DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

2014

The impact of MAO-A in cellular senescence

Pedro Emanuel de Magalhães Sequeiros

2014

The Impact of MAO-A in Cellular Senescence

Pedro Emanuel de Magalhães Sequeiros

DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

The impact of MAO-A in cellular senescence

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutor João Passos (Institute for Ageing and Health) e com supervisão académica do Professor Doutor Carlos Jorge Alves Miranda Bandeira Duarte (Centro de Neurociências e Biologia Celular)

Pedro Emanuel de Magalhães Sequeiros

2014

The present work was developed at the Institute for Ageing and Health, Newcastle University, under the supervision of João Passos and funded by European Union by the Erasmus programme.

Index:

List of abbreviations

53bp1	p53 Binding Protein1
CDK	Cyclin-dependent kinase
DDR	DNA damage response
DSB	Double-strand break
ECM	Extracellular matrix
Immuno-FISH	Immuno Fluorescence In Situ Hybridisation
IR	Irradiated
LTE	Long term experiment
MRC5	Fetal human lung fibroblasts
NIR	Non irradiated
OIS	Oncogene-induced senescence
PBS	Phosphate buffer saline
PD	Population doubling
PFA	Paraformaldehyde
ROS	Reactive oxygen species
SAHF	Senescence-associated heterochromatic foci
SASP	Senescence-associated secretory phenotype
SA-β-Gal	Senescence-associated β-galactosidase
SSB	Single-strand break
TAF	Telomere-associated foci
TIF	Telomere induced foci

Abstract: Aging, Cellular senescence and the possible role of MAO-A

Cellular senescence is a tumour suppressor and a contributor to age-related loss of tissue function and so ultimately related to the aging process itself. It has been classically characterised *in vitro* as the result of a DNA damage response (DDR) to uncapped telomeres. Senescence can also be induced by several other mechanisms, including oncogene activation, agents that alter chromatin structure or DNA damage, with oxidative stress being the prominent damaging agent. However, recent studies have revealed that senescence is in fact an intricate process, involving the sequential activation of multiple cellular processes, which have proven necessary for the establishment and maintenance of the phenotype. One of such multiple cellular process includes the generation of Reactive Oxygen Species (ROS) and their role in the establishment of the senescent phenotype, however the complexity of signalling pathways involved and its consequences for senescence *in vitro* and *in vivo* are far from being understood.

MAO-A is a mitochondrial enzyme which catalyses the oxidative deamination of monoamines and generates hydrogen peroxide, aldehyde and ammonia. Recently, it has been demonstrated that MAO-A contributes to heart failure during ageing, by generating increased ROS and activating p53. Moreover, it has been demonstrated that MAO-A expression increases in human fibroblasts undergoing replicative senescence.

For future work it becames clear, the need to study the impact of MAO-A in cellular senescence, and so, determining the impact of MAO-A in cellular senescence, mitochondrial dysfunction, ROS production and DNA damage response.

These studies will provide a better understanding of the complexixity of cellular senescence and its signaling pathaways and its relation with the aging process.

Keywords: Cellular Senescence; Aging; MAO-A; p53; ROS; DDR

Resumo: Senescência celular e o possível envolvimento da MAO-A

Senescência celular é um mecanismo de supressão tumoral e um contribuinte para a perda de função tecidual, a medida que envelhecemos, relacionado assim com o processo a que chamamos envelhecimento. Ele tem sido e caracterizado *in vitro*, como o resultado de uma resposta a danos no ADN em virtude de telómeros não disfuncionais. Senescência também pode ser induzida por vários outros mecanismos, incluindo a ativação de oncogenes, agentes que alteram a estrutura da cromatina ou danos no DNA, com o stress oxidativo a ser o agente de degradação mais importante.

No entanto, estudos recentes revelaram que a senescência é, de facto, um processo complexo, que envolve a ativação sequencial de vários processos celulares, que têm se mostrado necessários para o estabelecimento e manutenção do fenótipo. Um desses múltiplos processos celulares, inclui a geração de espécies reativas de oxigênio (ROS) e seu papel no estabelecimento do fenótipo senescente, no entanto, a complexidade das vias de sinalização envolvidas e suas consequências para a senescência in vitro e in vivo estão longe de serem compreendidos.

MAO-A é uma enzima mitocondrial que catalisa a desaminação oxidativa de monoaminas e produz peróxido de hidrogénio, aldeído e amónia. Recentemente, tem sido demonstrado que a MAO-A contribui para a insuficiência cardíaca durante o envelhecimento, através da geração de ROS aumentada e activação de p53. Além disso, tem sido demonstrado que a MAO-A expressão aumenta em fibroblastos humanos submetidos a senescência replicativa.

Para trabalhos futuros, é claro, a necessidade de estudar o impacto da MAO-A na senescência celular, e assim, determinar o impacto da MAO-A em senescência celular, disfunção mitocondrial, produção de ROS e resposta a danos do DNA.

Estes estudos proporcionam um melhor entendimento da complexidade da senescência celular e seus mapas sinalização e a sua relação com o processo de envelhecimento.

Palavras chave: Senescência celular MAO-A; p53; ROS; DDR: envelhecimento

Introduction

Ageing

Ageing can be defined by the progressive loss of physiological integrity, leading to impaired function and increased vulnerability to death and is characterized by changes in appearance, such as a gradual reduction in height and weight loss due to loss of muscle and bone mass, a lower metabolic rate, declines in certain memory functions, declines in sexual activity, a functional decline in senses, declines in kidney, pulmonary, and immune functions, declines in exercise performance, and multiple endocrine changes. [1] [2]

The phenotype of human aging is one in which practically any system, tissue or organ can fail [3]. Clearly, the incidence of a number of pathologies increases with age however the intricate process of ageing cannot be viewed as a disease or pathology, or at least as the standard way we look at it, because aging has to be viewed as an extremely, multifactorial, complex processes which interact simultaneously and operate at many levels of functional organization [4] .Unlike any other pathology or disease, as so eloquently Hayflick describes [5], age changes:

1. Occur in every multicellular animal;
2. Across almost all species barriers;
3. Occur in all members of a species specially after reproductive maturation;
4. Occur in all animals in protected environments like animals removed from the wild and protected by humans even when that species probably has not experienced aging for thousands years;
5. Occur in virtually all animate and inanimate matter (if you see ageing as a process of molecular change over time);
6. Have the same universal molecular etiology, that is, thermodynamic instability.

