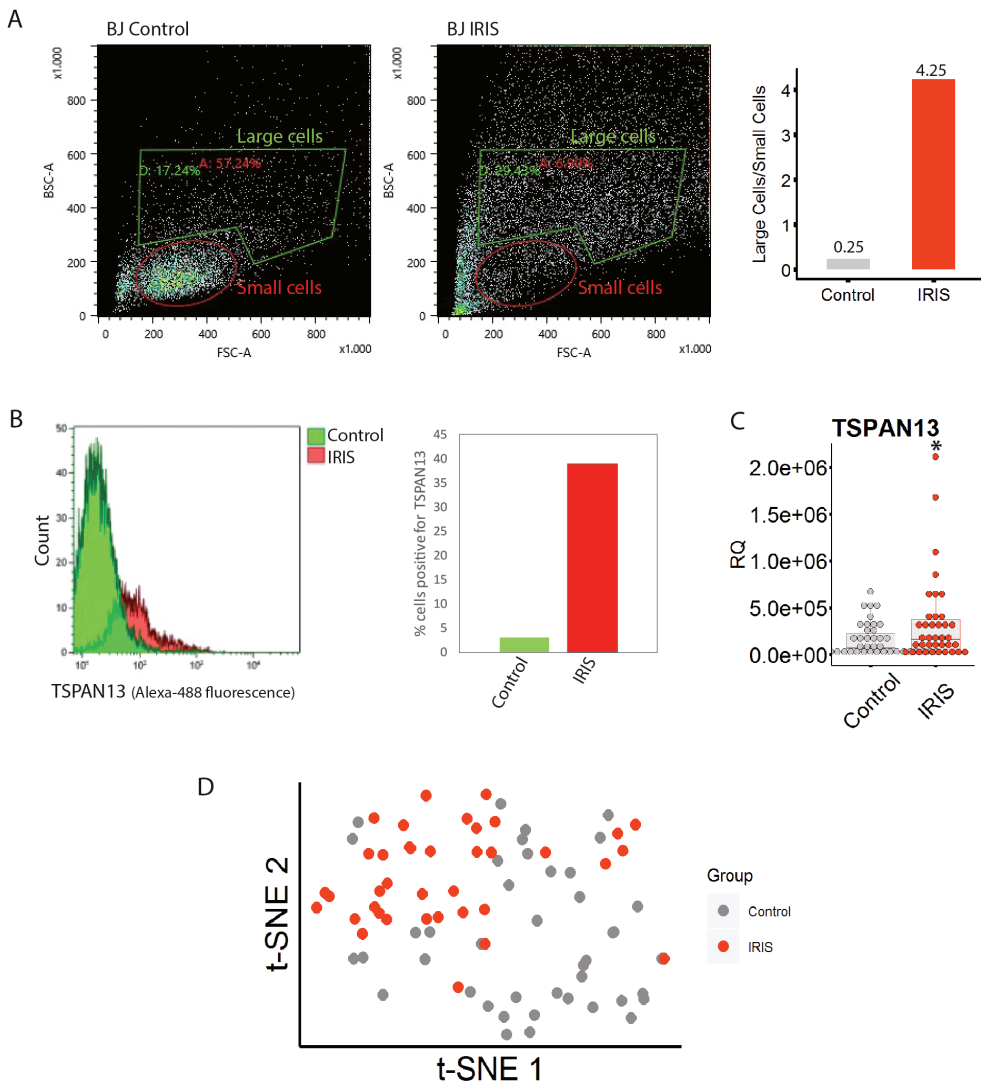


Figure 2. Intra-population Heterogeneity in Senescent Cells. **A.** BJ fibroblasts induced to Senescence by Ionizing Radiation (IRIS) show a more heterogeneous cell size than their proliferating counterparts (Control). Left and central panel show a representative image of the cell sorting marking the gating used to determine whether a cells was considered small (red) or large (green). The ratio of large/small cells in each sample was quantified in each sample type. The experiment was repeated two other times with similar results. **B.** Fluorescence-Automated Cell Sorting of TSPAN13(+) cells in proliferating BJ Control and IRIS. Left panel shows the histogram of both samples and right panel shows the quantification of positive cells with respect to the total cell number. **C.** Relative Quantities of TSPAN13 by single-cell qPCR calculated as recommended in [64]. TSPAN13 was upregulated in BJ IRIS ($pval \leq 0.05$). **D.** t-Stochastic Neighbor Embedding (t-SNE) plot using single-cell qPCR data for different genes (TSPAN13, MKI67, RB1, CDK4, IL6, MMP1, FGF2, LMNB1, PCA, GAPDH, BiP, Cav1) in BJ Control and BJ IRIS (perplexity: 20).

regulation of p21, p53 and PAI1 [49]. Despite being downregulated in senescent cells, EFNB3 or Ephrin B3 inhibition is one of the targets used for senolytics such as dasatinib and quercetin [50]. Indeed, the original article that used EFNB3 as a senolytic target mentions that the ephrins are not highly expressed, not even at the protein level [51]. Still, they hypothesized that the low expression of the ephrins facilitates the further downregulation by siRNAs or drug targets, enough to trigger apoptosis, which would perhaps not happen with other anti-apoptotic proteins with higher expression. Finally, the upregulation of CCND1 or Cyclin D1, which has been already reported [52], might be due to the increase of cells arrested in G1 phase.

In any case, we focused also on the pathways and functions that most senescent cells rely on. “Cell cycle”, “cytokine-cytokine receptor pathway”, “p53 pathway”, “Insulin-like growth factor pathway”, “extracellular matrix organization”, “secretion”, “lysosome” and “plasma membrane” were among shared features of the senescence program. Notably, some of the known hallmarks of senescence such as mitochondrial dysfunction and endoplasmic-reticulum stress [17] were not within the list and seem to be less universal. Unpublished results from our laboratory could not always demonstrate the activation of these pathways in all senescent samples. We hope that studies like the one we just presented will help fine tuning the list of common hallmarks of cellular senescence, and defining markers of specific senescent programs. Moreover, the presence of common pathways might help classifying cells as senescent. For instance, one of the foreskin samples (HFF Replicative Senescence, **Figure 1B** and **S2**) was quite different from the rest, with many of the pathways and GO terms important for other senescence-associated programs being absent in this dataset. Accordingly, the original report shows that only ~60% of the cells are SA- β gal-positive [53]. Although we considered that any sample with more than 50% of SA- β gal positive cells as senescent, the population is indeed not fully senescent and the signal of many of the pathways might be too diluted to be detected.

Finally, the intra-population heterogeneity of senescent cells also makes more difficult the development of markers. From gene expression to cell size, senescent cells are very heterogeneous. We encourage the assessment of intra-population heterogeneity when searching for new markers of cellular senescence and to use the right techniques to isolate and select single-cells of various sizes and morphologies. Previous studies have made use of microfluidics devices [25,54] to isolate senescent cells, a technique which might be pre-selecting for smaller cells and not reflecting the morphological variability of senescent cells. In our laboratory, some FACS experiments in which only smaller (comparable size to proliferating cells) senescent cells caused sometimes contradictory results in the single-cell qPCR data (data not shown).

Besides technical limitations, only two reports have studied senescent cells by using single-cell techniques so far [25,54]. We foresee that single-cell omics will give us a broader and clearer picture of the intra-cellular heterogeneity in senescent cells and refine our use of markers.

The study of pathways and features shared by senescent cells is necessary to generate a clearer definition of cellular senescence. Until now, the unique, universal and unequivocal marker of senescent cells is not more than a utopia. The field has claimed the need of a unique universal marker or at least a defined panel of markers to characterize senescent cells [2,17]. However, it is also possible that different markers are

necessary for different senescent programs. Indeed, while we find several pathways and molecular processes shared among senescence-associated programs, only few individual molecular effectors are conserved. In the cancer field, the heterogeneity of different cancers is acknowledged and the use of specific markers for different cancers is a common practice [55]. This realization has also posed the basis for the development of targeted therapies effective only on sub-types of cancer cells. The same could apply to cellular senescence, and perhaps the heterogeneity can be exploited for the development of therapeutic approaches aimed at specifically targeting detrimental senescent cells.

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Annex 1. Material and methods

RNA-sequencing Data

The list of public datasets as well as the selection criteria was previously published [18]. Briefly, we used datasets of primary fibroblasts without any genetic modification (with the exception of Ras overexpression in the case of oncogene-induced senescence datasets). The public datasets were obtained from the Gene Expression Omnibus repository under the accession numbers: GSE56293, GSE61130, GSE 664553, GSE63577 and GSE53356 [21,22,56–58] or from Array Express under the accession number E-MTAB-5403 [18]. The last one was formerly generated in our laboratory and included six samples of quiescent HCA2 fibroblasts besides the proliferating and senescent (IRIS) counterparts. Three new datasets of BJ fibroblasts induced to senescence by Doxorubicin, Palbociclib and Abemaciclib were added to the previous list. These datasets are not yet available but belong to an ongoing project in our laboratory (Wang *et al.*, in progress).

