Abstract

Aging is defined in an evolutionary biological context as a steady state decline in the specific fitness components of an organism due to internal physiological deterioration. The most profound and important contribution of modern biology to our understanding of aging is the discovery of conserved molecular mechanisms that can regulate ***senescence*** in vastly different organisms. In addition, it is now accepted that aging can be recapitulated at cellular level as well which shows almost all the typical signs of organismal aging viz. lac of regeneration, growth arrest, enhanced oxidative stress and inflammation. Cellular senescence is now accepted as a state of irreversible loss of proliferating capacity of somatic cells and is considered as an important tumour suppressor mechanism as well as the driver of organismal aging. These effects propose aging as a anti-cancer mechanism. However, current understanding from the area of cancer biology is that age associated inflammation and oxidative stress is pro-carcinogenic. These seemingly contradictory roles and 'antagonistic pleotropic' behavior of senescent cells is dependent on the development of the so-called senescent phenotype, where over-production of ***proinflammatory*** and ***pro-oxidant*** signals promote oncogenesis in non – senescent cells. However, the exact molecular and genetic mechanisms underlying this polar behavior remain incompletely understood.

It is now well established that irrespective of the cause or the driver, persistent DNA damage, which chronically accumulates with aging and initiated a cellular stress response termed DNA damage response (DDR) is essential for senescence process. DDR tunes the cell fate based on the extent of DNA damage, which could be to repair the DNA under low damage or to kill the cells in case of severe damage. However, it is the moderate unrepairable DNA damage which induces an interesting cell fate decision termed as ***cellular senescence***. The mechanisms how cells recognized the extent of DNA damage and cell fate decision still not understood clearly and it is also not clear as to what keeps the cells in persistent DNA damage state as there is DNA damage repair taking place at all times. One possible mechanism is ***Oxidative stress*** which is known to be high in senescent cells as well as is capable to triggering DDR.

Oxidative stress results from an imbalance between the production and elimination of Reactive oxygen species (ROS), as well as reactive nitrogen species (RNS). ROS and RNS, are products of normal cellular metabolism and play both deleterious and beneficial roles It is now well documented that ROS can induce DNA damage and drive and reinforce senescence. Elevation in ROS levels has been seen in various models of cellular senescence viz. genotoxic stress induced, telomere uncapping (replicative) or by activation of oncogenes. In addition, senescence-associated inflammatory phenotype further stimulated generation of mitochondrial ROS, thereby establishing a feed forward cycle of senescence state. However, no clear role for other free radical, RNS during cellar senescence and aging is established despite the fact that it is as mutagenic as ROS.

This objective forms my first line to investigate, where the role of RNS in initiation and maintenance of cellular senescence was investigated later in objective two, I investigate the role of ROS-RNS in maintain the free radical hemostasis in senescent cell. Give that free radicals are short-lived molecules their impact of cellular metabolism is completely anticipated and given that metabolically senescent cells are highly active, which can in turn cause higher production of free radicals, we investigated if this is what drives the feed forward loop of senescent cells.

However, our understanding of the metabolic reprogramming that occurs during cellular senescence is still in infancy, therefore my third objective was to identify and characterize the metabolome changes during cellular senescence and identify the contribution to free radicals in driving the feed forward loop in senescence. In the last chapter of my work, I screened to identify signaling molecules which two primary features of senescent cells viz. proliferation arrest and inflammation. Towards this I utilized a shRNA mediated gene expression knockdown approach to screen for protein kinases and phosphatases which regulated the senescence features during genotoxic induced cellular senescence. The sections below describe the salient findings from each of the work objectives to ultimately propose a modular molecular model for cellular senescence which can used to either eliminate senescent cells (senolytic) or used to suppressed inflammation associated with senescence (senotherapy).

Objective 1. Role of NO and ATM-ROS-iNOS axis in induction of cellular senescence by nitric oxide.

In the first part of my study, I report identification and characterization of nitric oxide as an inducing agent for cellular senescence. Physiologically, NO is produced by nitric oxide synthase and is highly reactive colorless gaseous free radical. Using specific NO donors, sodium nitroprusside (SNP) and DETA.NO, which spontaneously yield NO under aqueous conditions, it was shown that NO causes DNA double-strand breaks, which activates DNA damage response (DDR), which culminates in cellular senescence at one specific dose. The same phenotype was recorded when iNOS was overexpressed. The NO activated DDR was mediated through ATM kinase activation and resulted in expression or activation changes in many of the known markers for cellular senescence including phosphorylation of DDR pathway proteins like Chk2, 53BP1, H2A.X; alteration in cell morphology and shape; enhanced expression of senescence-associated molecular markers like p21, IL-8, NFκB; marked enhancement in activity of senescence-associated □-galactosidase; production of senescence-associated inflammatory molecules like IL-8 and IL-6, increased ROS level and etc.