Unlike ageing, there is no disease or pathology that shares these six qualities. Ageing is unique because no other pathology shares these six characteristics.

Ageing shows a broad phylogenetic distribution but is not universal, as some species show no age-associated increase in mortality or decline in fertility being such examples the freshwater *Hydra* and the immortal jellyfish *Turritopsis nutricula* [6], [7]

In recent years the ageing research expanded as the knowledge and theories which try to explain this process. They usually can be separated in two large groups. The evolutionary theories which try to explain why we age and the origins of this process as long as the differences in longevity between species and the Molecular/Celular damage based theories which try to explain how we age and mechanist of this process under the demise that ageing results in an accumulative, progressive and stochastic process of cellular damage. [8] [9]

Biological Level/Theory	Description
Evolutionary	
Mutation accumulation*	Mutations that affect health at older ages are not selected against.
Disposable soma*	Somatic cells are maintained only to ensure continued reproductive success; after reproduction, soma becomes disposable.
Antagonistic pleiotropy*	Genes beneficial at younger age become deleterious at older ages.
Molecular	
Gene regulation*	Aging is caused by changes in the expression of genes regulating both development and aging.
Codon restriction	Fidelity/accuracy of mRNA translation is impaired due to inability to decode codons in mRNA.
Error catastrophe	Decline in fidelity of gene expression with aging results in increased fraction of abnormal proteins.
Somatic mutation	Molecular damage accumulates, primarily to DNA/genetic material.
Dysdifferentiation	Gradual accumulation of random molecular damage impairs regulation of gene expression.
Cellular	
Cellular senescence-Telomere theory*	Phenotypes of aging are caused by an increase in frequency of senescent cells. Senescence may result from telomere loss (replicative senescence) or cell stress (cellular senescence).
Free radical*	Oxidative metabolism produces highly reactive free radicals that subsequently damage lipids, protein and DNA.
Wear-and-tear	Accumulation of normal injury.
Apoptosis	Programmed cell death from genetic events or genome crisis.

Table 1 *Classification and brief description of main theories of ageing*
Retrieved from Weinert B.T., Timiras P.S.; *J Appl Physiol VOL 95 OCTOBER 2003*

The evolutionary theories

Ageing increases an organism's vulnerability to diseases and death, which in Darwin´s terms is a contradiction because under the theory of nature selection, how could evolution favors a process which declines fitness and survival of a species?!

Evolutionary theories argue that aging results from a decline in the force of natural selection because in wild populations the cause of death it´s not the process of ageing but yes, external hazards such infection, predation, starvation or cold and so, wild animals simply do not live long enough to grow old. This concept is important to understand because the principal determinant in the evolution of longevity is predicted to be the level of extrinsic mortality and not the ageing process.[10]

Theories like the mutation accumulation proposed by Peter Medawar [11], the Antagonist Pleiotropy proposed by George Williams [12] and the disposable soma proposed by Thomas Kirkwood [13] try to explain why ageing occurs and how species have different lifespans taking into account this assumption **(Fig1).** If extrinsic mortality is high, life expectancy in the

wild is short, the force of selection attenuates fast, deleterious gene effects accumulate at earlier ages, and there is little selection for genes with beneficial effects at old ages[12] or high level of somatic maintenance[13]. So the opposite also happens, if the level of extrinsic mortality is low, selection is predicted to postpone deleterious gene effects and to direct greater investment in building and maintaining a durable soma (beneficial gene effects at old ages).

