

Critical Review

Reactive Oxygen Species as Mediators of Cellular Senescence

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Summary

Aging has often been viewed as a random process arising from the accumulation of both genetic and epigenetic changes. Increasingly, the notion that aging is a stochastic process is being supplanted by the concept that maximum lifespan of an organism is tightly regulated. This knowledge has led to a growing overlap between classical signal transduction paradigms and the biology of aging. We review certain specific examples where these seemingly disparate disciplines intersect. In particular, we review the concept that intracellular reactive oxygen species function as signalling molecules and that oxidants play a central role as mediators of cellular senescence.

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TELOMERES, SENESENCE AND OXYGEN

In the early 1960s, Hayflick and colleagues demonstrated that cultures of primary cells ceased proliferation *in vitro* after a finite number of replications (1). This concept, known as replicative senescence or the Hayflick limit, is widely believed to inhibit older, and presumably damaged cells, from continuous division. The postulated evolutionary benefit is that the Hayflick limit provides a simple mechanism to stop the transmission of accumulated mutations from mother to daughter cells. Numerous biochemical and genetic markers exist that can identify a senescent cell. These include specific alterations in cellular morphology as well as the development of an endogenous β -galactosidase activity (2). Controversy continues to exist regarding the relevance of cellular senescence to overall aging and whether or not the mechanisms underlying cellular senescence are reflective of what occurs at the level of the organism (3). With that said, our particular bias is that the understanding of cellular senescence will in the end provide important insight into

aging and that senescent cells directly or indirectly account for many of the phenotypes we routinely associate with aging. One of the most important recognized triggers of senescence is telomere attrition (4). Telomeres are specialized structures present at the end of chromosomes and evidence suggests that each round of normal replication results in their progressive shortening. In certain specialized cells such as germ cells and stem cells, the presence of the enzyme telomerase can maintain telomere length even though these cells continuously divide throughout the organism's life span. The importance of telomerase is underscored by the observation that forced expression of the enzyme in primary cells can forestall normal replicative senescence (5). In most dividing cells, telomere length gradually declines and can trigger the senescent program when the length of the telomere reaches a certain minimum critical threshold. Although for human cells, each division appears to erode approximately 100–200 base pairs of telomere length, evidence suggests that this number is not rigidly fixed. For instance, when cells are exposed to high levels of oxidative stress, the amount of telomere attrition per cell division significantly increases (6). As such, telomere length is not merely a reflection of the absolute number of cell divisions but integrates both cumulative oxidative stress and replicative history.

Telomere attrition is clearly not the only stimulus for replicative senescence since mouse cells possess very long telomeres but still undergo senescence in culture. Interestingly, a recent study demonstrated that mouse embryonic fibroblasts (MEFs) grown at near physiological oxygen concentrations (3%) do not senesce, while senescence was routinely observed when MEFs were grown under standard tissue culture conditions of 20% oxygen (7). These authors also demonstrated that mouse cells were particularly sensitive to higher oxygen conditions and accumulated significantly more DNA mutations than either human cells cultured under standard conditions (i.e., 20% oxygen) or mouse cells that were grown in a 3% oxygen environment. Such observations complement earlier studies demonstrating that senescence

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could be delayed by culturing human cells in low oxygen conditions (8).

INTRACELLULAR OXIDANTS AS MEDIATORS OF SENESCENCE

The relationship between intracellular oxidants and the senescent program has been strengthened by the observation that treatment with exogenous hydrogen peroxide can trigger certain primary cells to rapidly enter senescence (9, 10). Interestingly, the concentration of hydrogen peroxide appears important as very high concentrations of ROS appear to trigger apoptosis while lower concentrations appear to favor senescence (9). The addition of these sub-lethal concentrations of hydrogen peroxide induces a number of biochemical changes in cells, most notably, a rise in the level of the tumor suppressor protein p53 and the induction of a p53-dependent cell cycle regulator p21/waf-1 leading to G1 arrest (9).

The fact that exogenous hydrogen peroxide could initiate senescence and the growing appreciation that hydrogen peroxide and other reactive oxygen species (ROS) can act as intracellular signaling molecules (11) raises the possibility that high levels of ROS within cells, perhaps generated through normal aerobic metabolism could regulate entry into senescence. One example suggesting this might be the case comes from observations made from the overexpression of the Ras oncogene in normal diploid cells.

