Multiple facets of p53 in senescence induction and maintenance

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More than half a century ago, cellular senescence was first reported by Hayflick and Moorhead¹ as a durable cell cycle arrest of cultured human fibroblasts after a defined number of population doublings. The number of population doublings of normal cells in culture, known as the Hayflick limit, depends on the species used to establish the cell culture. In addition, this number is related to a positive correlation with the life span of the animals,² with higher limits observed in cultures derived from long-lived animals. Taken together, these results suggest a strong link between a limited proliferative capacity in culture and the processes observed in organismal aging. Although the physiological importance of cellular senescence in organismal aging has long been unclear, it should be noted that a recent study successfully identified senescent cells in aging tissues of humans.³ Senescence biomarkers associated with aging and its related pathology are easily detectable in human tissues in vivo.⁴–⁸ For example, the number of cells positive for senescence-associated β-Gal is markedly increased in atherosclerotic lesions of the coronary arteries⁹ and in benign prostatic hyperplasia.¹⁰ In addition, the number of senescent fibroblasts exponentially increases with age in skin tissues of baboons.¹¹ The essential role of senescence in aging and age-related disorders is further supported by the observation that clearance of p16-positive senescent cells delays aging-associated disorders and extends lifespan.¹²,¹³

In addition to organismal aging, cellular senescence is also regarded as a tumor suppression mechanism, functioning in this role by limiting proliferation of cells with aberrant DNA structures in vivo. Precancerous tissues show evidence of senescence, and this is suppressed during cancer progression.⁷,¹⁴–¹⁷ This pattern has been generally observed in multiple tumor types.⁷,¹⁴,¹⁶ A DNA damage response (DDR) to DNA double-strand breaks (DSBs), one of the major causes of senescence, is activated in precancerous lesions but not in cancers, although evidence for the presence of DSBs was found in both precancerous and cancerous lesions.¹⁴,¹⁵,¹⁸ These observations suggest that senescence functions as a tumorigenesis barrier in human precancerous lesions.

Permanent cessation of cell proliferation is a hallmark of senescent cells, but these cells also show specific characteristics,¹⁹,²⁰ and recent observations have extended the role of senescence to biological processes other than cancer and aging, such as development²¹ and tissue repair.²² Senescent cells secrete a diversity of growth factors and pro-inflammatory cytokines, known as the senescence-associated secretory phenotype (SASP). These factors are pleiotropic and induce various local activities, such as recruitment of inflammatory cells and alterations of the tissue microenvironment and likely act as a double-edged sword either facilitating or inhibiting tumorigenesis (Fig. 1). Considered collectively, although the
profound role of senescence in diverse biological processes including cancer is evident, the molecular basis underlying the induction of senescence and maintenance of senescent phenotypes has remained elusive. We will discuss this subject in the present review.

Senescence-inducing stimuli

Senescence is now believed to be induced by a diversity of stimuli. The limited proliferative potential of animal cells is generally well-understood to be a consequence of telomere shortening.

In addition to canonical DNA damage, oncogene activation or DNA replication stress by overexpression of cyclins also triggers senescence induction. For example, expression of oncogene-mutated Ras (Ras12) in primary fibroblasts results in p53- and p16-dependent permanent cell cycle arrest, a phenotype morphologically and functionally indistinguishable from replicative senescence.

Oncogene activation likely results in reactive oxygen species or in hyperactivation of DNA replication, leading to a DDR, and consequently, p53 activation.

Retinoblastoma 1 (pRb) inactivation mediated by p16 is also known to ensure durable cell cycle arrest, but is unlikely to be regulated by a canonical DNA damage response. Rather, it is stimulated by other stress mechanisms including the p38–MAPK pathway. In contrast, p16 is markedly suppressed by Polycomb-group genes such as Bmi-1 whose deficiency results in premature senescence and whose overexpression allows for immortalization. Collectively, the p16-pRb pathway can function in most forms of senescence either alone or in combination with the p53–p21 pathway in a manner dependent on the stressor or cell type.

Mechanisms of senescence induction

A prevailing model suggests that all senescence-inducing stimuli ultimately activate a DNA damage response, which in turn activates both checkpoint kinase–p53 pathways and the p38–MAPK pathway. Activated p53 transcriptionally upregulates p21, which in turn suppresses cyclin-dependent kinase 2 (Cdk2)-mediated pRb inactivation, consequently preventing S-phase entry. The p38–MAPK pathway upregulates p16, which prevents Cdk4- or Cdk6-mediated pRb inactivation. This model therefore proposes that in senescent cells, the cell cycle is arrested at G1 through pRb-mediated inhibition of E2F-dependent transcription. Although the observation that both p53 and pRb family pocket proteins are essential for senescence induction supports this model, the fact that mere activation of p53 and pRb are insufficient for senescence induction argue against it. Therefore, the specific mechanisms and the phase at which senescent cells exit the cell cycle are as yet unclear.