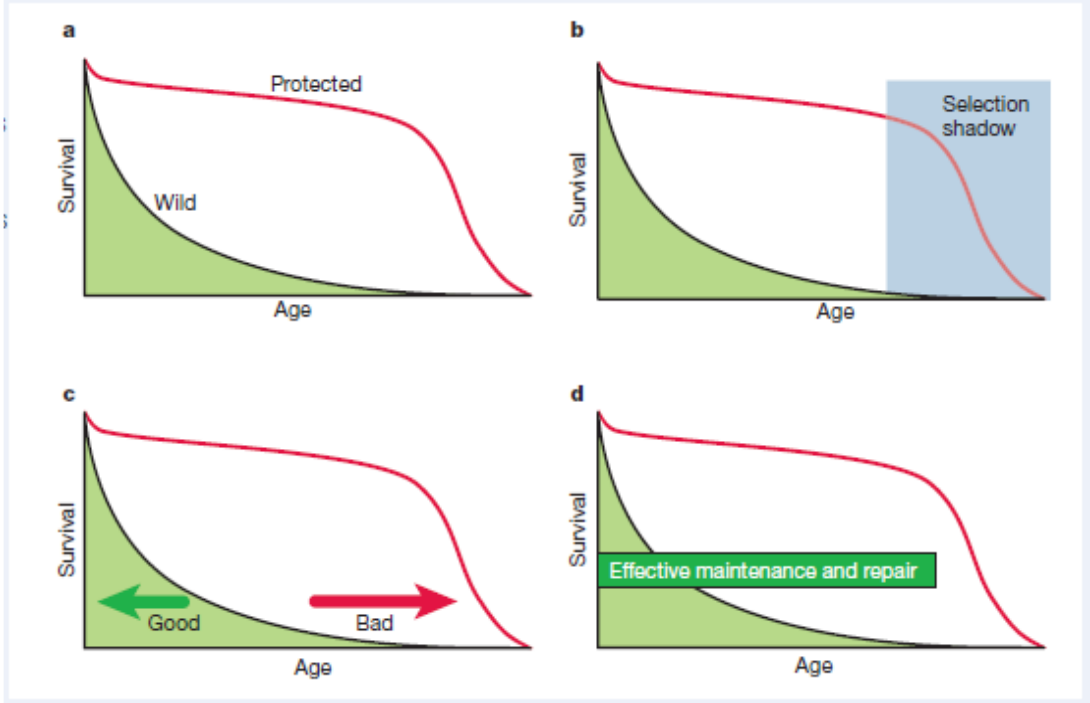


Fig.1 **a**, Extrinsic mortality in wild environments occurs to an extent that senescence-associated mortality is rare, undermining any idea that genes specifically for ageing have evolved.
b, The 'selection shadow' at older ages may permit an accumulation of late-acting deleterious mutations (mutation-accumulation theory).
c Pleiotropic genes that benefit organisms early in life will be favoured by selection even if they have bad effects at later ages (pleiotropy theory).
d, Selection pressure to invest metabolic resources in somatic maintenance and repair is limited; all that is required is to keep the organism in sound condition for as long as it might survive in the wild (disposable-soma theory).
Retrieved from " Kirkwood T.B.L, Austad S.N., "Why do we age?" Nature, vol. 408, 9 November 2000"

All this theories could all account for the life history theory which studies the changes in organisms from conception to death, focusing on the schedule of reproduction maturity and survival. The r/K selection is a life history simplified model proposed by Robert MacArthur and Edward Wilson [14]. It hypothesis that natural selection and so, selective pressures drive evolution in one of two generalized directions: *r*- or *K*-selection. Organisms *r*-selected will favor early maturity onset, high fecundity, rapid development, small body sizes, and a short lifespan. This is the case of semelparous species like the pacific salmon (genus *Oncorhynchus)* in which

individuals reproduce only once and then enter in a massive decrease of fitness ultimately leading to death. Removal of the gonads allows this species to live much longer with the cost of not being able to reproduce [15]. One the other hand organisms *K*-selected will favor delayed development, few offspring larger body sizes, and a longer lifespan. This is obvious the case of our species. This theory shows a clear tradeoff between fecundity, growth and longevity.[16]

Molecular/Celular damage based theories

This class of theories of aging is based on the concept that damage, either due to normal toxic by-products of metabolism or inefficient repair/defensive systems, accumulates throughout the entire lifespan and causes aging. Protein Damage (error catastrophe theory) [17], cellular senescence and free radical theory [18], are some some examples of such theories.

Ageing Hallmarks

Aging research has experienced an unprecedented advance over recent years, and now is known that this process is ruled by genetic and biochemical pathways conserved in evolution.[1]

Each hallmark should ideally fulfill the following criteria:

1. It should manifest during normal aging;
2. Its experimental aggravation should accelerate aging; and
3. Its experimental amelioration should retard the normal aging process and hence increase healthy lifespan

Each hallmark is interconnected with each other, implying that experimental amelioration of one particular hallmark may impinge on others. [1]

Some hallmarks like the processes of cellular senesce and it´s relation with mitochondrial dysfunction and genomic instability specially in telomeres will be described in more extent on this introduction (Fig.3)

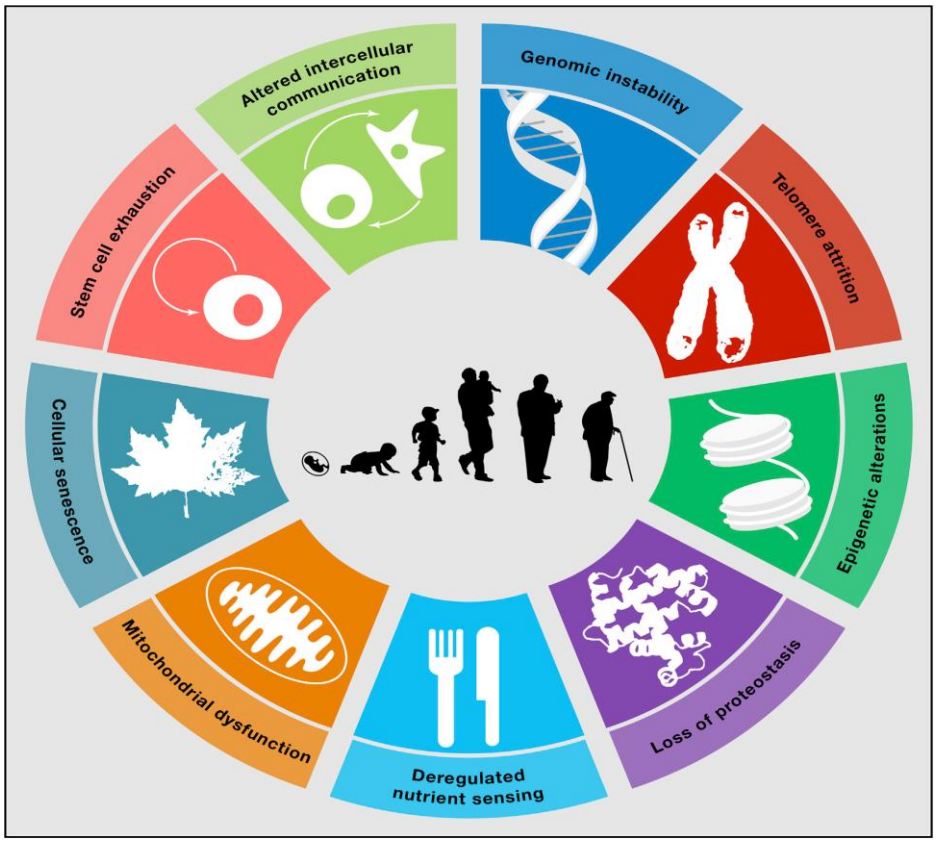


Fig 2. The scheme enumerates the nine hallmarks described in this Review: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication.
Retrieved from Otin C.L., Blasco M.A., "The Hallmarks of Aging" Cell 153, June 6, 2013

Cellular senescence

Cellular senescence can be defined as the irreversible loss of division potential in somatic cells in a response to cellular stress, and is marked by a number of distinct phenotypic changes including protein aggregation in endoplasmic reticulum, enlarged dysfunctional mitochondria and nonfunctional lysosomes [18], [19]. Despite entering in a permanent cell cycle arrest, these cells remain viable and metabolically active for long time periods [20].