Given that nitric oxide reacts rapidly with superoxide anion and generate a short-lived highly reactive molecule called peroxynitrite, and senescence induction by NO also showed the increased levels of ROS, therefore I investigated and established that senescence induced through nitric oxide is independent of ROS and peroxynitrite. Further, I mapped that signaling cascade and identified that ATM-ROS axis plays a role in NO mediated senescence and thereby established a mechanistic model for NO mediated senescence (Figure.1). Interestingly, I observed that during induction of senescence state by nitric oxide or any other agent the expression levels of iNOS increased significantly. Using this observation I probed if iNOS expression by itself can drive senescence and for this I utilized an inhibitor based iNOS activity derepression approach and demonstrated that sustain generation of NO even through iNOS is capable for inducing senescence. Overall, for the first time it was established that NO can induce DNA damage and activate ATM-dependent cell cycle arrest and senescence, which is independent of ROS, but a physiologically probable process.

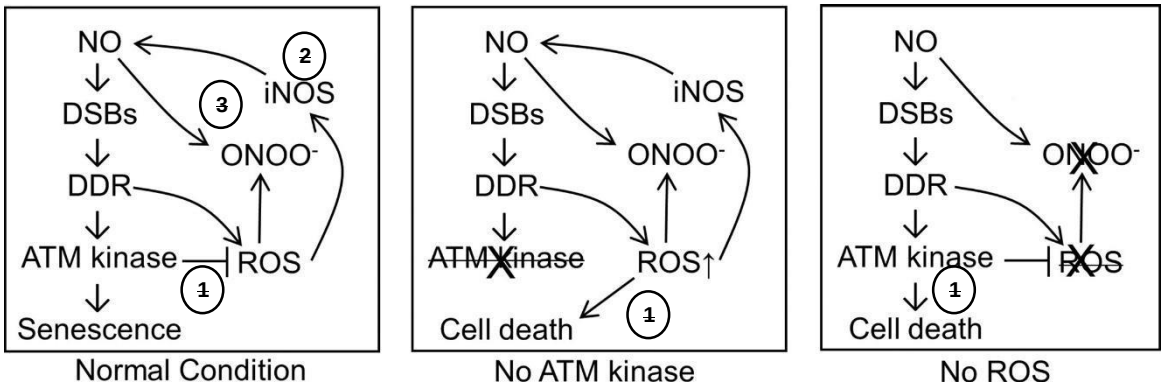

Figure 1. Models of regulation of senescence and cell viability through NO, ROS and ATM kinase. The left panel represents the cascade in normal cells, where NO induces senescence through DSB → DDR → ATM kinase activation. Here step (1) represents ATM kinase mediated suppression of ROS induction, which maintains cell viability; step (2) represents ROS mediated iNOS induction which strengthens the DDR and maintains senescence and step (3) represents quenching of ROS thorough reaction with NO, by forming peroxynitrite (ONOO −) which is short lived. The middle panel represents a situation where the ATM kinase is knocked down/inhibited, here because of an increase in ROS levels (Step 1), which are kept in check by ATM kinase; the cell dies after exposure to NO. The right panel represents the situation where ROS is quenched: here exposure to NO leads to cell death as NO is not quenched through a reaction with ROS and leads to a strong activation of the ATM kinase (Step 1) leading to cell death.

Objective 2. ROS-RNS homeostasis stabilizes Hif1 to regulate senescent cell survival.

In objective 1, we found that NO can induce cellular senescence as well as the NO levels increases during cellular senescence like ROS. Given that role of ROS is senescence is controversial as well as inconsistent, we hypothesised that other free radical, NO might also be compensating for ROS redundancy in initiation and maintenance of senescence. Using this presupposition, in the second objective of my study I probed for the roles of ROS and NO are critical during initiation of senescence when the cells are insulted with the DNA damaging agents or during the maintenance of senescent state of the cells. To confirm the previous finding, I investigated the expression of nitric oxide in cells insulted with DNA damaging agents and found that both level and expression of iNOS increased during genotoxic stress induce senescence. By quenching ROS and inhibiting iNOS during the initial state, I recorded that either ROS or NO is critical for inducing senescence. However, it was observed that quenching ROS and inhibiting iNOS simultaneously senolyticly killed senescent cells. Further, to identify the molecular players which regulate ROS and NO homeostasis in senescent cells, shRNA mediated gene expression knockdown was done for more than 50 genes which modulate the ROS – RNS levels. In the screen, the transcription factor ***HIF-1*** was found to regulate the levels of both ROS and NO in senescent cells.

It was found that simultaneous quenching of ROS and inhibiting iNOS stabilized PHD2 (prolyl hydroxylase) which facilitated Hif1 degradation, inducing a programmed cell death process in the senescent cells called ***Necroptosis***, which was validated by microarray analysis, protein levels changes and even in animal model of aging using irradiated mice. In animals fed with NaLC and iNOS inhibitor, necroptosis of damaged cells was recorded which protected the injured animals for radiation injury.

The findings from this study show that senescent cells need either ROS or NO for the retention of persistent DNA damage and survival of senescent cells. Overall, I was able to establish that Hif1⍰ stabilization during cellular senescence is critical for regulation of senescent cells survival and stabilization of survival state of senescence cells and induction of inflammatory phenotype (Figure. 2).