Ras proteins are extensively studied biological switches that stand at the crossroads of multiple signaling pathways (12). These molecules act as binary elements, being in the 'off' configuration when bound to GDP and in the 'on' state when bound to GTP. Interest in Ras and the related family of small GTPases was initially sparked by the observations made over 20 years ago that these proteins are frequently mutated in human cancers. Numerous examples have demonstrated that expression of Ras genes encoding single amino acid mutations that lock Ras proteins in the 'on' configuration are capable of transforming immortalized cells such as the routinely used cell line, NIH 3T3 cells. As mentioned above, the vast majority of cell culture work with Ras proteins has been performed by transient or stable expression of the gene product in the background of an immortalized cell line. In contrast to these observations, experiments where activated forms of Ras were expressed in primary cells did not stimulate cell proliferation but rather resulted in the rapid induction of senescence (13). As with the previous results with exogenous hydrogen peroxide, these observations underscore that senescence can be triggered independently of cell division. Ras expression resulted in increased levels of both p53 and p16 (Ink4a) and in rodent cells, inactivation of either of these two proteins/pathways prevented senescence (13). More recent experiments have emphasized that Ras-induced p16 (Ink4a) accumulation may be particularly critical for senescence in human cells (14).

The observation that non-replicative senescence could be triggered by either exogenous hydrogen peroxide or by Ras expression and that both strategies induced a G1 arrest and increased expression of p53 and p16(Ink4a) suggested that these two senescence-inducing strategies might share common mechanisms. This notion was also consistent with previous observations that small GTPases such as Ras and Rac could increase the level of ROS in immortalized cells (15, 16). Similar to these observations made in immortalized cells, expression of Ras in normal human diploid fibroblasts resulted in an increase in intracellular hydrogen peroxide levels (17). The source of this increase appeared at least in part to derive from the mitochondria. Scavenging with the hydrogen peroxide antioxidant N-acetylcysteine (NAC) or lowering the level of ambient oxygen (to 3% or 1% O₂) was sufficient to block the induction of p53 expression and rescued the cells from Ras-induced senescence (17).

The above results suggest that ROS act downstream of Ras proteins in the induction of senescence. These results are particularly interesting given the role of ROS in aging (18). As mentioned, Ras expression in diploid fibroblasts led to an accumulation of p53, and this p53 induction appears to require the generation of ROS. Little was known concerning the molecular intermediaries that existed between Ras and p53. A recent report however has placed the FAD-dependent oxidoreductase seladin-1 as one such intermediary (19). The authors uncovered this connection by performing a genetic suppressor screen in mammalian cells asking for genes which when suppressed, allowed for an escape from Ras-induced senescence. They identified seladin-1 (also known as DHCR24) as one such gene. This gene product had been previously studied in two disparate contexts. The first is that the enzyme is involved in cholesterol synthesis and in particular, the conversion of desmosterol to cholesterol. Indeed a rare inborn error of cholesterol metabolism called desmosterolosis has been described in which patients inherit, in a recessive manner, two mutated and presumed non-functional seladin-1/DHCR24 alleles (20). Seladin-1 had also been previously described in the context of another screen, in this case, for gene products differentially expressed in normal brains compared to brains obtained from individuals with Alzheimer's disease. Interestingly, these results demonstrated that expression of seladin-1 was significantly down regulated in Alzheimer brains (21). These authors also demonstrated that increased seladin-1 expression appears to protect cells from oxidative stress.

The notion that knockdown expression of seladin-1 allowed for an escape of Ras-induced senescence greatly extended the biological importance of this gene product (19). Interestingly, Ras-expression or direct oxidative stress caused the redistribution of seladin-1 from the cytosol to the nucleus where it can directly bind to both Mdm2 and p53. These molecular interactions lead to an accumulation of p53 in the setting of either Ras expression or direct oxidant challenge. These authors

also demonstrated that the interaction of seladin-1 with p53 and the protein's ability to effect cholesterol metabolism were separable, with only the former being important for senescence induction. Nonetheless, it is curious that a single gene product would regulate both cholesterol metabolism and p53 levels. Indeed, the authors speculated that further analysis might reveal important connections between these two aspects of seladin-1 function especially given the highly conserved nature of seladin-1 from plants to humans. Taken together, these results suggest that seladin-1 represents an important effector molecule in the cellular senescence program and again demonstrates that a rise in intracellular ROS may be an important signal that triggers senescence (see Fig. 1)