This cell state was first described by Hayflick and his colleague [21]. They discovered that human embryo fibroblasts cells could only divide a finite number of times (around 50 times) in cell culture. The number of divisions that cells complete upon reaching the end of their replicative life span has been termed the Hayflick limit. Now it is known this replicative life is

intimately related to telomeres and it´s shortening in each cell division, function as a replicometer and triggering replicative senescence[22], [23].(Fig.3)

The senescence arrest is considered irreversible because no known physiological stimuli can stimulate senescent cells to reenter the cell cycle except by biological manipulations. for examples inhibition of certain proteins involved in signaling pathways which maintain the permanent cell cycle arrest and so, the senescent state. [25]

The permanence of the senescence growth arrest enforces the idea that the senescence response evolved at least in part to suppress the development of cancer [24], which is the opposite cell senescent state (continuous proliferation). So, cell senesce is a programmed response which acts as a tumor suppressive mechanism [18].

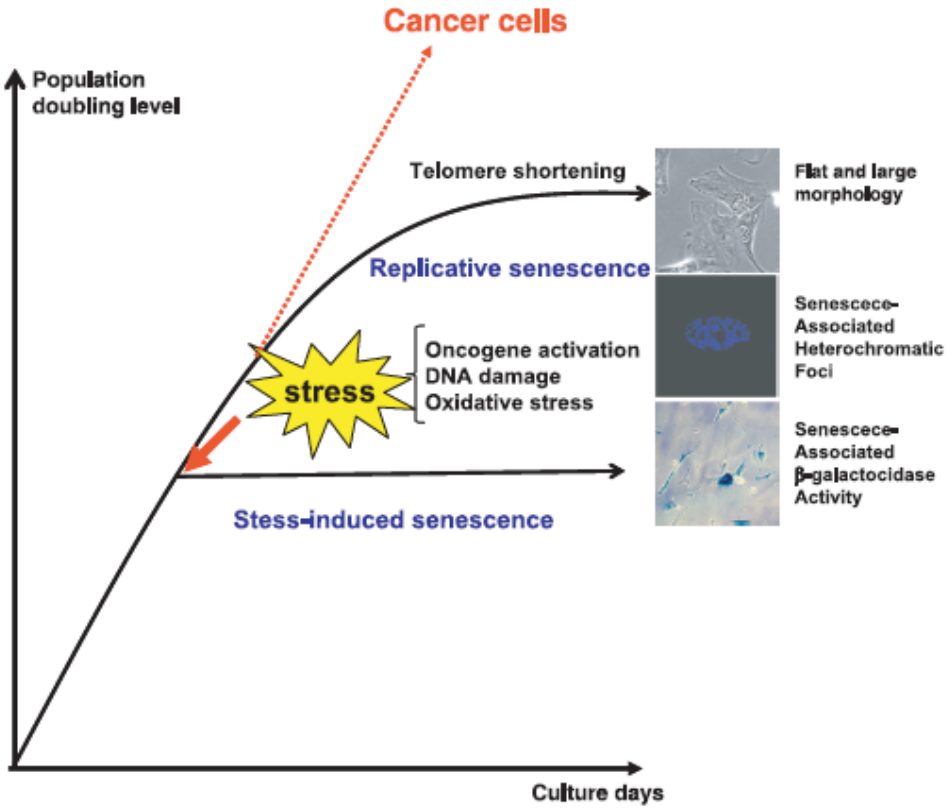


Fig.3 Cellular senescence. Normal human fibroblasts enter a state of irreversible growth arrest after a finite number of cell divisions *in vitro* caused by telomere shortening but cancer cells appear to bypass this replicative limit and proliferate indefinitely.
Cellular senescence can also be induced prematurely by a number of cellular stresses such as oncogenic stimuli, oxidative stress, and DNA damage, before reaching their limits of replicative life span.
Senescent cells are characterized by a large and flat morphology, senescence-associated acidic galactosidase activity, and senescence-associated heterochromatic foci.
Retrieved from Naoko Ohtani, David J. Mann and Eiji Hara Cellular senescence: Its role in tumor suppression and aging Cancer Sci, May 2009, vol. 100, no. 5, 792–797

The induction of cellular senesce

Now it is known the Hayflick limit and replicative live span is not the only stimulus which leads *to cell senescence* such as DNA damage, damage to chromatin structure, oxidative stress and oncogenic signaling [26] Here is the description of some of the causes and stimulus that leads to this cellular state (fig.4):

Telomere-dependent senescence

Telomeres are stretches of repetitive DNA and associated proteins that cap the ends of linear chromosomes and protect them from degradation or fusion by DNA-repair processes [27].

The structure of mammalian telomeres are thought to end in a large circular structure, termed a t loop [28] and they are associated a proteins that bind to form a complex known as shelterin [85]. Because standard DNA polymerases cannot completely replicate DNA ends - the end-replication problem - cells lose base pairs of telomeric DNA during each cell division and telomeres become shorter and shorter until become dysfunctional.

