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Review

Never-ageing cellular senescence

Müge Ogrunc *, *Fabrizio d'Adda di Fagagna*

IFOM Foundation – FIRC Institute of Molecular Oncology Foundation, Milan, Italy

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ABSTRACT

Cellular senescence was historically discovered as a form of cellular ageing of *in vitro* cultured cells. It has been under the spotlight following the evidence of oncogene-induced senescence *in vivo* and its role as a potent tumour suppressor mechanism. Presently, a PubMed search using keywords 'cellular senescence and cancer' reveals 8398 number of references (by April 2011) showing that while our knowledge of senescence keeps expanding, the complexity of the phenomenon keeps us – researchers in the field of cancer biology – fascinated and busy. In this short review, we summarise the many cellular pathways leading to cellular senescence and we discuss the latest experimental evidence and the questions emerging in the field.

1. Introduction

1.1. *The evolution of cellular senescence concept*

In the early 1960s, Leonard Hayflick reported that primary human fibroblasts isolated from embryonic lung tissues cease to proliferate after a limited number of population doublings (PD 50 ± 10).[1] This maximum proliferative lifespan of fibroblasts is still known as the Hayflick limit, and we use the term replicative cellular senescence, coined by Hayflick, to describe this cellular condition of ceased proliferation. Since then, for the last five decades, the original definition of cellular senescence has withstood the test of time and the subject has become more and more relevant in the contexts of ageing and cancer.

The loss of cell division potential and change in morphology of human lung and skin fibroblasts were primarily proposed as a model for ageing at the cellular level.[2] Later, what Hayflick described as cellular 'ageing' became equally relevant to understand the events that follow oncogene activation and lead to cellular transformation.[3,4] We now know that, in addition to reflecting the loss of normal tissue homoeostases as a consequence of ageing, cellular senescence acts as a natural tumour suppressor mechanism.

2. Pathways triggering the senescence programme

2.1. *Telomeres: The end-replication problem appears to be more complicated than the length*

Telomeres, the physical ends of linear chromosomes, are composed of a variable number of 5′-TTAGGG-3′ repeats in vertebrates.[5] These long tracts of double-stranded DNA sequences are followed by the presence of single-stranded

* *Corresponding author:* Address: IFOM Foundation – The FIRC Institute of Molecular Oncology Foundation, via Adamello 16, 20139 Milan, Italy. Tel.: +39 02 574303 325; fax: +39 02 574303 231.
E-mail address: muege.ogrunc@ifom-ieo-campus.it (M. Ogrunc).
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repeats termed as G-overhangs. This structure is crucial in terms of end-protection of telomeres, since G-overhangs can serve as a primer for telomerase and contribute to the formation of a structure called T-loop (for telomere loop) which results from the invasion of G-overhang into duplex region of telomere.[6] This structure together with a number of proteins that make up the so-named shelterin complex[7] provides a protection to chromosome ends by blocking Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) DNA repair machineries to distinguish them from DNA double strand breaks (DSBs), as well as blocking Ataxia Telangiectasia Mutated (ATM) signalling.[8–11] When telomeres shorten below a critical length they lose the functions of the shelterin complex and thus they are recognised as DSBs[12] and activate the DNA damage response pathway (DDR).[13] Shelterin protein complex binds and protects the chromosome ends from DDR. It also coordinates telomere maintenance by telomerase. Telomere uncapping that results from deregulation of components of the shelterin complex also initiates a DDR.[14] It will be interesting to investigate whether the expression, the stability or functions of individual components of the shelterin complex are altered in physiologically relevant processes. Indeed there is already some evidence of their alteration being detected in some tumours.[15]

Cellular senescence can be induced by a number of exogenous or endogenous stresses and historically telomere shortening was proposed to be the main mechanism leading to replicative cellular senescence establishment.[16] The inability of standard DNA polymerases to fully replicate linear DNA molecules causes the estimated loss of around 50 to 200 base pairs during each cycle of replication at the 5′ end of the lagging strand.[16] Hence, the end-replication problem causes gradual shortening of chromosomes at every DNA replication round and subsequent cell division, eventually leading to DDR activation at critically short telomeres.[12,13] Therefore, replicative senescence was proposed as a terminal arrest of cells with critically short telomeres lengths, thus acting as a tumour suppressive mechanism to prevent cells from being immortal. However, the exact critical length of such dysfunctional telomeres is still not fully clear, while it is clear instead that it varies widely in different species ultimately confounding our understanding of what ultimately triggers DDR activation and senescence establishment.