To determine at which phase senescent cells exit the cell cycle, we first used fluorescent ubiquitination-based cell cycle indicator-based live-cell imaging analysis during senescence induction. Interestingly, most human diploid cells treated with several senescence-inducing stimuli showed degradation of the S/G2 phase indicator, and accumulation of the G1 phase indicator without entry into mitosis. These results suggest that cells treated with senescence-inducing stimuli undergo mitotic skipping and show tetraploidy at G1 phase before entering into durable states of proliferative arrest. Similarly, p21-mediated inhibition of Cdk1 and Cdk2 at G2 phase was proposed to prematurely activate the anaphase-promoting complex/cyclosome

Fig. 1. Antagonistic roles of senescent cells in tumorigenesis in vivo. In response to various stimuli such as replicative stress, oncogenic activation, DNA damage, and oxidative stress, normal cells undergo senescence. Senescence has long been considered to function as an antitumorigenesis barrier in human precancerous lesions. This barrier is likely erected by inducing durable cell cycle arrest in cells with tumorigenic potential, activation of immune checkpoints, and ECM remodeling. However, it has now become apparent that senescent cells secrete various pro-inflammatory cytokines and growth factors, namely the oncogenic senescence-associated secretory phenotype, which promotes chronic inflammation, angiogenesis, catastrophic tissue homeostasis, and even invasion and proliferation of cancer cells. NK, natural killer.
(APC/C)\textsuperscript{Cdh1} to degrade various APC/C substrates, resulting in long-term growth arrest at G\textsubscript{2} phase in the response to DNA damage\textsuperscript{(37,38)}. In addition, the 4N genome is also apparent in multiple cell types including fibroblasts passaged extensively in vitro as well as in p16\textsuperscript{Rb44A}-expressing satellite cells collected from skeletal muscle of aged mice\textsuperscript{(39,40)}. Very interestingly, we also found that human melanocytic nevi with BRAF mutations, a typical feature of senescence in vivo\textsuperscript{(41)}, were tetraploid G\textsubscript{1} cells, and these cells presumably resulted from mitotic skipping in vivo\textsuperscript{(36)}. We then attempted to elucidate the underlying mechanism. We first explored changes in the expression of the proteins involved in regulation of G\textsubscript{2}/M transition. Surprisingly, all the proteins required for mitotic entry that were tested had almost completely disappeared at a point that coincided with mitotic skipping, suggesting that a loss of mitotic proteins is the major cause of cells evading mitosis. This loss of mitotic regulators as well as mitotic skipping are dependent on functional p53. Most notably, we found that only transient expression of p53 at G\textsubscript{2}, but not at G\textsubscript{1} or S phase, is sufficient for a mitosis skip and subsequent induction of senescence. Interestingly, transient induction of p21 at G\textsubscript{2} failed to induce senescence, although it prematurely activated APC\textsuperscript{Cdh1} which degrades various proteins involved in mitotic initiation at G\textsubscript{2} phase, functioning as a factor in mitotic skipping as reported previously\textsuperscript{(37,38)}. In addition, p53 suppressed transcription of mitotic regulators through inactivation of pRb family pocket proteins (pRb, p107, and p130)\textsuperscript{(42)}. Finally, transient expression of both constitutively active forms of cadherin 1 CDC20 homologue 1 (Cdh1) and pRb at G\textsubscript{2} induced mitotic skipping and senescence independent of p53. Taken together, we conclude that activation of p53 at G\textsubscript{2} is a critical determinant of senescence induction through its premature activation of APC\textsuperscript{Cdh1} and inactivation of pRb family pocket proteins (Fig. 2).\textsuperscript{(37)} As p53 activation in p53-deficient tumor in vivo can produce complete tumor regression through induction of senescence and their clearance by immune responses\textsuperscript{(43)}, collaborative activation of both APC\textsuperscript{Cdh1} and pRb pathways in cancer cells might be a cue to develop an innovative therapeutic method for p53-deficient cancer.