For every sample collected, we performed a quality control and trimming of the reads if necessary (FastQC software v0.11.5) and aligned to the GRCh38 genome (STAR-2.5), as described before [18]. Only protein-coding genes were used in downstream analysis.

Meta-analysis of Fibroblasts

The heterogeneity of the data was evaluated using a Principal Component Analysis (PCA) plot analysis (R software 3.5.1 and R-package “ggfortify” 0.4.5) of the log-transformed normalized counts for the protein-coding. After doing the meta-analysis, a

new PCA plot was built using only the genes of the signature.

For the meta-analysis, we first performed a differential expression analysis (DESeq2 [59]) for every set of samples separately. Datasets that contained more than one cell type were analyzed only one cell type at a time. We then used the Fisher P-value combination method as described [60]. Genes with an adjusted p-value ≤ 0.01 were included in the signature. Genes that were also differentially regulated in the quiescence samples (adjusted p-value ≤ 0.01 and sign of the fold change while compared to proliferating cells in the same direction than senescence) were removed from the signature. Only genes with a fold change ≤ 2 were used to build a heatmap (R-package “pheatmap” 1.0.10).

Pathway Analysis and Comparison between samples

Enriched KEGG and Reactome pathways and GO terms in the differentially expressed genes within the signature of senescence for fibroblasts (435 genes) were evaluated using the online tool “Over-representation analysis” of the Consensus Path DB-human (<http://cpdb.molgen.pmg.de>) [61,62] for every sample and its corresponding control separately. For every set of samples, upregulated and downregulated genes were evaluated separately. Heatmaps were created showing pathways that were present in at least 9/13 samples.

Cell culture

Human foreskin fibroblasts BJ (male) were purchased from ATCC (Manassas, Virginia, USA, Cat: CRL-2522) and regularly monitored for mycoplasma contaminations (once/2 weeks). All cells were cultured in 5% O₂, 5% CO₂ and 37C in DMEM (Thermo Fisher Scientific, Waltham, Massachusetts, USA, Cat: 31966-047) enriched with 10% fetal bovine serum (FBS, GE Healthcare Life Sciences, Chicago, Illinois, USA, Cat: 758092, Origin: South America, Batch: 41213-C05) and 1% penicillin/ streptomycin (Lonza, Switzerland, Cat: LO DE17-602E).

In order to induce senescence by ionizing radiation, cells were subjected to a 10Gy dose of γ -radiation using a 137-Cesium source and medium was refreshed every 2 days. Irradiated cells were harvested by trypsinization on day 10 after senescence-induction. For chemotherapy-induced senescence, cells were treated either once for 24 hours with 250 nM doxorubicin (Tebu-bio, BIA-D1202-1), or 8 times for 24 hours in consecutive days with either 1 μ M palbociclib (Sigma-Aldrich, PZ0199) or 1 μ M abemaciclib (MedChem Express, HY-16297). In all drug-treated cases, RNA was collected at both 1 day and 8 days post drug removal. On the day of harvest, a subset of cells was re-plated for validation of senescence. A sample of proliferating cells of the same population doubling was used as control for IRIS. For doxorubicin, palbociclib and abemaciclib, proliferating cells treated 8 times for 24 hours with the vehicle alone (water in volumes equal to the ones used for palbociclib or abemaciclib). All experiments were performed with cells younger than 40 population doublings.

Validation of Senescence by Senescence-associated (SA)- β -galactosidase assay

Cells were plated in a 24-well plate, fixed in a mixture of glutaraldehyde and formaldehyde (2%/2%) for 10-15 min and stained over- night with an X-Gal solution as

described in [63]. Cells were counter-stained with a 1 mg/ml 40,6-diamidino-2-phenyl-indole (Sigma-Aldrich, St. Louis Missouri, USA, D9542) solution for 20 min. Images were acquired at 100X magnification, and the number of cells counted by the software ImageJ (<http://www.rsweb.nih.gov/ij/>). The number of positive cells was counted manually in blind and the percentage of positive cells was calculated.

Isolation of RNA

Once the full treatment (if applicable) was performed, cells were collected in 350 μ l of RNA Lysis buffer RLY (Bioline, BIO-52079) and 3.5 μ l of β -mercaptoethanol using a cell scraper. Samples were stored at -80C until processed.

Total RNA was extracted from treated cells via a ISOLATE II RNA mini kit (Bioline, Cat: BIO-52073) following manufacturer's instructions. The extracted RNA was quantitated using a Nanodrop and RNA quality was measured via BioAnalyzer RNA chip (Agilent).

Same procedure accounts for BJ cells in cells used for RNA-sequencing (BJ treated with doxorubicin, Abemaciclib or Palbociclib) or the ones used for qPCR after sorting according to TSPAN13 levels (IRIS).

Library preparation and RNA-sequencing

Poly-A tail selection was used to enrich for messenger RNA (mRNA) using the NEXTflex Poly(A) Beads kit (Bioo Scientific Corp, cat #512980). RNA-sequencing library preparation was carried out using NEXTflex Rapid Directional qRNA-Seq kit (Bioo Scientific, cat# 5130-05D). In brief, mRNA was fragmented using a cationic buffer and then submitted to first and second strand synthesis, followed by adenylation, ligation and 15 cycles of PCR. Library quality and size distribution were validated on a Qubit (Thermo Fisher Scientific) and an Agilent High Sensitivity DNA chip. Clusters for sequencing were generated on the cBot (Illumina). Paired-end sequencing was performed at 400 M reads per lane of the flow cell on an Illumina HiSeq 2500. Average Quality scores for the completed run across all samples was >30, with an average of 10 million reads for each pooled sample. The read length was 76 bp.

Raw sequencing data were demultiplexed according to library-specific barcodes and converted to fastq format using standard Illumina software (bcl2fastq version 1.8.4). The resulting reads were mapped to the human reference genome (GRCh38) using Bowtie2 (version 2.2.4). Sequencing data is not yet publicly available.

Fluorescence Automated Cell Sorting for TSPAN13

Cells were harvested 10 days after ionizing radiation (10Gy) using accutase for 13min and stopping the reaction with 1%BSA/PBS. Cells were then harvested in fresh 1%BSA/PBS.

Isolation of single-cells for single-cell qPCR and reverse transcription

Cells were trypsinized, collected in sorting buffer (PBS + 2% FBS) containing propidium iodide (Sigma-Aldrich, Cat: P4864) to assess viability and put on ice right before sorting. Cell aggregates were removed by filtering with a 40 μ m cell strainer and

single-cells were sorted with a Sony SH800 sorter in 96-well plates containing 5 μ l of RLT buffer (Qiagen, Cat: 79216) + 1% β -mercaptoethanol. Every plate contained at least two wells with 32-cell positive controls and two wells with 0-cell negative controls. Collected cells were frozen on dry ice immediately after sorting and then transferred to a -80C freezer until processed (never more than one month later).

RNA was cleaned using 11 μ l per well (1:2.2 ratio) of Agencourt RNAdvance Cell v2 beads (Beckman, Cat: A47942) and washed twice with ethanol before eluting in 5 μ l of RNase free water. 1.5 μ l dNTPs (10mM) and 0.15 μ l custom oligo-dT (100 μ M) containing cellular barcodes and Unique Molecular Identifiers (UMIs) were added to the clean RNA and the mixture was incubated for exactly 3 min at 72C. We had 12 different versions of the oligo-dT containing different cellular barcodes. Then, the following reagents were added: 1M betaine (Affymetrix, Cat: J77507.AB), 5mM of dithiothreitol (Thermo Fisher Scientific, Cat: 18064071), 10mM MgCl₂ (Sigma-Aldrich, Cat: M8266), 0.6 μ M of custom-made TSO-B, 15 units of RNase OUT (Thermo Fisher Scientific, Cat: 10777019) and 150 U of Superscript II Reverse Transcriptase together with the Superscript II first-strand buffer (Thermo Fisher Scientific, Cat: 18064071) to a final volume of 15 μ l. Reverse transcription was performed at 42C for 90 min and terminated by an enzyme inhibition step at 70C for 15min and a hold at 4C.

Targeted preamplification and single-cell qPCR

Targeted cDNA pre-amplification was performed using 6 μ l of cDNA, 1x iTaq Universal SYBR Green Supermix (Bio-Rad, Cat#175-5125) and a primer pool consisting of a collection of primers of cell-cycle associated genes whose sequences were kindly provided by Daniel Andersson (Sahlgrenska Cancer Center) and adding primers for TSPAN13 and BiP (see **Table S3**) all to a final concentration of 0.04 μ M. The PCR program for the targeted pre-amplification consisted of 3min at 95C, followed by 19 cycles of amplification (95C for 20s, 60C for 3min and 72C for 20s), a step of 72C for 10min and a hold at 4C. Pre-amplified samples were chilled on ice and diluted 1:20 in RNase free water before performing a qPCR.