Functional telomeres prevent DNA repair machineries from recognizing chromosome ends as DNA double-strand breaks (DSBs), and elicit the DNA damage response (DDR). The DDR enables cells to sense damaged DNA, particularly double-strand breaks (DSBs), and to respond by arresting cell-cycle progression and repairing the damage if possible. However, as telomeres become dysfunctional (either by telomere shortening or other kind of induction of DNA damage), DNA is recognized as DNA damage, forming DNA damage foci, in this case these telomere-associated foci (TAF), and the DDR is activated and cell division is arrested without the attempt to repair telomeres [29], [30]. These TAF are long lived and can occur within telomeric repeats irrespectively of telomere length or telomerase activity [51]. So, dysfunctional telomeres appear to be irreparable and as a consequence, cells with such telomeres experience persistent DDR signaling and p53 activation which reinforce the senescence cell state [30], [31]

The end-replication problem and shortening of telomeres can be circumvented by the enzyme telomerase, the reverse transcriptase that can replenish repetitive telomeric DNA de novo [32]. Most normal, human cells do not express TERT (being the exception germ line, cancer and stem cells), and telomere shortening is not prevented [33]. However, ectopic TERT expression in normal human cells prevents telomere shortening and senescence caused by the end-replication problem. [34]

Genomic Damage

Many cells undergo senescence in response to severely damaged DNA, regardless of the genomic location DNA DSBs, such as those induced by ionizing radiation, topoisomerase inhibitors, and other agents, are especially potent senescence inducers Many types of cytotoxic chemotherapies are severe DNA-damaging agents that can induce senescence in both tumor cells and surrounding normal cells [35], [36].

These lesion in DNA are known to generate persistent DDR signaling, the same kind in telomere dependent senescence. This chronic DDR contrasts sharply with the response to mild DNA damage, which generates a transient growth arrest and transient DDR signaling. Persistent DDR signaling is generally identified by the long-term presence of nuclear DNA damage foci. [35]

Oncogene-induced senescenc/ Mitogens and Proliferation-Associated Signal

Cellular senescence can also be induced by strong and unbalanced mitogenic signals [37], consistent with its role in suppressing tumorigenesis.. The first report of oncogene-induced senescence showed that an oncogenic form of H-RAS (H-RASV12), which chronically stimulates the mitogen-activated protein kinase (MAPK) signaling pathway, provokes senescence in normal cells [38]. Subsequently, other members of the RAS signalling pathway (for example, RAF, MEK, MOS and BRAF), as well as pro-proliferative nuclear proteins (E2F‑1), were shown to cause senescence when overexpressed or expressed as oncogenic stimulus [39].

The mechanism for this oncogene induction senescece also involves DNA damage and DDR and sometimes DDR independent signalling patahways like the p38MAPK [40].

Epigenomic Damage

Changes in the epigenome like chromatin organization like the formation of repressive heterochromatin at several loci for proliferative genes -which usually involves E2F target genes - and chromatin relaxion and consequent activation of the tumor supressos p16 are ways of inducing senescence. senescence-associated heterochromatin are formed in this process. [41]

Under some circumstances, epigenomic perturbations can elicit a DDR in the absence of physical DNA damage by activating the protein ATM of the DDR. [42]

Oxidative stress

Oxidative stress and the accumulation of intracellular reactive oxygen species (ROS) play an important role in the induction of senescence. For example it has been shown that Human

fibroblasts when grown at 40-50% oxygen undergo to senescent state but the same does not happen when the cells are cultured in a low ambient oxygen conditions (2 - 3%). [43]
The same results were achieved in experiments where ROS levels were increased either by inhibition of anti oxidant enzymes or by hydrogen peroxide treatment.[44]

The increase in internal ROS production leads to the oxidation of biomolecules like DNA, proteins and lipids. This oxidation, cause specially damage in telomeres and accelerates telomere shortening through the induction of single strand breaks [45] [46]. This rise the possibility that ROS promote senescence through telomere dysfunction. Another possibility is that it is the induction of damage to genomic DNA by ROS that accelerates the senescence of these cells through p53 and p21 activation [35]. More important, this p53 and p21 activation can lead to the production of more ROS, creating a feedback positive loop [58] [81].

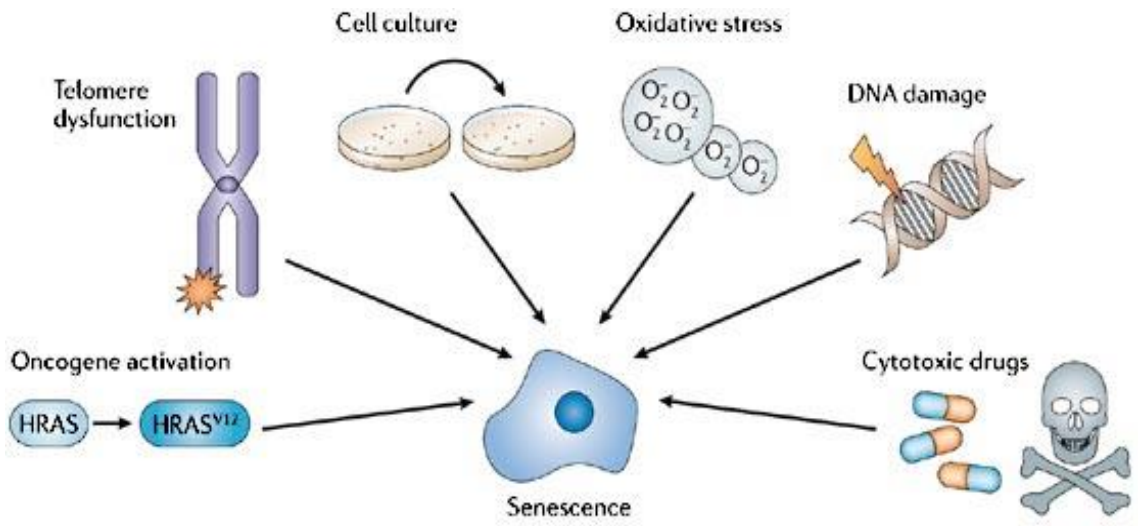


Fig.4 The signals activating senescence. Multiple types of stress can induce cells to undergo senescence. The combined levels of stress determine how rapidly the entry into senescence will occur. Senescence functions as a self-defence mechanism to prevent the proliferation of potentially damaged cells. In some instances, the same stimulus might induce either senescence or apoptosis, but the mechanisms that govern the decision to engage one or the other are not known