A causative link between DDR signalling at individual telomeres and senescence was analysed using a single-cell detection method to detect upstream DDR events and cell cycle checkpoint in HDFs at a near-senescent stage.[13] Here, a significant inverse correlation between BrdU incorporation and the presence of one or few telomere dysfunction-induced foci (TIFs) was observed. These data suggest that even a single telomere-associated DNA damage event is sufficient to induce a long-term growth arrest. As the persistence of these foci seems to be the inherent feature of senescence-inducing DNA damage, this begs the question of why this DNA damage is not repaired. Answering this question will provide the essential molecular mechanism that establishes DNA damage-induced cellular senescence and ultimately why they senesce.

The realisation that telomeres are fragile sites,[17] hard to replicate chromosomal regions, unveils an additional layer of complexity and suggests that telomere shortening and DDR activation may occur also during standard telomeric DNA replication. Ataxia Telangiectasia and Rad3-related protein (ATR) involved in resolving replicational stress has an important role in the suppression of telomere fragility and recombination.[18,19]

A recent study on DNA adducts has indicated that telomeres are more sensitive to the formation of UV-induced cyclobutane pyrimidine dimers (CPD).[20] Telomeres somehow tolerate these CPDs and nucleotide excision repair is prevented at telomeres.[20] The rate of oxidative DNA damage has also been correlated with the amount of telomere loss during successive rounds of DNA replication.[21,22] A study explored this further in a mouse model carrying a deletion of the 8-oxoguanine DNA glycosylase (Ogg1) gene, involved in the elimination of oxidised bases via base excision repair. As telomeric repeats, being rich in guanine, are prone to oxidative damage, Ogg1-deficient mice have increased oxidative guanine lesions in telomeres *in vivo* and display accelerated telomere shortening under high oxygen conditions (20%) without impacting on the enzymatic activity of the telomerase.[23] A further impact of the oxidative stress on telomere regulation is the nuclear export of telomerase, which has been shown to be induced by high levels of oxidative stress.[24]

Therefore, it appears that additional events, and not only progressive telomere shortening caused by the inherent limits of the DNA replication machinery, may contribute to telomere shortening or DNA damage generation at telomeres, with consequent replicative senescence establishment.

2.2. *Oncogene activation – opening Pandora's box*

Upon exposure to various oncogenic stimuli normal mammalian cells can respond by activating DNA damage response (DDR) pathway. This response may commit cells to programmed cell death (apoptosis) in some cases[25] or may induce them to enter cellular senescence.[26] Hence, in addition to the first observation of cellular senescence, which was the *in vitro* replicative exhaustion of human fibroblasts, other diverse forms of cellular stress also lead to a cell condition in which the cells remain alive and metabolically active, yet irreversibly arrested. Oncogene-induced cellular senescence (OIS), also called premature senescence, was first observed in normal fibroblasts by the ectopic overexpression of oncogene H-RAS^{G12V} expression.[26] This apparently telomere-independent type of senescence is shared with other mutant forms of RAS family proto-oncogenes and its downstream effectors. Mutations leading to the activation of oncogenes in K-RAS^{G12V},[27] BRAF^{V600E},[28] or inactivation of the tumour suppressors, such as in PTEN[29] and NF1[30] can trigger cellular senescence *in vitro* and *in vivo* in a variety of host tissues. Further studies into these key observations lead to the proposal of DDR activation as a critical barrier to tumourigenesis, by forcing cells to stop their aberrant proliferation.[31,32] At the molecular level, OIS is regulated by two major tumour suppressor pathways, p53 and Rb. However, the requirements and dependency of these key players of both pathways, and their relative contributions seem to be dependent on the type of stress and cellular context.

Although the activation of DDR by oncogene-induced hyper-replication has been shown by some groups as the key mediator of oncogene-induced senescence,[31,32] other reports underline the independency of DDR in terms of senescence induction by some leukemogenic fusion proteins, such as BCR-ABL and CBFB-MYH11.[33] Here, p38 kinase pathway followed by p16 induction plays a crucial role in the commitment to senescence. These data indicate that multiple pathways may be involved in senescence. While involvement of the p53 axis in human fibroblasts through the activation of DDR pathway has been well-demonstrated, in murine cells both ARF and p53 appear to be important in OIS establishment as inactivation of either p53 or ARF bypasses senescence in murine systems. Thus different mechanisms of senescence establishment and maintenance may make different contributions in different species. Murine models remain one of the best systems to prove causality of the mechanisms that in human samples can often only be proposed. However, mice retain their own specificities that occasionally must be taken into account. As an example, it is possible that the contribution of ARF in OIS may obscure the role of DDR in senescence control. Indeed, ATM in some murine models seems to display a more limited role in Ras-induced senescence compared to human systems.[34] Intriguingly however, ATR suppression seems to synergise with endogenous levels of oncogenic k-Ras in the induction of a variety of tumours.[35] So, it is possible that ATR is an important genetic determinant of senescence establishment and tumour suppression in mice.