Each qPCR assay mix contained 1x iTaq Universal SYBR Green Supermix (Bio-Rad, Cat#175-5125), 0.4 μ M of the primer of interest (same used on the targeted pre-amplification) and 2 μ l of the 1:20 diluted pre-amplified sample. The qPCR program consisted of 2min at 95C, 40 cycles of amplification (95C for 5sec, 60C for 20sec and 70C for 20sec), 65C for 5sec and 95C for 5min, followed by a hold at 4C.

Analysis of single-cell qPCR data

We used a reported algorithm [64] to analyze single-cell qPCR data. In brief, we first manually chose wells in which the melting temperature did not correspond to the right one for the particular assay analyzed (determined using 32-cell positive controls and 0-cell negative controls), that had more than one melting temperature or that presented atypical amplification curves. Samples run in different batches were normalized according to two inter-plate calibrators (two samples run in every plate to measure technical variability among plates). Samples that had missing values and/or very high C_q (≥ 35) for the majority of the assays were excluded from further analysis. Equally, assays for which all values were missing for every cell were excluded from the analysis.

Relative quantification of data was calculated as explained in [64]. Statistics (mean and SD) were calculated in logarithmic scale, but linear scale was used for plots presented (R-package “ggplot2” 3.1.0). Visualization of high-dimensional data was done using the t-stochastic neighbor embedding (t-SNE) method [65] (R-package “Rtsne” 0.15).

Annex 2. Supplementary Information

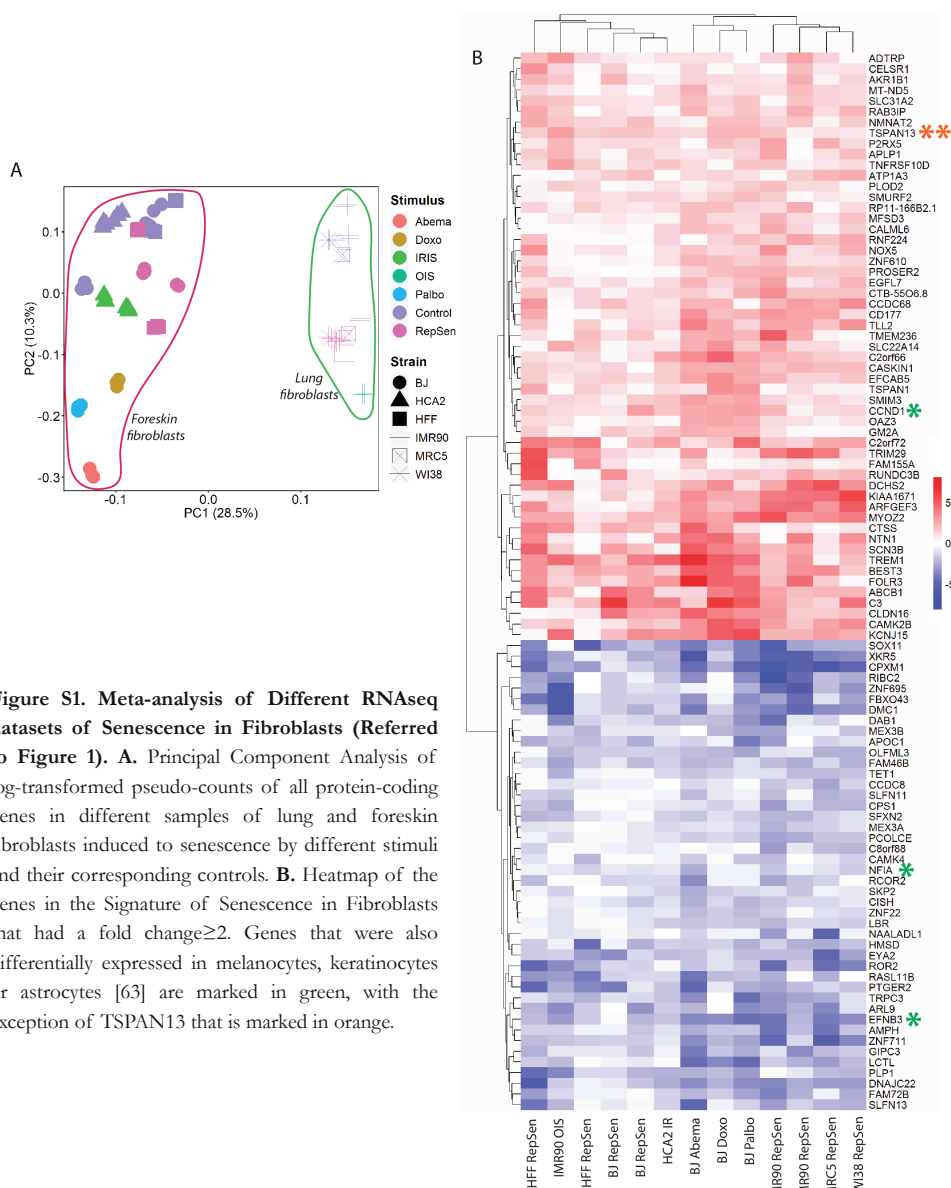
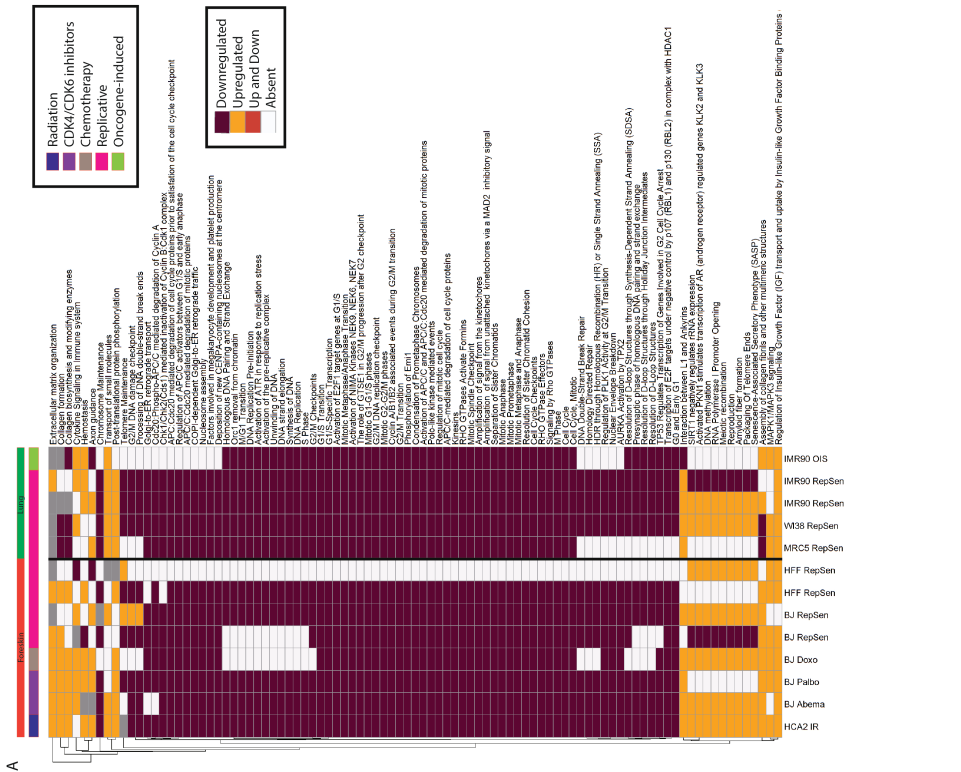
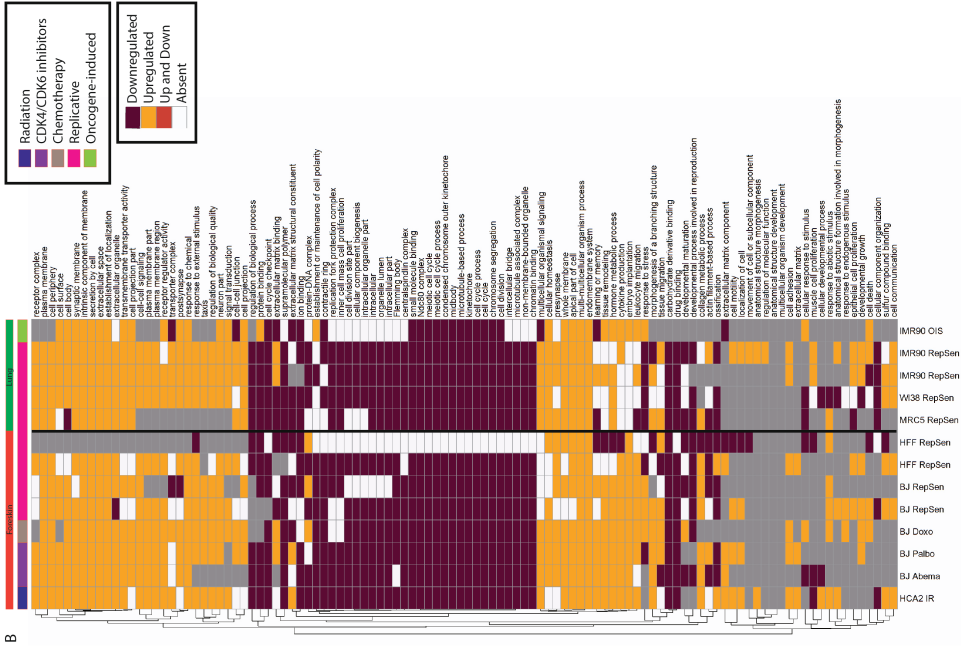