**Mechanism of senescence maintenance**

A recent body of evidence has led to the proposition that senescence is a dynamic and multistep process\textsuperscript{(44)}. The properties of senescent cells gradually evolve during each process, although durable arrest of cell proliferation is an essential feature. The evolving of senescence involves profound transcriptional changes. Implicated in these changes is a conspicuous subset of genes involved in the SASP. A change in the expression level of p53 during the senescence process is likely a critical event in evolving such properties because p53 has antagonistic effects on the SASP at least in part by suppressing the activity of p38-MAPK, which is required for nuclear factor-\(\kappa\)B activation\textsuperscript{(45)}. This notion is supported by the observation that p53\(^{+/−}\) mouse embryonic fibroblasts enter into a senescence-like state in spite of loss of the residual wild-type allele during long culture\textsuperscript{(46)}, suggesting that p53 might not be required for the evolving of senescence once cells skip mitosis. These findings suggest that establishment of an SASP requires coordinated suppression of p53 during the senescence process.
We found that F-box only protein 22 (Fbxo22), a less-characterized F-box protein, is highly expressed in senescent cells based on microarray analysis.\(^{(47)}\) The induction of Fbxo22 on exposure of a cell to senescence-inducing stimuli is p53-dependent but occurs at a relatively late phase of senescence. The SCF\(^{\text{Fbxo22}}\) complex was found to ubiquitylate p53 in vitro. In addition, Fbxo22 depletion and overexpression in cells resulted in an increase and decrease in the level of p53, respectively. Although most F-box proteins are reported to interact with substrates through a phosphorylation-dependent degron,\(^{(48)}\) Fbxo22 interacts with p53 through a carboxyl-terminal domain (CTD) on the p53 protein.\(^{(49-51)}\) In a phosphorylation-independent manner. Conversely, Fbxo22 binds to p53 and lysine-specific demethylase 4A (KDM4A) through its FIST-N and FIST-C domains, respectively. These findings prompted us to speculate that Fbxo22 acts in concert with KDM4A to couple the demethylation and ubiquitination of p53 for a selective degradation. Hence, the depletion of Fbxo22 specifically would increase the level of K370-dimethylated p53 but not that of the acetylated version. Importantly, Fbxo22 knockout mice were smaller with almost half the body weight of their control littermates, reminiscent of the phenotype of knockout mice for Skp2, an E3 ubiquitin ligase for CDK inhibitor p27.\(^{(52)}\) Analyses of the p53 protein level of these mice showed that p53 was markedly accumulated in all tissues tested. Considered together, we conclude that the SCF\(^{\text{Fbxo22}}\)–KDM4A complex is a novel bona fide E3 ubiquitin ligase for methylated p53. This SCF\(^{\text{Fbxo22}}\)–KDM4A-mediated degradation of methylated p53 is necessary for SASP as well as p16 induction in durably arrested cells (Figs 3,4).

Fig. 3. Schematic representation of SCF\(^{\text{Fbxo22}}\)–lysine-specific demethylase 4A (KDM4A)-dependent degradation for methylated (Me) p53 in senescence. F-box only protein 22 (Fbxo22) is highly expressed in senescent cells in a p53-dependent manner. SCF\(^{\text{Fbxo22}}\) forms a complex simultaneously with p53 and KDM4A through FIST-N and FIST-C domains on Fbxo22. SCF\(^{\text{Fbxo22}}\)–KDM4A-mediated ubiquitylation (Ub) and degradation of methylated p53 is essential for the induction of senescence-associated secretory phenotype (SASP) and p16 senescent cells.

Fig. 4. Proposed model for induction and maintenance of senescence by fine-tuned regulation of p53. On activation of a DNA damage response by a senescence stimulus, the level and activity of p53 abruptly increase at G2 phase, leading to a mitosis skip and subsequent durable arrest of proliferation (early stage of senescence). Activated p53 then transcriptionally induces F-box only protein 22 (Fbxo22), and the SCF\(^{\text{Fbxo22}}\)–lysine-specific demethylase 4A (KDM4A) complex ubiquitylates (Ub) methylated (Me) p53, leading to its degradation. Downregulation of p53 is essential for the induction of senescence-associated secretory phenotype (SASP) and p16 in senescent cells (late stage of senescence).
methylated p53 is competed by acetylation of p53 CTD and its interaction with PHF20 (Fig. 3). 42

Future directions
We identified a “mitosis skip” as an essential part of senescence induction, generating tetraploid G1 cells with one nucleus as a key event. However, the molecular basis underlying the irreversible cell cycle arrest found in tetraploid G1 cells remains elusive. Given that p16 is a prerequisite for suppressing DNA replication in tetraploid G1 cells, elucidation of the regulatory mechanisms underlying the transcriptional induction of p16, especially in the context of the high-order chromatin structure, could provide important clues to understanding the inability of senescent cells to proliferate in response to any form of mitotic signaling.

Several lines of evidence strongly suggest that factors controlling the level and timing of p53 activation at G2 phase play a principal role in senescence induction. In this respect, most progeroid syndrome patients show a mutation in the genes responsible for specific DNA repair systems and inefficient DNA repair likely increases the duration of G2 checkpoint activation, leading to an increase in populations of G2 cells. Therefore, investigation of the relationship between the duration of G2 checkpoint activation and senescence sensitivity in progeroid patients will provide clues for understanding the rational basis for the pathogenesis of progeroid syndromes. Although the role of senescence in aging-associated phenotypes in vivo is still not clear, involvement of p53 function in organismal aging has already been proposed by the observation that augmentation of p53 response in mice showed early aging-associated phenotypes. Thus, fine-tuned regulation of p53 activity likely plays a critical role in organismal aging and lifespan.

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Disclosing Statement
The authors declare have no conflict of interest.

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