The role of DDR in OIS in mouse model was probably most formidably challenged in a mouse model in which senescence is triggered by PTEN loss.[36] In this system, while inactivation of single allele triggers enhanced proliferative rates, inactivation of both alleles determines abrupt senescence establishment. This occurs in the absence of DNA replication. Most importantly, DDR activation, mainly the ATM branch, is below detection. Crucially, ATM inactivation fails to rescue senescence. Although at face value these results seem to indicate that indeed OIS can be established in the absence of DDR signalling, and seem to challenge the causative role of DDR genes in OIS control, it is worth considering that ATR, rather than ATM, is likely to be the most acutely sensitive DDR kinase and may be engaged by a very early DNA replication origin firing event (which may expose DNA replication origins and single-stranded DNA even in the absence of actual DNA replication) caused by a strong mitogenic event such as PTEN loss. This hypothesis is consistent with the observation that PTEN-loss induced senescence may be rescued by a TOR inhibitor (rapamycin). Such a compound has recently been demonstrated to counteract ATR signalling in yeast.[37] We thus propose that the present evidence does not yet exclude the possibility of the engagement of some branches of DDR pathways in PTEN-loss induced senescence.

A hallmark of senescent cells is the condensation of chromatin. OIS cells display senescence-associated heterochromatin foci (SAHF), which have been proposed to repress proliferative E2F-target genes[38] involving lysine 9 methylation of histone H3 and complex formation with HP1 at their promoters. Initial work linked SAHFs primarily to p16/pRB functions, later studies have shown that the efficiency of SAHF formation can be dependent on p53.[39] What comes as a surprise is a later stage event in SAHF development, the translocation of HIRA to PML bodies, which has been suggested to be neither p53 nor RB dependent.[39] We have recently observed that SAHF are markers of oncogene activation (and likely oncogene-induced DNA replication stress) rather than specific markers of the senescence conditions. Indeed both cultured cells and human tumours can proliferate and display proliferative markers despite SAHF formation.[40] This suggests that SAHF may have additional, still unanticipated functions, independent from their ability to control proliferation and expression of proliferative genes as initially proposed.[38]

The senescence condition is also associated with the activation of the expression of a number of inflammation-associated genes.[41–43] Indeed, senescent cells have a robust secretory activity, known as a senescence-associated secretory phenotype (SASP), which has been put in relation to persistent DDR signalling.[44] Senescent cells secreting matrix metalloproteinases and inflammatory cytokines alter the surrounding tissue structure and some of them, such as interleukin-6 (IL-6) and IL-8, can reinforce the senescence phenotype in an autoparacrine manner,[42,43] while at the same time also stimulate their clearance by the immune system.[45] This, however, is at odds with the demonstrated role of SASP in stimulating the proliferation of transformed cells.[46] Probably, these observations can be reconciled in the 'senescent cells: good citizens bad neighbours' paradigm proposed by Judith Campisi.[41]

2.3. *Reactive oxygen species – a cause or a consequence?*

Reactive Oxygen Species (ROS) have been linked to cellular ageing since the postulation of 'The Free Radical Theory of ageing',[47] based on the idea that free radicals may cause damage, including DNA damage, leading to mutations, cancer and ageing. The production of ROS is an inevitable biochemical consequence of oxygen metabolism, which is essential for the life of aerobic species. Antioxidant systems, via both enzymatic (superoxide dismutases, catalases, peroxidases) and non-enzymatic (small molecules like vitamin C, glutathione) defenses, maintain a controlled balance against oxidative stress within the cell by conversion of such oxidants into harmless more reduced molecular species. It has been proposed that both the rates of telomere shortening and replicative senescence can be modulated by simply modifying the amount of oxidative stress,[48] which leads to DNA breaks accumulating at telomeres.