Figure S1. Meta-analysis of Different RNAseq datasets of Senescence in Fibroblasts (Referred to Figure 1). **A.** Principal Component Analysis of log-transformed pseudo-counts of all protein-coding genes in different samples of lung and foreskin fibroblasts induced to senescence by different stimuli and their corresponding controls. **B.** Heatmap of the genes in the Signature of Senescence in Fibroblasts that had a fold change ≥ 2 . Genes that were also differentially expressed in melanocytes, keratinocytes or astrocytes [63] are marked in green, with the exception of TSPAN13 that is marked in orange.

Figure S2 (next page). Main pathways and Gene Ontology terms that are used in different senescence programs (Referred to Figure 1). Heatmaps showing enrichment of Reactome pathways (**A**) or Gene Ontology terms (**B**) in different fibroblast samples induced to senescence by different stimuli. In every case the colors mark whether every KEGG pathway was enriched in up-regulated genes (yellow), down-regulated genes (purple) or in both (pink) ($p\text{-val} < 0.01$). IR: Ionizing Radiation; Abema: Abemaciclib; Palbo: Palbociclib; Doxo: Doxorubicin; Repsen: Replicative Senescence; OIS: Oncogene-Induced Senescence.



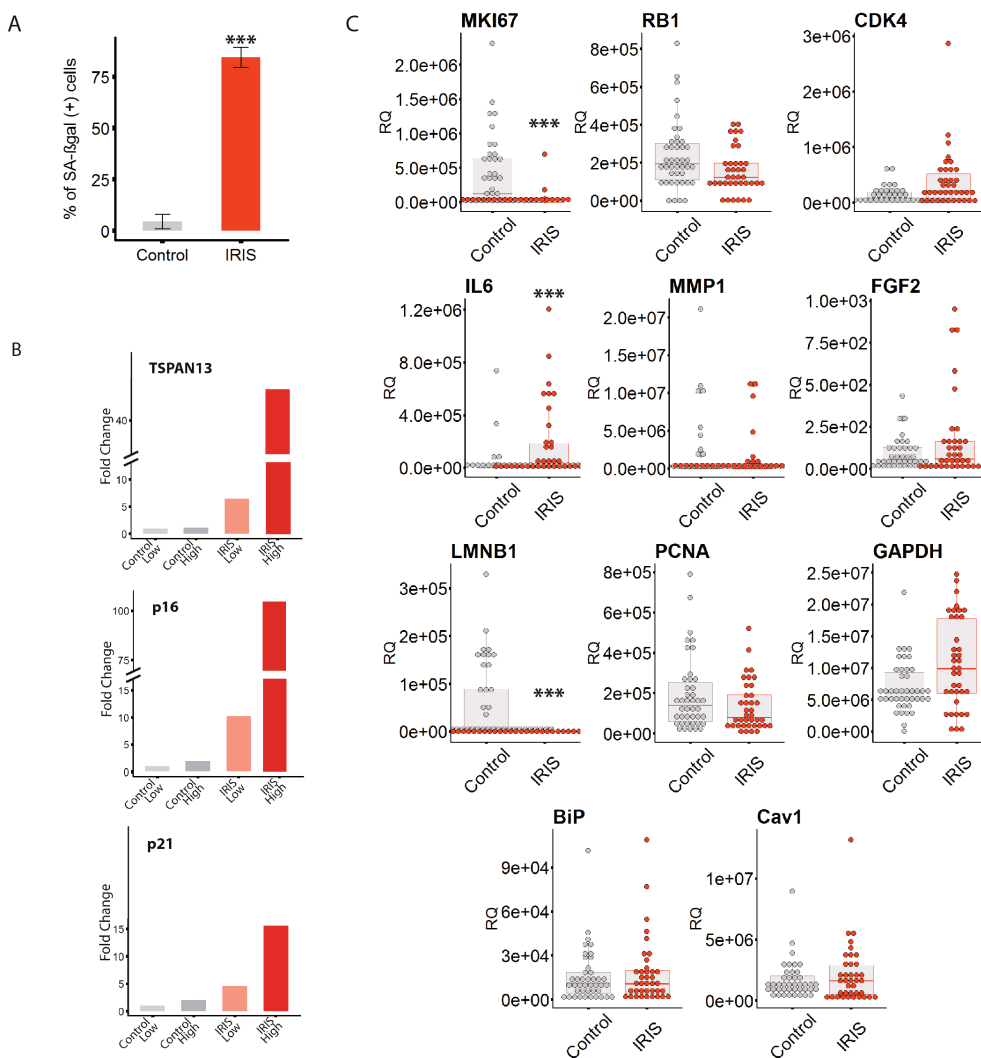


Figure S3. Intra-population heterogeneity of BJ Fibroblasts induced to Senescence by Ionizing Radiation (Referred to Figure 2). **A.** Quantification of Senescence-Associated β -galactosidase staining in Irradiated (IRIS) and Proliferating (Control) BJ fibroblasts. **B.** Bulk qPCR for TSPAN13, p16 and p21 in BJ Control and BJ IRIS sorted by low or high TSPAN13 on their plasma membranes. **C.** Relative Quantities measured by Single-cell qPCR for multiple genes in BJ Control and BJ IRIS. Assays included genes involved in cells cycle (MKI67, RB1 and CDK4), senescence-associated secretory phenotype (IL6, MMP1 and FGF2), nuclear and chromatin changes (LMNB1 and PCNA), metabolism (GAPDH), Endoplasmic Reticulum Stress (BiP) and changes in plasma membrane (Cav1). ***: p val ≤ 0.001 .

Table S2. Frequency of lysosomal genes upregulation in the different Senescence Programs

Gene	Description	Frequency
NAPSA	napsin A aspartic peptidase	11
ARSB	arylsulfatase B	10
ATP6AP1	ATPase H ⁺ transporting accessory protein 1	9
GLA	galactosidase alpha	9
SORT1	sortilin 1	9
LAMP2	lysosomal associated membrane protein 2	8
HEXB	hexosaminidase subunit beta	8
LAPTM4A	lysosomal protein transmembrane 4 alpha	8
ABCA2	ATP binding cassette subfamily A member 2	8
M6PR	mannose-6-phosphate receptor, cation dependent	7
CTNS	cystinosis, lysosomal cystine transporter	7
MCOLN1	mucolipin 1	7
ARSA	arylsulfatase A	7
CTSD	cathepsin D	7
SUMF1	sulfatase modifying factor 1	7
SMPD1	sphingomyelin phosphodiesterase 1	7
LAMP3	lysosomal associated membrane protein 3	6
CTSZ	cathepsin Z	6
ACP5	acid phosphatase 5, tartrate resistant	6
AP3B2	adaptor related protein complex 3 subunit beta 2	6
ASAH1	N-acylsphingosine amidohydrolase 1	6
MAN2B1	mannosidase alpha class 2B member 1	6
DNASE2	deoxyribonuclease 2, lysosomal	6
CTSB	cathepsin B	6
HGSNAT	heparan-alpha-glucosaminide N-acetyltransferase	6
GNPTG	N-acetylglucosamine-1-phosphate transferase subunit gamma	5
SLC17A5	solute carrier family 17 member 5	5
CD68	CD68 molecule	5
CTSK	cathepsin K	5
IDS	iduronate 2-sulfatase	4
AP3M2	adaptor related protein complex 3 subunit mu 2	4
CLN5	CLN5, intracellular trafficking protein	4
CTSH	cathepsin H	4
FUCA1	alpha-L-fucosidase 1	4
GALC	galactosylceramidase	3
ATP6V0A4	ATPase H ⁺ transporting V0 subunit a4	3
NAGLU	N-acetyl-alpha-glucosaminidase	3
NPC2	NPC intracellular cholesterol transporter 2	3
IDUA	iduronidase, alpha-L-	3