Collado M., Serrano M., " The power and the promise of oncogene-induced senescence markers *Nature Reviews Cancer* **6**, 472-476 (June 2006)

Cellular senescence hallmarks

Besides permanent cell cycle arrest, senescent cells are characterized by a myriad of attributes (Fig 5) which includes:

- Apoptotic resistance - what determines whether cells undergo senescence or apoptosis is still unknown but the nature and intensity of the damage as well as cell type accounts for such choice [47]. However once cells became senescent they acquire resistance to certain apoptotic signals. the mechanism itself is not known but there is evidence that p53 is a master regulator on this process [18]
- Altered gene expression - CDKIs p21 (also termed CDKN1a, p21Cip1, Waf1 or SDI1) and p16 (also termed CDKN2a or p16INK4a) are the most common altered expressed proteins in senescent cells. These CDKIs are components of tumour-suppressor pathways that are governed by the p53 and retinoblastoma (pRB). Ultimately, p21 and p16 maintain pRB in a hypophosphorylated and active state. [18]

  Also, senescent fibroblasts overexpress proteins like cytokines, chemokines, growth factors and proteases that remodel the extracellular matrix or mediate local inflammation. This phenotype of senescent cell is known as the senescence-associated secretory phenotype (SASP) [48]. One particular fact of SASP is that can induce (and be activated) by reactive oxygen species (ROS). In fact, there is evidence suggesting that ROS and the SASP cooperate to induce and stabilise the senescent phenotype. Cytokines like IL-6 and IL-8 are involved in this process [83], [84].
- Senescent markers - Histochemical assay for β-galactosidase activity was the first widely used marker to detect senescent cells and is still used in cell culture and vertebrate tissues [49] but the specificity of this assay has been questioned and now, other markers such as H2AX (γ-H2AX) and p53-binding protein-1 (53BP 1) which are present in senescence associate DNA damage foci or in telomere induced/associated foci (TIF/TAF) are widely used. [18] [51]

  Also proteins that are overexpressed in senescent cell like p16 and p21 can serve as markers.[18]

  Some senescent cells can also be identified by the cytological markers of contain senescence-associated heterochromatin foci (SAHF) [41]

  DEC1 (differentiated embryo-chondrocyte expressed‑1), p15 (a CDKI) and DCR2 (decoy death receptor-2), are promising additional markers.[50]

In the recent years, work has been made to create a framework for quantitative assessment of markers for senescence, markers like γH2AX foci, p21 and Ki67 [82]. In this this, some results are based in this assessment.

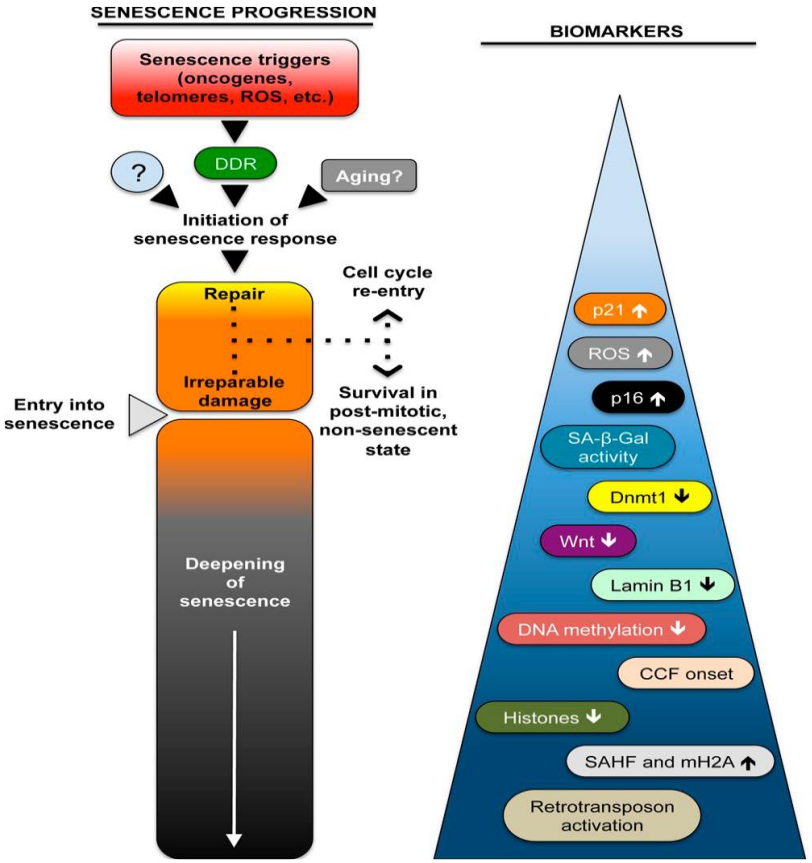


Fig.5 A schematic representation of the processes that lead to the establishment of cellular senescence as well as biomarkers. The progression of senescence has been separated into several components: (1) triggering events; (2) initiation of the senescence response; (3) entry into senescence; and (4) a further deepening of senescence phenotypes. This listing is not meant to be comprehensive and similarly, the order is not meant to imply the chronological acquisition of these features.
Retrieved from Baker D.J., Sedivy J.M., Probing the depths of cellular senescence J. Cell Biol. Vol. 202 No. 1 11–13

Cellular senescence pathways and DDR

The processes by which cells repair damage to DNA and coordinate repair with cell cycle progression are collectively known as the DDR (Fig 6). In cases in which the damage cannot be repaired, prolonged cell cycle arrest can lead to senescence or the induction of apoptotic signals.