ROS have been suggested to induce cellular senescence by causing direct oxidative DNA damage.[49–51] However, ROS are also known to positively modulate cell proliferation and act as second messenger molecules involved in mitogenic signal transduction.[52,53] As an example, NAPDH oxidases are membrane-associated enzymes that produce superoxide ($O2^{-}$) and/or hydrogen peroxide (H_2O_2), implicated in regulation of cytoskeletal remodelling, gene expression, proliferation, differentiation, migration and cell death.[54–56] Superoxide is chemically one electron reduced form of oxygen. The chemical conversions of highly reactive superoxide species to a less reactive but more soluble hydrogen peroxide make it easier to

penetrate through membranes quickly working as a secondary messenger signalling molecule.[57]

Depending on the concentration, exogenous H_2O_2 was shown to affect positively or negatively cells' growth and cell cycle kinetics.[58] Growth factors stimulate the production of ROS[59] and expression of oncogenic Ras also has been demonstrated to induce ROS generation.[52] Indeed, H_2O_2 can mimic the activity of growth factors.[60] The dual role of ROS in DNA damage inducers and mitogenic mediators is further complicated by the recent indication that ROS control DDR signalling.[61] Indeed, using a systems biology approach, it was shown that there is a dynamic feedback loop, which is triggered by DDR and p21, leading to mitochondrial dysfunction and ROS production in order to reinforce DDR activation.[62] Oxidative stress can be additionally modulated also by cellular genes such as the *seladin-1*, an oxidative stress sensor and a regulator of p53, contributing to p53-mediated Ras-induced senescence.[63] Lately, DDB2 joined the list of genes induced by ROS leading to OIS by repressing the antioxidant system and reinforcing persistent ROS accumulation within the cells.[64] These studies show that ROS may be instrumental to trigger p53-dependent OIS by regulating gene expression and functions rather than merely generating DNA damage. However, the precise mechanism and downstream paths will need further investigative efforts. Oxidation of nucleotide pools by ROS has also been recently proposed to control senescence induction.[65]

Apparently, ROS is a mediator of OIS and participates both in the initiation and further maintenance of the senescence status of the cells. However, there are many unanswered questions. Which is the biologically relevant source of ROS in OIS? Does the source of ROS that is activated upon oncogene activation and the one that locks the cells in the senescent state remain the same? Most importantly, how do ROS impact on cell-cycle progression?

A limitation to the study of ROS in cells is our ability to detect and measure ROS and to monitor their targets. Most of the techniques to visualise or quantify ROS are limited by several restrictive factors, one of which is their short half-life. Moreover, most of the probes used for detection or quantification also promote further free radical production increasing background noise to signal ratio.[66] Fortunately, the emerging studies in the field of live imaging and the usage of specialised reporter probes, such as reduction–oxidation sensitive GFP,[67,68] may help us to get more insight information about how ROS are regulated within cells or whole animals.

3. Conclusions and perspectives

From the discovery of cellular senescence in cultured cells to the observation of its *in vivo* accumulation in various human pre-malignant lesions,[69] there is mounting evidence suggesting that senescence is a powerful natural anti-tumour mechanism. However, the proper clinical use of therapy-induced 'accelerated senescence'[70] both in terms of prevention, and progression, or recurrence of human cancers is still not completely understood. PTEN-loss-induced cellular senescence (PICS) has been proposed to provide an alternative 'pro-senescence' therapy approach.[36] Combination of pharmacological inhibitors against PTEN and Mdm2 may promise a novel cancer therapy. However, the cautionary marks that need to be considered are that the proposed Mdm2 inhibitor and p53 activator Nutlin3 may, reportedly, trigger a DNA damage response upon treatment in cancer cells by slowing DNA repair.[71] Thus, while the activation of DDR by Nutlin3 may lead to a positive feedback loop to elevate p53 activity, it may also result in further mutagenic events.

The mechanisms underlying the bypass of senescence response in the progression of tumours as well as the identification of multiple biomarkers in tissues will pave the road for successful clinical decisions. In a recent study, researchers introduced a 'senescence scoring', combining a DNA damage associated and a modified secretory senescence signature as a network of senescence-associated gene interactions rather than individual identified biomarkers.[72] Therefore, they were able to identify differential expression of damage associated and secretory senescence pathways in a context-dependent manner. Such bioinformatic analysis could improve our understanding in tumour prognosis or response to the treatment.

On the other hand, recent studies reveal a dark side of cellular senescence, which is associated with the secreted inflammatory factors, and may alter the microenvironment in the favour of tumour progression.[73] Therefore, it would be beneficial to modulate the SASPs.