Chapter 6

GNS	glucosamine (N-acetyl)-6-sulfatase	3
GAA	glucosidase alpha, acid	3
CLTB	clathrin light chain B	3
ATP6V0A1	ATPase H+ transporting V0 subunit a1	2
LGMN	legumain	2
GNPTAB	N-acetylglucosamine-1-phosphate transferase subunits alpha and beta	2
ATP6V0B	ATPase H+ transporting V0 subunit b	2
ACP2	acid phosphatase 2, lysosomal	2
ARSG	arylsulfatase G	2
AP3S2	adaptor related protein complex 3 subunit sigma 2	2
ATP6V0D1	ATPase H+ transporting V0 subunit d1	2
PSAP	prosaposin	2
AP1G2	adaptor related protein complex 1 subunit gamma 2	2
CTSO	cathepsin O	2
PLA2G15	phospholipase A2 group XV	1
MANBA	mannosidase beta	1
AP1M2	adaptor related protein complex 1 subunit mu 2	1
CD63	CD63 molecule	1
ATP6V0D2	ATPase H+ transporting V0 subunit d2	1
AP1S3	adaptor related protein complex 1 subunit sigma 3	1
CTSS	cathepsin S	1
TPP1	tripeptidyl peptidase 1	1
GLB1	galactosidase beta 1	1
CTSF	cathepsin F	1
GBA	glucosylceramidase beta	1
SGSH	N-sulfoglucosamine sulfohydrolase	1
LAMP1	lysosomal associated membrane protein 1	1
LITAF	lipopolysaccharide induced TNF factor	1
GM2A	GM2 ganglioside activator	1
IGF2R	insulin like growth factor 2 receptor	1
NEU1	neuraminidase 1	1
HEXA	hexosaminidase subunit alpha	1

Table S1. Signature of Senescence in Fibroblasts and **Table S3. Primers used for scqPCR** are available upon request

An abstract painting with a vibrant pink background. In the bottom left, there are two large, rounded green shapes with dark spots, resembling cacti or rocks. To the right, there are dark, textured, layered shapes in shades of grey, black, and brown, suggesting a rocky or mountainous landscape. The overall style is expressive and textured.

Chapter 7

General Discussion and Future Perspectives

Cellular damage caused by insulting stimuli can lead to a stable proliferation arrest known as cellular senescence [1]. Accumulation of senescent cells in an individual's body is recognized as one of the hallmarks of aging [2] and its contribution to age-related diseases has been repeatedly reported [3–7]. Great advances have been made to understand the mechanisms by which cellular senescence leads to aging [8]. Moreover, therapies targeting the negative aspects of cellular senescence are under development [9]. However, we are not yet able to unequivocally recognize senescent cells *in vivo*. Even in cell culture, the markers of cellular senescence are often not exempt of ambiguity [1,10].

Researchers in the field recognize that the current markers of cellular senescence are far from ideal, in particular when used individually [1,10]. The present work demonstrates the lack of universality of the current markers. We compared the transcriptome of different senescence-associated programs, different senescence-inducing stimuli and different cell types exposed to them [11]. We found 55 new marker candidates that presented consistent differential expression, independent of cell type and stimulus used to induce cellular senescence. To our surprise, most of the common senescence-associated markers did not make it to this list because they were neither specific nor universal. Notably, in most cases, the 55 genes that constituted the “core” senescence-associated signature presented variable fold changes, sometimes low for some of the cell types. In such cases, a simple qPCR experiment with limited number of samples (as most of them are) would not be able to detect those differences. When working with whole tissues, the power of such markers would decrease further due to low percentage of senescent cells, and thus get confounded with other sources of changes in gene expression. This problem could be avoided by analyzing single-cells, but as discussed in Chapter VI, the field studying single-cell senescence is just starting.

Chapter III and V discuss more technical issues with the reproducibility and validity of the markers of cellular senescence. The culture conditions, the design of the assays and the performance itself is often not optimal when trying to detect senescent cells [12]. Senescence-associated markers are already imperfect and ambiguous, but the technical variations, and in some cases true mistakes, only exacerbate their unreliability (Hernandez-Segura, in press).

In Chapter VI we explore further the heterogeneity of senescent cells. In one hand, we make evident that different senescence-associated programs often use the same pathways or biological functions, despite using different molecular effectors. This could explain why markers testing functionality of pathways or biological functions (such as Senescence Associated β -galactosidase, SA- β gal, testing for high lysosomal content) are sometimes more universal than defined protein markers (such as p16 expression), albeit not necessarily more specific. We propose along with others, that simultaneous use of multiple markers should be used to assess cellular senescence [1,10]. Perhaps an approach would be to choose few genes from each of the top pathways that we found essential for cellular senescence and use them in combination for testing (“cell cycle”, “cytokine-cytokine receptor pathway”, “p53 pathway”, “insulin-like growth factor pathway”, “extracellular matrix organization”, “secretion”, “lysosome” and “plasma membrane”). The test of multiple markers can be time-consuming so more research needs to be done to find the appropriate combination.

In that same chapter, we also dealt with a new level of heterogeneity: the one found

within a senescent population. Technical issues inherent to the single-cell transcriptomics, such as low starting materials, low capture of mRNA, and amplification biases, pose a challenge in the study of single-senescent cells. Many of the markers that are used at the population level are undetectable at the single-cell level. Furthermore, senescent populations are heterogeneous [13]. Indeed, even TSPAN13, which was one of the universal markers found in Chapter IV, was not upregulated in every single-senescent cell. Ideally, single-cell RNA-sequencing could identify genes with lower heterogeneity in their expression. Unfortunately, we could not include the results of that approach in the current thesis, but at least the first report on single-cell qPCR and intra-population heterogeneity in cellular senescence [13], along with our own research, have initiated the field.

Our results also uncovered deep basic questions that need to be addressed in the senescence field. The next paragraphs will develop them further.

What defines cellular senescence?

In Chapter II we described the hallmarks of cellular senescence [1]. As discussed, none of those hallmarks are universal or specific for cellular senescence. Thus, it is fair to ask what makes a cell senescent? Cellular senescence is often defined as an “irreversible” proliferative or cell cycle arrest [8,14,15], that renders cells insensitive to mitogens [16]. However, the “irreversibility” of cellular senescence has been challenged. Different studies claim that cellular senescence can be reversed since cells can actually escape senescence [17–19]. Melanoma, for example, is a malignancy that emerges when a small fraction of the senescent melanocytes that formerly constituted a nevus are able to escape the arrest [20]. Perhaps it is more adequate to define cellular senescence as a “stable” rather than irreversible cell cycle arrest.

The stability of the arrest still poses questions for the definition of cellular senescence. What does stable arrest mean? How long does a cell need to stop dividing in order to be considered senescent? Dai and Enders [21] made some early efforts to determine the time point at which the arrest becomes stable. They showed that induction of p16, one of the main molecular drivers of the proliferation arrest and a common cellular senescence marker, in U2-OS cells for one day would be enough to arrest proliferation. However, once the levels of p16 went back to basal levels, the cells resumed the cell cycle. This was not the case for cells in which p16 was induced for six days, where the vast majority of these cells remained arrested even after p16 levels returned to basal levels. However, as many things in cellular senescence, this timeline does not apply to every situation. Data from our laboratory (Wang, B. *et al.*, in revision) shows that p53-mutated cancer cells and primary cells with a disrupted p53 functions exposed to p16 analogues for eight days stopped proliferating, but only transiently. After removal of the drug, these cells resumed proliferation. Furthermore, in some cases the assessment of a stable arrest is not even technically feasible (e.g. fixed samples) or simply not adequate (e.g. non-dividing cells).

Post-mitotic cells do not divide, so evaluating cell cycle arrest in these cell types is redundant. If cellular senescence is only defined as a stable cell cycle arrest, all post-mitotic cells should be considered senescent, but that is not the case. Moreover, there are reports of post-mitotic cells acquiring senescence-like features besides the stable arrest [22,23]. Data from our laboratory demonstrates that iPSC-derived cardiomyocytes

(which are post-mitotic) increase expression of p16, p21, secreted factors and also present mitochondrial dysfunction and functional impairment when exposed to senescence-inducing doxorubicin (Ovchinnikova *et al*, in preparation). Is that cellular senescence? To make it more complicated, those cardiomyocytes were positive for SA- β gal even before the treatment with doxorubicin. Were those cells senescent before the treatment started? Then how can the functional impairment and the secretory phenotype occurring after doxorubicin treatment be explained? Cellular senescence goes beyond the cell cycle arrest and the appearance of other features is what turns a “stably arrested” cell into a senescent one, the question is to set up the rules or boundaries on when a cell can be said to be senescent.