THE SIGNAL TRANSDUCTION OF SENESCENCE

The activation of p53 through Ras is not the only signaling pathway through which rapid cellular senescence is induced. Recent evidence suggests that constitutive activation of Akt results in accelerated senescence of endothelial cells (22). This result is of particular interest because extensive analysis of model organisms such as *C. elegans* has demonstrated that in worms, a pathway involving insulin/insulin growth factor (IGF) receptors is an important determinant of life span (23). In the worm, transduction of the insulin/IGF pathway involves the sequential activation of the nematode orthologs of phosphatidylinositol-3-kinase (PI3K) and Akt. The activity of Akt appears in turn to regulate the subcellular distribution and hence activity of the transcription factor Daf-16.

Daf-16 belongs to the family of Forkhead transcription factors and its closest mammalian ortholog is Foxo3a. Analysis of the targets of both Daf-16 and Foxo3a revealed that both the worm and mammalian genes appear to transcriptionally up-regulate antioxidant defenses including both superoxide dismutase (24, 25) and catalase (26, 27). In the worm, mutations that prolong life appear to inhibit Akt activity leading to increased Daf-16 nuclear localization (and hence activity) and subsequent increased oxidative stress resistance. In this context, it is interesting to note that in cell culture, constitutive expression of Akt led to an inhibition of Foxo3a transcriptional activity leading to a rise in intracellular ROS (22). The rise in ROS was further demonstrated to induce a senescence-like arrest via a p53-dependent pathway. Once again the addition of NAC blocked the induction of p53 by Akt in a similar fashion as we have previously discussed that this antioxidant blocked Ras-induced p53 expression and subsequent senescence.

Although the previous results suggest that ROS act upstream of p53, there is also evidence that oxidants can act downstream of p53. It has been appreciated for some time that high level expression of p53 in cells increases the level of intracellular ROS (28, 29). Indeed, some of the transcriptional targets of p53 appear to directly generate ROS (29), and the

apoptotic effects of p53 can be rescued by NAC treatment (28). Subsequent to these studies it was observed that in some cells, although high levels of wild type p53 induced apoptosis, slightly lower levels of p53 expression could trigger a senescent-like growth arrest (30). Again the degree of p53