In the case of senescence response, it is characterized by activation of sensor kinases (ATM/ATR, DNA-PK), formation of DNA damage foci containing phosphorylated histone H2A.X (γH2A.X) and ultimately induction of checkpoint proteins, such as p53 (TP53) and the CDK

inhibitor p21 (CDKN1A), which contribute to cell-cycle arrest. The described signaling pathway continues to actively contribute to the stability of the cell-cycle arrest long after induction of senescence [51]

So the induction of cell senescence is established and maintained through the DDR by the p53/p21 pathway but also by the p16INK4a/pRB pathway and the p38 pathway as we already seen have some role in this response.

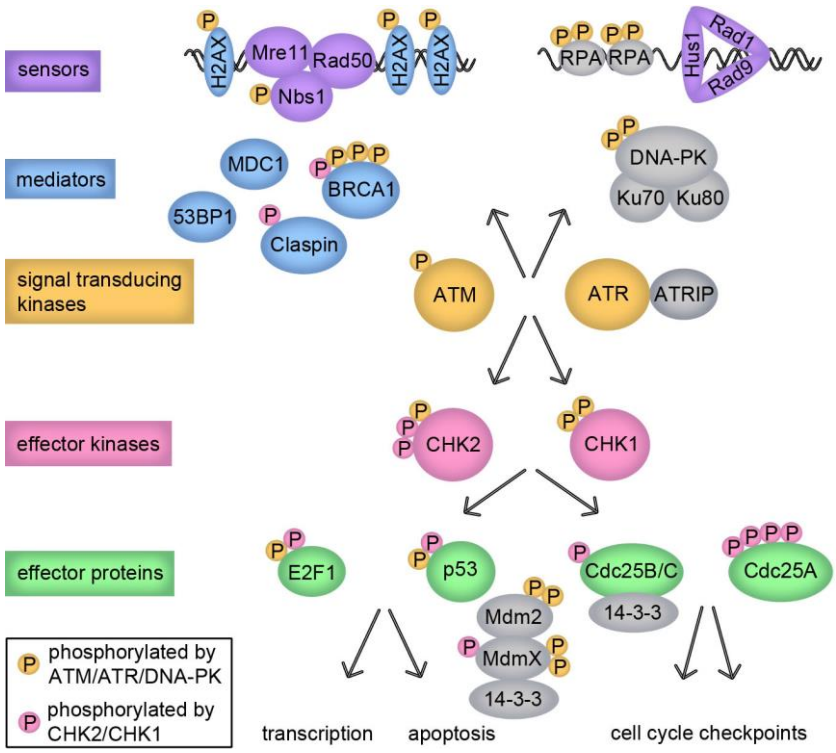


Fig.6 A simplified view of the cellular response to DNA damage. Single and double stranded DNA breaks signal through the sensors (MRN and 9-1-1) shown in purple, mediators (H2AX, BRCA1, MDC1, 53BP1) shown in blue, signal transducing kinases (ATM, ATR) shown in yellow, effector kinases (CHK2, CHK1) shown in pink, and effector proteins (E2F1, p53, Cdc25) shown in green, leading to gene transcription, apoptosis, and cell cycle arrest.
Retrieved from Freeman A.K., Monteiro A.N.A. "phosphatases in the cellular response to DNA damage" Cell Communication and Signaling 2010, 8:27

The p16/Rb pathway (fig.7)

Rb mediates regulation of the cell cycle at the transition from first gap phase (G1) to DNA synthesis phase (S phase). Rb is hypophosphorylated during G1/G0 and is bound to E2F whereby the activity of E2F is inhibited. When Rb is phosphorylated it releases E2F which mediates of a variety of genes necessary for G1 to S progression and replication. [52]

Phosphorylation of Rb is mediated by cyclin dependent kinases (CDK). One of them, the CDK4/cyclin-D is activated by mitogenic signaling through the RAS pathway. There are proteins called cyclin dependent kinase inhibitors that can inhibit the CDKs. One of them is p16 which

inhibits phosphorylation of Rb and thereby G1 to S progression by inhibiting CDK4/cyclin-D. p16 can in turn be regulated transcriptionally by several proteins and seems to be a sensor for cellular stress [52]

In the last years, evidence for an important role by the p16/Rb pathway during the induction of senescence. has been shown. [53][54]

The p53/p21 pathway (fig.8)

p53 the "guardian of the genome" acts as an integrator for various signals and can mediate cell cycle arrest, apoptosis or even differentiation. This protein is mutated in 50% of all tumors. There are several mechanisms that regulate the activity of p53. The DNA damage-ATM/ATR-Chk1/Chk2 pathway activate p53 by phosphorylation leading to displacement of the cellular protein MDM2, a inhibitor of p53 MDM2 can also be regulated by p19ARF, which inactivates MDM2 leading to an increased activity of p53 Activity of p53 can be modulated by protein modifications and many other protein interactions. [55]

One of the activated proteins that mediate the cell cycle arrest downstream of p53 is p21.

p21 is a member of the "Cip/Kip" family of cyclin dependent kinase inhibitors (CDKI) that inhibits CDK2/cyclin-E) and to a lesser extent CDK4/cyclin-D. p21 is believed to be the main target for cell cycle arrest downstream of p53. [52]

Evidence for an important role by the p53/p21 pathway during the induction of senescence, has been shown. Mouse embryonic fibroblasts (MEF) lacking p53 [56], or when is inhibited in Human cells [57] do not senescence.