Cellular senescence is also considered a stress response and that is, in part, what differentiates it from the post-mitotic state. But then, what about developmental senescence which is also programmed [24,25]? Or senescence caused by analogs of p16 that do not induce any measurable damage (Chapter VI)? The use of markers becomes of great importance to answer those questions and this is where the senescent field is falling short. Cellular senescence needs a clearer, more consistent and measurable definition, even if some of the phenotypes that are now considered senescence-associated have to be omitted. We already proposed the test of the main pathways involved in cellular senescence as a way to classify cells as senescent or non-senescent. However, as discussed in Chapter VI, from our knowledge in the cancer field we know that there are certain features that all samples share (incontrollable proliferation in the case of cancer and cell cycle arrest in the case of cellular senescence) and still, not every cancer or senescent program is the same. Consequently, not every cancer uses the same markers or treatments [26] and it may be that different senescence-associated programs will also require especial markers and treatments.

Does it make sense at all to look for universal markers of cellular senescence?

Defining cellular senescence does not necessarily imply that cells need to present every senescent marker that there is. It may be that cellular senescence is just a general name given to different programs and we need different markers for each of them. Certain features need to be fulfilled in every senescent population, but it is becoming clear that different senescence-associated programs use specific molecular effectors to achieve the same end goal. Moreover, we and others have exposed the dynamicity of the senescent phenotype and of its secretome [11,27,28], so that testing for a particular marker might be too restrictive. As a way to deal with the heterogeneity of the senescence-associated programs, we and others have proposed the use of multiple markers to validate cellular senescence [1,10–12]. How many markers must a cell have to be considered senescent? In practice, the test of multiple markers would probably be an intensive job and, as mentioned in Chapter II, there is no consensus assay for some of those hallmarks. We proposed a list of pathways, all discovered through transcriptional expression, but the specific assays and the number of positive tests that a sample/cell needs to fulfill to be considered senescent requires further investigation.

As mentioned, another possibility and one that I personally support, is the use of different markers for each senescence-associated program. The challenge would be to determine the source of senescence induction so the right markers can be used. In cell

culture or even in animal experiments where the source of damage is known, this is quite straight-forward, although even then a fraction of the population may be primary senescent and another a result of paracrine senescence [29]. Similarly, in certain situations in humans, such as chemotherapy-induced senescence, the stress causing induction of senescent cells is clear [30]. However, conditions such as naturally occurring aging, the source of damage is highly variable and heterogeneous. If markers for different senescence-associated programs were discovered and regularly used, it would be possible to determine the main source(s) of cellular senescence. Testing for different cell types would be more challenging at this moment, when most of the literature uses fibroblasts and few other cell types. Importantly, there is some evidence that only a subset of senescent cells in a population is the main producer of cytokines and chemokines that constitute the secretory phenotype [13]. Markers for that subpopulation would be of special interest.

More extended projects would help address many of the questions stated in this discussion. Moreover, this thesis largely focused in the transcriptome of senescent cells. The study of other levels of gene expression such as epigenetics or protein levels, will probably shed light in other possibilities and also in new challenges. All this ambiguity in the definition of cellular senescence and the heterogeneity that we have discussed in the whole thesis leads us to a third question.

Do we know all the sources of heterogeneity in senescent cells?

In Chapter IV we studied two of the most obvious sources of heterogeneity at the population level: the senescence-inducing stimulus and the cell type subjected to it [11]. Already at this level we saw a high amount of inter-population heterogeneity in the senescent transcriptome, particularly cell type-dependent. However, for senescence-associated markers the intra-population heterogeneity also hinders the recognition of senescent cells. In tissues or *in vivo* it is even more difficult to measure such markers. Many of them are not specific, so the presence of a certain marker is often not strong enough evidence of cellular senescence. Co-staining or simply co-expression of multiple markers might not be adequate either if we consider that not all senescent cells will express all the markers (Chapter VI and [13]). Moreover, the same tissue is composed by different cell types, some exposed to the same extrinsic damaging stimuli and some with their particular intrinsic stimuli and immersed in a particular microenvironment. It cannot be assumed that a certain marker or the co-appearance of a couple of them is a proof of senescence without taking into account the context.

To our knowledge, no study has assessed intra-population heterogeneity in complex tissues so far. Moreover, most studies assessing the intra-population heterogeneity in cultured cells have focused on one or few markers at a time [31–33]. Only a recent article [13] and our own work in Chapter VI have described the heterogeneity of multiple markers simultaneously. Despite this, the sources of this heterogeneity have been overlooked.

One possible source of intra-population heterogeneity of senescent cells is the cell cycle stage at which each individual cell receives an insult. For instance, cells receiving an insult in G1, right before they go through the G1/S checkpoint, probably react very different from cells that receive the damage in the G2/M phases [34–36]. Few experiments have synchronized cells before inducing cellular senescence and showed

the differential response of cells according to cell cycle stage [36,37]. This source of heterogeneity is more relevant in unsynchronized cultures and it probably account for part of the intra-population heterogeneity observed. Studying this cell stage-dependent senescence-associated program would be particularly helpful to identify senescent cells in complex tissues, particularly in ones containing large amounts of dividing cells. Moreover, the epigenetic landscape of each cell at the moment of receiving an insult may account for the different response to it [38], a phenomenon that, even if it is partially related to cell type and cell cycle stage [39,40], is not well studied in the context of cellular senescence.

Another source of heterogeneity are the specific organelles, proteins or even the genomic loci receiving the damage. Although this might be a stochastic process, the consequences are not. For instance, it is known that DNA double-strand breaks can directly lead to cellular senescence [41]. However, not every break is equally damaging: a break in a repetitive region is more difficult to repair than in a non-repetitive one [42,43]. It is probable that different types of damage follow a distinctive path to cellular senescence. Most of the senescence-inducing stimuli used now affect multiple cell functions. Studying every possibility of damage in the DNA and in any particular molecule would be impossible. Yet, understanding the extent to which this heterogeneity accounts for the lack of success of current markers would help to choose more appropriate ones or the right combination of them.

A limiting factor on the study of intra-population heterogeneity derives from technical obstacles of the methodologies used to study it. The continuous advancements in the single-cell field will lessen these issues.

Why is it important to have appropriate markers of cellular senescence if current senotherapies work?

Despite our feeble definition of cellular senescence and the lack of proper markers, we are able to successfully target senescent cells [44]. In recent years the link between cellular senescence and aging became directly evident thanks to two genetically-modified mice in which p16-positive (senescent) cells can be tagged and continually removed [45,46]. These mice age healthier, presenting less age-related diseases than the control groups in which p16-positive cells are not removed [6,7,47–49]. Such experiments in humans would be unethical, so drugs or therapies targeting senescent cells and mimicking those effects are needed. Multiple senotherapies have been developed in the last years, all of them either eliminating senescent cells (senolytics) or targeting some of the negative aspects of it [9]. However, current senolytics use only a specific aspect of cellular senescence as a target. For instance, dasatinib targets the dependent-receptors EFNB (ephrins), a branch of the anti-apoptotic machinery [53]. Recently, it was published that HSP90 inhibitors have a senolytic effect by disrupting the PI3K/AKT anti-apoptotic pathway [54]. ABT-263 and ABT-737 are two well-known senolytics that inhibit certain members of the BCL2-family of anti-apoptotic proteins [50–52]. This variation in target is probably necessary since senescent cells are known to use anti-apoptotic mechanisms, but not all senescent cells use exactly the same molecular effectors [55]. Furthermore, in Chapter IV, we found BCL2L2 as one of the genes in the core signature of senescence, thus demonstrating its universality. ABT-263 and ABT-737 actually target BCL2L2 (together with other members of the BCL2-family) [50,51].

However, senescent cells are not the only ones using these anti-apoptotic pathways, so that these therapies have major side effects. Specifically for ABT-263 thrombocytopenia has been reported as a major side effect [56]. Few other senolytics take advantage of other senescence-associated features [57].

The knowledge we could gain in the different senescence-associated programs and the markers that recognize them would help in the selection of better targets and more specific therapies. There is a possibility that certain sub-types or sub-populations of senescent cells are more damaging than others [13] and they could be the focus of therapies. It could be, for instance, that markers will help us recognize and target senescent cells producing inflammatory cytokines, while maintaining senescent cells that are beneficial in wound healing. It could also be that the senescent version of certain cell types are also particularly damaging and therefore should be the main targets. A deeper knowledge in understanding senescence-associated programs and markers will also help to monitor and evaluate the success of any type of senolytic.

This thesis is only a small contribution to the demystification of the senescence phenotype. Many aspects of cellular senescence remain unexplored and therefore the future of the field is promising and exciting.