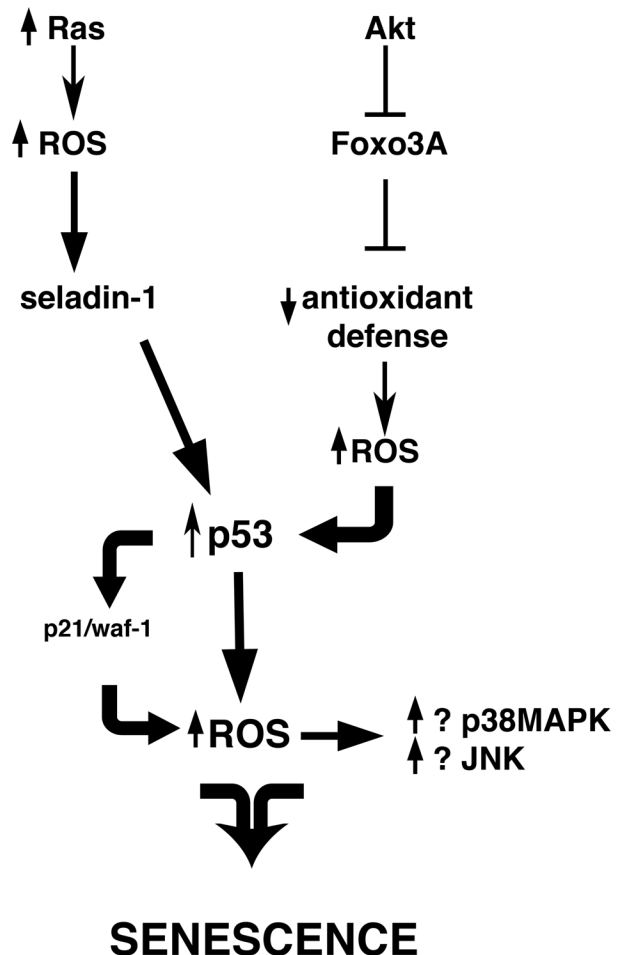


Figure 1. ROS as mediators of senescence. A simplified diagram of two signal transduction pathways that can trigger senescence in a ROS-dependent fashion. One pathway begins with the forced high-level expression of an activated form of Ras. This leads to an increase in ROS and appears to proceed through the FAD-oxidoreductase seladin-1 that physically binds to p53 and regulates the level of the tumor suppressor in a redox-dependent fashion. The other pathway described in endothelial cells involves the forced expression of the protein kinase Akt. Activation of this kinase inhibits the Forkhead transcription factor Foxo3a. This leads to reduced antioxidant defenses (e.g., MnSOD) and a rise in ROS. This Akt-dependent increase in intracellular ROS levels results in higher p53 activity. The role of seladin-1 in this pathway is unknown. Evidence suggests that ROS can function both upstream and downstream of p53. See text for details.

expression appeared to correlate with the level of ROS generation and the ultimate cellular decision to undergo apoptosis or senescence. In many ways these observations are reminiscent of the previously discussed experiments with exogenous hydrogen peroxide where it was also observed that the level of hydrogen peroxide stimulation determined whether apoptosis or senescence ensued (9). The continuum between apoptosis and senescence was also recently strengthened by the observation that members of the Bcl-2 family of anti-apoptotic gene products could under certain conditions rescue p53-induced senescence (31). Expression of these antiapoptotic genes also suppressed the p53-induced rise in ROS. These authors also provided interesting observations suggesting that the redox activation of the p38 MAPK pathway was an important effector of p53-induced senescence. Interestingly, some groups have previously implicated activation of p38 MAPK in Ras-induced senescence (32), while others have suggested activation of the JNK pathway is more important (33).

Finally, the p53 transcriptional target, p21/waf-1 can also induce cellular senescence when overexpressed (34). Similar to what was observed with p53 expression, increased forced expression of this cyclin dependent kinase inhibitor also resulted in a rise in intracellular ROS. Again, treatment with NAC reversed the p21-induced growth arrest arguing that elevated ROS play a causative role in senescence induction.

Although the above examples are exclusively from *in vitro* demonstrations, a recent fascinating report hints at a role for ROS in a model of what may be viewed as *in vivo* cellular senescence. The syndrome ataxia telangiectasia caused by mutations in the ATM gene results in a constellation of symptoms including immunodeficiency, premature aging, cancer predisposition and neurological degeneration. A mouse model of the disease resulting from a knockout of the *Atm* locus reproduces some, but not all, of these symptoms (35, 36). Recently, a more careful analysis of these mice revealed that after approximately 6 months, *Atm* $-/-$ mice begin to exhibit progressive bone marrow failure secondary to a failure of self-renewal of the hematopoietic stem cell (HSC) population (37). Further examination of the HSC cells from *Atm* $-/-$ mice revealed that these stem cells demonstrated significantly elevated levels of basal ROS levels, implying that ATM is somehow involved in the regulation of intracellular ROS. This notion has been previously suspected based on prior work with the *Atm* $-/-$ mouse (38). Interestingly, elevated levels of ROS in HSC resulted in an increase in p16(Ink4a) expression. Similar to what was observed in Ras- or p53-induced senescence, the defect in HSC biology could be rescued by the administration of NAC to *Atm* $-/-$ animals. Taken together these results suggest that in the absence of ATM, HSC have elevated levels of ROS and fail to self-renew. This lack of self-renewal represents a model of cellular senescence since in its absence, HSC rapidly age and are depleted.

The results with the *Atm* $-/-$ mouse, as well as those discussed above, add to a growing literature that appears to suggest two important themes. The first is that many of the genes involved in cellular transformation and tumor formation appear to also be involved in the regulation of intracellular ROS. This extends not only to the Ras oncogene and the tumor suppressors ATM and p53 but also to other candidate transforming genes such as c-myc (39). This link between cancer causing genes and intracellular ROS has important implications in tumor formation and possibly to the relationship between the steep rise in cancer incidence with increasing age. It also underscores the hypothesized biological importance of senescence as a tumor suppressor mechanism (40). Secondly, in normal cells, a rise in ROS appears to regulate specific intracellular events including an increase in the level of p53 and in some cases p16 (Ink4a) as well as the activation of redox sensitive MAPK pathways. We believe these observations argue against a model in which oxidants function in a random and stochastic manner and suggest instead a specific signaling role for ROS in the induction of cellular senescence. While the relevance of cellular senescence to overall aging remains controversial, we believe these observations may also have important implications for how free radicals contribute to organismal aging.

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