DDR appears to be instrumental not only in the establishment, but also in the maintenance of several aspects of the senescence programme (Fig. 1). Since widely used traditional cancer therapy relies on destruction of tumours by cytotoxic treatment, understanding the details of OIS within the context of DDR signalling may provide us invaluable information for translating our basic research knowledge into successful clinical outcomes. According to the three-stage carcinogenesis model proposed previously for Ras-induced tumours using a dose-dependent mouse model, low levels of Ras activation promote cellular proliferation and are neither sufficient for cellular transformation nor senescence. The second stage leads to increasing the levels of Ras, and turns hyperplasic lesions to the oncogenic threshold signal, which activates DDR providing senescence barrier[74] The third stage is the bypass of senescence as a result of inactivation of tumour suppressor pathways (Fig. 2). In line with this model, two recent studies showed that the p53- a well-known downstream target of DDR pathway- is activated only when oncogene activation signalling reaches a critical threshold in these activated-kras mice model settings.[75,76] Hence, restoring p53 has no effect in benign tumours because the activity is not enough to engage the p53 system. However, in the later stage malignant tumour cells, reactivated p53 eliminates cells. Previous analysis of physiological levels of expression of $kRas^{G12D}$ mouse models questioned the presence of OIS as an artefact of kRas overexpression.[77] However, two new studies demonstrated that senescence does occur in MEFs expressing physiological levels of oncogenic kRas in mice, either in the absence of WT1[78] or suppression p16 tumour suppressors[79] in lung and pancreatic mouse models, respectively. At low levels of oncogenic signalling, either the signal amplification of the oncogene or the loss of negative regulators of the oncogene is [77] necessary for the development of the senescence phenotype. Combination targeted therapy, which keeps the senescence

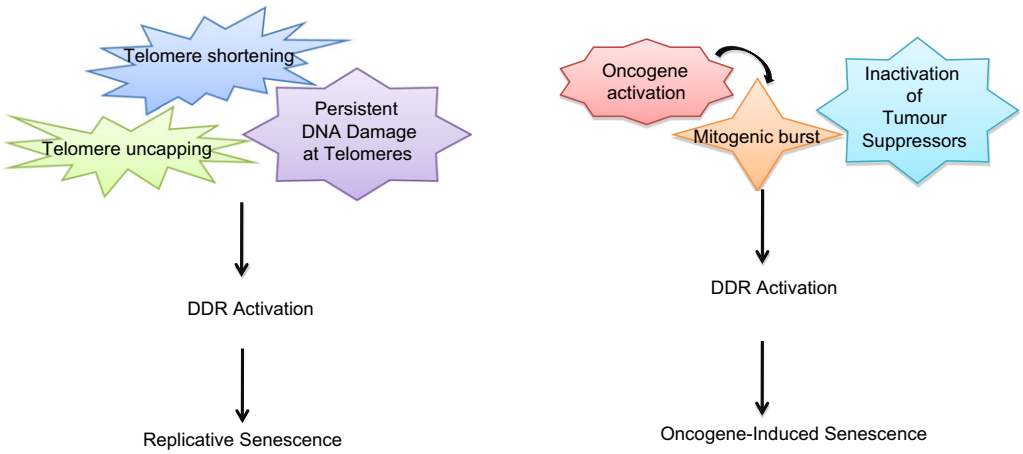

Fig. 1 – Main roads to cellular senescence. While telomere maintenance is a critical regulator of replicative senescence, multiple pathways can lead to DDR activation and oncogene-induced cellular senescence.

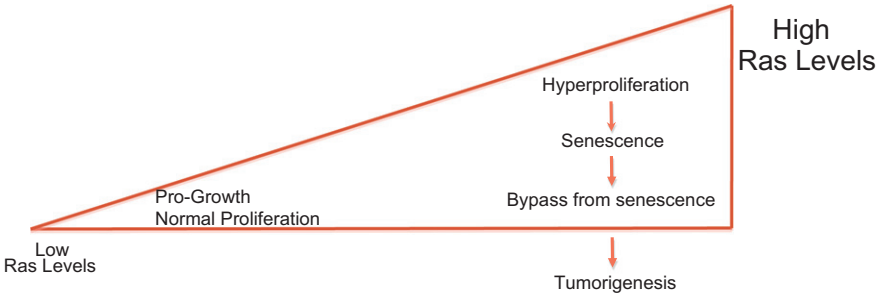

Fig. 2 – Dose-dependent oncogenic signalling outcomes. While acquired Ras mutations are pro-growth signals that promote proliferation, the level of activation is an important predictor in terms of the outcome *in vivo*.

associated secretory phenotype under control and restores tumour suppressors, could be a promise for future.

Conflict of interest statement

None declared.

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