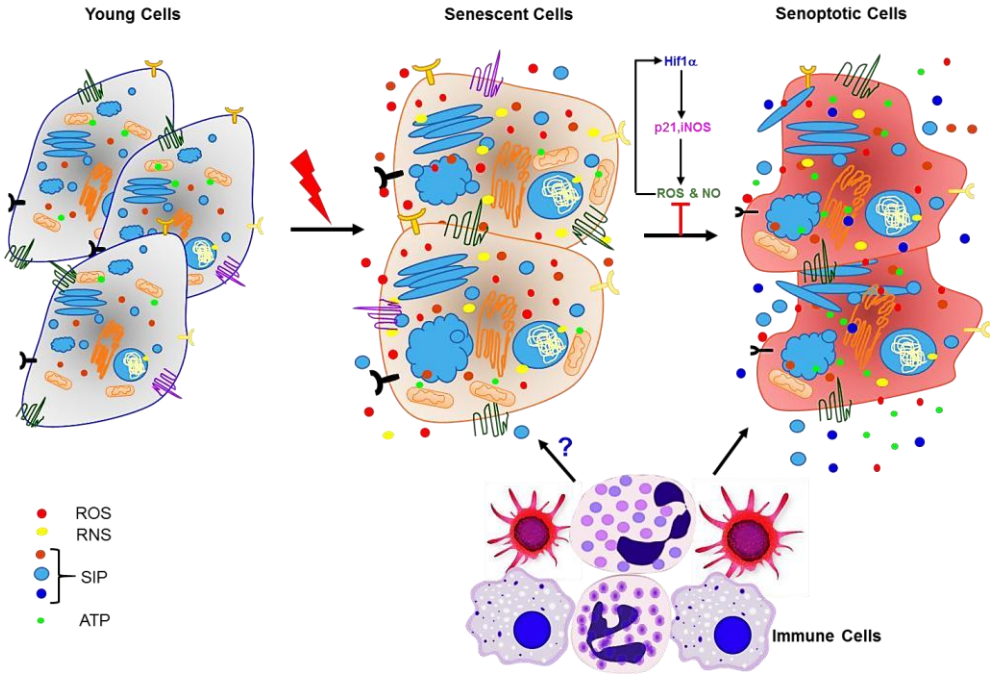

Figure 2. Model of regulation of senescent cell viability, clearance, and survival by Hif1⍰. Cells enter senescence state in response to DNA damage, depending on the dose of insult. Intracellularly ROS and NO levels are regulated by Hif1⍰ stabilization and causes persistent DDR. ROS quenching and iNOS inhibition leads to the destabilization of Hif1⍰ and trigger senolytic effect which kill senescent cells.

Objective 3. Metabolome analysis of senescent cells.

While we recorded changes in the free radical homeostasis during cellular senescence and identified regulatory role of HIF1⍰⍰⍰one possible and highly possible effect of these changes would be high metabolic activity observed in the senescent cells. Towards this the metabolome profile of senescent cells was examined to identify the changes which takes place during senescence. Changes in both intracellular and extracellular metabolites were examined and various changes such as increased glycolysis, change in amino acids uptake and metabolisms as well significant change in fatty acid biosynthesis was recorded. Some of the observed changes reflected the metabolic changes which are recorded during physiological changes as well, such as increase in intracellular level of glucose, presence of myoinositol which could explain alternation in key biological processes during senescence, including cell volume regulation, cell signaling (in phosphoinositide-mediated processes), phospholipid production, and energy consumption. In addition, generation of these molecules could also explain formation of advanced glycated end products (AGEs) whose levels increase in aging.

In addition, fatty acid biosynthesis was altered, for example, the cholesterol level significantly increased in senescent cells which can be due to increased oxidative stress in senescence cells, and it was known that cholesterol can act as an antioxidant agent and form oxy-cholesterol to quench ROS. On the other hand, the amino acid metabolism showed significant difference between senescent and non-senescent cells, for instance, level of proline significantly increased, it is known that proline helps to preserve intracellular glutathione pool, which is major redox buffer of the cells. The metabolic changes were confirmed by changes in the expression of gene involved in fatty acid biosynthesis like ACACA, FASN, and inhibitor of lipolysis like PLIN as well as genes involved in the metabolism of glucose like GCK, HK2, FBP1 and GYS were significantly overexpressed. Further, these observations were validated in animal model of aging as well and mice which exposed to ionizing radiation showed elevated levels of cholesterol than control animals. Overall, these finding show dramatic changes in metabolome profile of senescent cells, which consequently, explain several observations made in aged animals which were unexplained till date.

Objective 4. Identification and characterization of kinases and phosphatases which regulate senescent phenotypes.

Age-dependent signaling changes are still not fully understood and the kinases and phosphatases which regulate the senescent cell viability and inflammation are still not understood. Towards this wide shRNA screening protocol based on determining (i) cell proliferation index, (ii) cell metabolism index using Alamar blue assay and (iii) senescence inflammatory phenotype was utilized. The screening allowed us to identify a few phosphatase families which regulated SASP, cell growth, and metabolisms and few selected candidates from these families were selected for further analysis. Overall findings from the screening allowed us to identify multiple signaling components which regulate various phenotypes of senescent cells.