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Chapter 7

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An abstract painting with a vibrant pink background. On the right side, there are dark, textured brushstrokes in shades of grey, black, and brown. In the bottom left corner, there are two large, rounded green shapes with dark spots, resembling cacti or succulents. Several smaller, bright orange-red spots are scattered in the lower middle area.

Chapter S

Annexes: Summaries, *Curriculum Vitae* and Publications

Summary for the lay-man

Aging is the decline in fitness that individuals suffer with time and that eventually leads to death. However, anyone can attest that different people age differently as well. Indeed, while most old humans (and animals) experience hearing loss, reduced vision, loss of strength, cardiovascular diseases, diabetes, osteoarthritis, neurodegeneration and cancer, these disorders do not occur in every individual and certainly not at the same rate. Although genetic predisposition is partly responsible for the differences observed, our “lifestyle” is also an important contributor. Exposure to sunlight, chemicals found in cigarette and pesticides, unhealthy food, lack of exercise and even the secondary effects of medicines used to treat other medical conditions can all cause damage to our cells and organs. The more exposed you are to damaging stimuli, the more damage your cells and organs get. Even though our cells have mechanisms to combat and repair this damage, occasionally the harm is big enough that it cannot be repaired. Some cells with unrepaired damage simply die, while others remain alive but with some precautions to avoid spread of the injury. These damaged cells that do not die, are called “senescent cells”. Senescent cells avoid the spread of the damage by not dividing anymore, preventing the birth of dysfunctional progeny that could become cancer. Senescent cells also send signals to neighboring ‘normal’ cells to communicate the problem and to further delimitate the damage. However, with time senescent cells accumulate in the organism, and become an obstacle for the regeneration of tissue eventually leading to dysfunction. Furthermore, the signals sent to neighboring cells, also put the immune system in permanent alert, which causes a chronic state of inflammation and eventually triggers age-related diseases. Therefore, cellular senescence is one of the main causes of aging.

The goal of this thesis was to study the main features of senescent cells. Many different damaging factors (UV-light, metabolism, pesticides, drugs) can cause senescence, but all (or most) senescent cells share some features. As mentioned, the major feature of a senescent cell is its inability to divide. Also, most senescent cells show changes in shape, structure and metabolism which can be used as “markers” for their identification. However, the shape and the metabolism are influenced by many other factors, not only senescence. Therefore, all these features are not unique to senescent cells, and finding a single or “universal” marker that unequivocally identifies senescent cells is not yet possible.

In this thesis, we have interrogated what genes make senescent cells different from “normal” (non-senescent) cells. The task is daunting because each cell has around 20 thousand genes that need to be evaluated and because senescent cells are very heterogeneous. For instance, a cell that became senescent by being exposed to UV-light (sunlight) does not look the same than a cell that entered senescence because of a drug. Similarly, a senescent cell from the lung does not look the same than a senescent cell from the skin. Yet, we found new processes and genes that are common and we discussed their potential as novel markers for cellular senescence. However, we also highlighted the variability that exists on different types of senescence and even from one senescent cell to the other. We suggested that it may be better to find markers separately for each type of senescence instead of a universal marker.

Our research is important in view of the therapeutic potential of senescent cells.

If we were able to recognize senescent cells with adequate markers, we could either eliminate them from the body or modify them to avoid their negative effects. Until now, different groups of scientists have eliminated senescent cells and they have been able to prevent the appearance of some age-related diseases in mice and even to make them live longer. However, those strategies are not yet applicable to humans or they have many undesirable side effects. Moreover, the current techniques are not completely efficient because there is much that we do not know about senescent cells. We hope that the results of this thesis and the advancements in the study of cellular senescence will help finding better therapies to eliminate senescent cells and their bad effects in the body. Preventing age-related diseases would ultimately have a positive effect in the economy and in the well-being of humans.

Nederlandse Samenvatting

Vertaling door Simone Brandenburg en Gertrud Kortman

Veroudering is de verminderde fitheid waarmee personen die ouder worden te maken krijgen en deze veroudering leidt uiteindelijk tot de dood. Echter, het is een ieder bekend dat elk individu op een andere manier het verouderingsproces doormaakt. De meeste oude mensen (en oude dieren) ervaren gehoorverlies, verminderd zicht, verminderde spierkracht, hart- en vaatziekten, suikerziekte, artrose, neurodegeneratie en/of kanker, maar dit gebeurt niet in hetzelfde tempo in de gehele populatie. Hoewel genetische aanleg deels verantwoordelijk is voor de verschillen die we zien in de populatie, de levensstijl die we onszelf aanmeten heeft zeker ook een belangrijke rol in deze. De mate van blootstelling aan zonlicht en aan chemische stoffen, zoals in sigaretten en pesticiden, ongezonde voeding, gebrek aan beweging, als ook de secundaire effecten van medicijnen voor het behandelen van andere medische condities, kunnen allen schade toebrengen aan onze cellen en organen. De meer blootstelling aan schadelijke stimuli, de meer schade wordt toegebracht aan de cellen en organen. Zelfs al hebben onze cellen een afweermechanisme om de schade te bestrijden en te repareren, soms is de schade te groot om nog gerepareerd te worden. Sommige cellen met onrepareerbare schade gaan gewoon dood, terwijl andere wel blijven leven, maar met enige voorzorgsmaatregelen, zodat verspreiding van de schade beperkt blijft. Deze beschadigde cellen, die dus niet doodgaan, noemen we ‘senescent’ cellen. De senescent cellen vermijden de verspreiding van de schade, doordat deze cellen stoppen met delen. Daarbij voorkomen ze dat de beschadigde cellen nakomelingen krijgen die disfunctioneel zijn en kanker zouden kunnen veroorzaken. Senescent cellen zenden ook signalen uit naar nabijgelegen ‘normale’ cellen om het probleem te communiceren en daarmee de schade nog beter in te perken. Echter, mettertijd stapelen de senescent cellen zich op in het organisme en ze beginnen een obstakel te vormen voor weefselregeneratie, wat uiteindelijk leidt tot disfunctie. Verder zorgen de signalen, die naar nabijgelegen cellen worden verzonden, er ook voor dat het immuunsysteem in een staat van permanente alertheid gaat verkeren, welk zorgt voor een chronische ontsteking en dit kan eventueel verouderingsgerelateerde ziekten veroorzaken.

Het doel van dit proefschrift was om de belangrijkste kenmerken van senescent cellen te bestuderen. Veel verschillende schadelijke factoren (UV-licht, metabolisme, pesticiden, medicatie) kunnen leiden tot ‘senescence’, maar uiteindelijk hebben alle (of de meeste) senescent cellen gemeenschappelijke delers. Zoals vermeld, een belangrijk kenmerk van een senescent cel is het onvermogen om nog te kunnen delen. En ook vertonen vrijwel alle senescent cellen een verandering in vorm, structuur en metabolisme, welke kunnen worden gebruikt als ‘markers’ voor hun identificatie. Echter, de vorm en het metabolisme van een cel worden ook beïnvloedt door vele andere factoren en niet alleen door senescence. Al deze kenmerken zijn daarom niet uniek voor senescent cellen en om één unieke ‘marker’ te vinden welk eenduidig alleen voorkomt in senescent cellen is nog altijd niet mogelijk.

In dit proefschrift hebben we onderzocht welke genen nu een senescent cel onderscheiden van een ‘normale’ (‘niet-senescent’) cel. Deze taak was ontmoedigend, aangezien elke cel ongeveer 20.000 genen heeft die gecheckt moeten worden en omdat senescent cellen erg heterogeen (ongelijksoortig) zijn. Bijvoorbeeld, een cel die

senescent is geworden door blootstelling aan UV-licht (zonlicht) ziet er niet hetzelfde uit als een cel die senescent is geworden door een medicijn. En zo ziet een senescent cel van een long er niet gelijk uit als een senescent cel van de huid. Toch hebben we nieuwe processen en genen gevonden die gemeenschappelijk zijn en hiervan hebben we hun potentieel beschreven als nieuwe ‘markers’ voor cellulaire senescence. Echter, we hebben ook de variabiliteit uitgelicht, die bestaat tussen de verschillende soorten van senescence en zelfs tussen de ene senescent cel en de andere. We hebben gesuggereerd dat het wellicht beter is om markers te vinden voor iedere soort senescence apart i.p.v. één ‘universele marker’.

Ons onderzoek is belangrijk wanneer we kijken naar het therapeutische potentieel van senescent cellen. Als we senescent cellen zouden kunnen herkennen met adequate markers, dan kunnen we deze óf verwijderen uit het lichaam, óf deze aanpassen, zodat hun negatieve effecten teniet worden gedaan. Tot op heden hebben verschillende wetenschappers senescent cellen verwijderd en daarmee hebben ze voorkomen dat sommige leeftijdsgelateerde ziekten in muizen verschijnen en dat deze muizen zelfs langer leven. Echter, deze strategieën zijn nog niet toepasbaar bij mensen of ze hebben onwenselijke bijwerkingen. Bovendien zijn de huidige technieken niet geheel efficiënt, omdat we nog heel veel niet weten over senescent cellen. We hopen dat de resultaten van dit proefschrift en de vooruitgang in de studie naar cellulaire senescence zal helpen met het vinden van betere therapieën om senescent cellen en hun slechte effecten te verwijderen uit het lichaam. Het voorkomen van leeftijdsgelateerde ziekten zal uiteindelijk een positief effect hebben op de economie en het welzijn van de mens.

Resumen en Español

El envejecimiento es la decadencia paulatina del estado físico de los individuos y que, eventualmente, conduce a la muerte. Sin embargo, cualquiera es testigo de que cada individuo envejece de una manera distinta. A pesar de que la mayoría de los humanos (y animales) viejos sufren de pérdida del oído, visión reducida, debilitamiento, enfermedades cardiovasculares, diabetes, osteoartritis, neurodegeneración y cáncer, estas enfermedades no aparecen en toda la población o, por lo menos, no al mismo tiempo. Aunque la predisposición genética es, en parte, responsable de estas diferencias, nuestro estilo de vida contribuye también a ellas. La exposición a la luz del sol, productos químicos del cigarro y pesticidas, comida chatarra, falta de ejercicio e incluso los efectos secundarios de medicinas usadas para tratar otras condiciones médicas pueden causar daño a nuestros órganos y células. A mayor exposición a estímulos dañinos, mayor es el daño a células y órganos. Si bien nuestras células tienen mecanismos para combatir y reparar estos daños, ocasionalmente el deterioro celular es tan grande que no puede ser revertido. Algunas de estas células que no pueden reparar el daño simplemente mueren, mientras que otras permanecen con vida pero tomando algunas precauciones para prevenir la propagación de la lesión. Estas células dañadas que no mueren son llamadas “células senescentes”. La estrategia que usan las células senescentes para evitar la propagación del daño, es dejar de dividirse, previniendo así el nacimiento de una progenie disfuncional que podría convertirse en cáncer. Las células senescentes también mandan señales a las células “normales” vecinas para así comunicar el problema y delimitar aún más el daño. Sin embargo, durante toda la vida, los seres vivos van acumulando células senescentes en sus organismos, lo que obstaculiza la regeneración de tejidos y, eventualmente, causa el malfuncionamiento de órganos. Además, las señales que las células senescentes envían a células vecinas, también ponen en alerta permanente al sistema inmune, lo que causa un estado de inflamación crónica que desencadena las enfermedades asociadas al envejecimiento. Por tanto, la senescencia celular es una de las principales causas del envejecimiento.

El objetivo de esta tesis era estudiar las características principales de las células senescentes. Muchos factores dañinos (radiación ultravioleta, metabolismo, pesticidas, drogas) pueden causar senescencia. No obstante, todas (o la mayoría de) las células senescentes comparten ciertas características. Como se mencionó, la característica principal de las células senescentes es que no pueden dividirse. Así mismo, la mayoría de las células senescentes muestran deformaciones y cambios en su metabolismo. Estas características pueden ser utilizadas como “marcadores” para la identificación de células senescentes. Sin embargo, la forma celular y el metabolismo son influenciados por muchos otros factores, no sólo por la senescencia celular. Por tanto, estas características no son únicas de las células senescentes y hasta ahora no existe un marcador único o “universal” que identifique inequívocamente a todas las células senescentes.

En esta tesis hemos investigado qué genes hacen a las células senescentes diferentes de las células normales. Este objetivo es intimidante, puesto que cada célula tiene alrededor de 20 mil genes que necesitan ser evaluados y también porque las células senescentes son muy diferentes unas a otras. Por ejemplo, una célula que se convierte en senescente por la exposición a la luz ultravioleta no es igual que una célula que se convirtió en senescente por una droga. De igual manera, una célula senescente del pulmón no es

igual que una célula senescente de la piel. Aun así, en nuestra investigación encontramos nuevos procesos y genes que son comunes a diferentes tipos de senescencia y discutimos su uso potencial como nuevos marcadores de senescencia celular. Sin embargo, también resaltamos la gran variabilidad que existe en diferentes tipos de senescencia e incluso entre una célula senescente y otra. Finalmente, sugerimos que quizá sea mejor buscar marcadores independientes para cada “tipo” de senescencia en lugar de un marcador universal.

Nuestra investigación es importante en vistas del potencial terapéutico de las células senescentes. Si fuéramos capaces de reconocer a las células senescentes con los marcadores adecuados, podríamos eliminarlas o incluso modificarlas para evitar los efectos negativos que tienen en el cuerpo. Hasta ahora, diferentes grupos de científicos han eliminado las células senescentes y han sido capaces de prevenir la aparición de varias enfermedades asociadas al envejecimiento en ratones e incluso de alargarles la vida. Sin embargo, estas estrategias no son aplicables a humanos todavía o tienen muchos efectos secundarios indeseables. Además, las técnicas actuales no son completamente eficientes porque hay mucho que desconocemos sobre las células senescentes. Esperamos que los resultados de esta tesis y los avances en el estudio de la senescencia celular ayudarán a encontrar mejores terapias para eliminar a las células senescentes y sus efectos negativos. La prevención de las enfermedades asociadas al envejecimiento tendría un efecto positivo en la economía y, sobre todo, en el bienestar de los seres humanos.

Curriculum Vitae

Alejandra Hernández Segura was born in Puebla, Mexico, on May 13th, 1988. In 2006 she obtained a full scholarship from the Fundación Jenkins to study Clinical Biochemistry at the Universidad de las Américas Puebla (UDLAP). During the last years of the bachelor and until the start of her master program she did some short stays in different diagnostic and research laboratories in Mexico and the Netherlands, she worked as an intern at the Clinic of Obesity and Diabetes from the Institute of Social Security Services for Workers of the State of Puebla (ISSSTEP) and worked as a biology/chemistry/physiology teacher in different high schools in Puebla and in a summer camp at the Center for Talented Youth from the Johns Hopkins University, USA. In 2010 she graduated from the bachelor with Magna cum laude (with honors).

In 2012 she enrolled to the top master program Medical and Pharmaceutical Drug Innovation from the University of Groningen but supported by a Mexican scholarship from the National Council for Science and Technology (CONACYT). There she performed her first research project at the European Research Institute for the Biology of Ageing (ERIBA) under the supervision of Prof. dr. Peter Lansdorp and Prof. dr. Ellen Nollen. There, she developed a method to isolate single cells from *C. elegans* adult worms and to perform single-cell DNA sequencing on them. Her second research project was performed in the department of Human Genetics from the University Medical Center Groningen (UMCG) under the supervision of Prof. dr. Cisca Wijmenga and Dr. Vinod Kumar. This project consisted on analyzing the transcriptome of activated gut lymphocytes from celiac disease patients.

Alejandra finished her master's degree with Cum laude in 2014 and she worked for six months as an invited lecturer at the UDLAP, in Mexico. In 2015 she went back to the Netherlands and started her PhD supervised by Prof. dr. Peter Lansdorp and funded by a personal scholarship from the Graduate School of Medical Sciences (GSMS) from the University of Groningen. However, after her supervisor moved to Canada one year later, she decided to switch projects. Since beginning of 2016, she was supervised by Prof. Marco Demaria, where she studied the transcriptional heterogeneity of cellular senescence. She published four articles and reviews and holds a patent related to her research. Since November 2018 she works as a Data Scientist in Intermax Datascience/Grace Systems, in Utrecht, The Netherlands.

Publications

Publications

1. Hernandez-Segura, A., De Jong, T. V, Melov, S., Guryev, V., and Campisi, J. (2017). Unmasking Transcriptional Heterogeneity in Senescent Cells. *Curr. Biol.* 27, 2652–2660. Available at: <http://dx.doi.org/10.1016/j.cub.2017.07.033>.
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5. Wang, B., Brandenburg, S., Hernandez-Segura, A., van Vliet, T., Krizhanovsky, V., Ohtani, N. and Demaria, M. (*in preparation*). Inhibition of CDK4/6 causes a p53-dependent senescent state with mild secretory phenotype.

Patent

Biomarkers for Cellular Senescence. European Patent Office: 17160165.1-1408.
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An abstract painting with a vibrant pink background. In the bottom left, there are two large, rounded green shapes with dark spots, resembling cacti or rocks. To their right, there are several smaller, bright orange-red spots. On the right side of the image, there are large, dark, textured shapes in shades of black, grey, and brown, which look like craggy rock formations or perhaps the wings of a bird. The overall style is expressive and textured, with visible brushstrokes.

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