Reactive oxygen species (ROS) are possible mediators of the senescence response downstream of p53/p21. It seems that, p53-and p21-induced senescence has been shown to be at least partly dependent on ROS. [58][59]

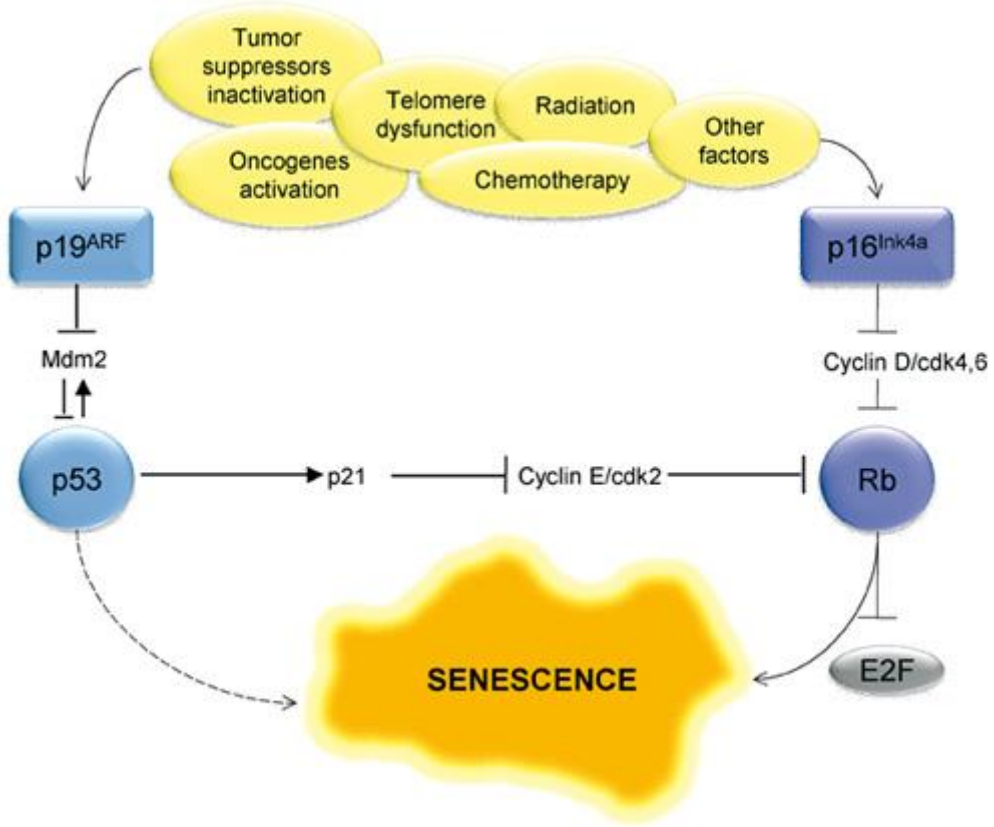


Fig.7 Senescence activation models. Rb can be lineally activated through p53-p21 pathway, through p16-Rb, or through both pathways at the same time according to specific combinations and/or the severity of the factors that cause stress
Noack L.C., Corredor M.C.S., Clavijo S.R.R., "The Dual Role of Senescence in Tumorigenesis" *Int. J. Morphol., 28(1)*:37-50, 2010.

The p38 pathway (fig.8)

The p38 pathway is one of the mitogen-activated proteinkinase (MAPK) pathways and c-Jun N-terminal kinase (JNK) pathways. [60]

In normal non-transformed cells, oncogene activation may lead to senescence by this pathway. For example in oncogene induced senescence (OIS) the p16 cyclin-dependent protein kinase (CDK) inhibitor and the transcription factor p53 are two major effectors that enforce growth arrest and cell senescence.

Activation of p38 leads to increased expression of p16INK4A during OIS [61]. Other studies indicate that p53 is a downstream effector of p38 pathway, mediated by ras induced senescence. [62] Also ROS seems to have a paper in p38 activation and OIS. [63]

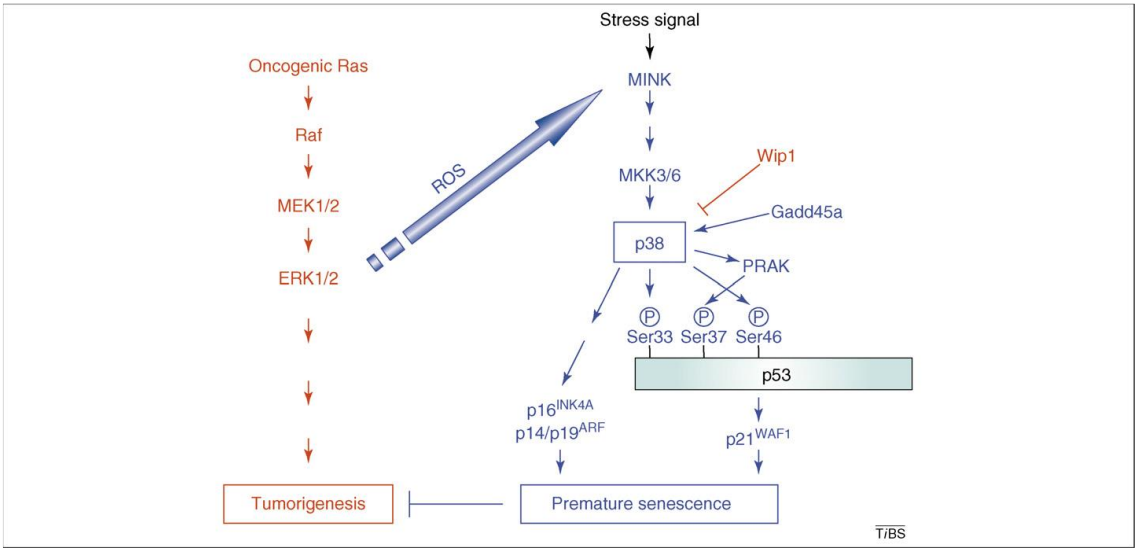


Fig.8 The p38 pathway and OIS. Signal-transduction pathways mediating the role of p38 in OIS are shown. Oncogenic ras, and possibly other oncogenes, induces sequential activation of the tumorigenic Raf–MEK–ERK MAPK pathway and the stress-induced MINK–MKK3/6–p38–PRAK MAPK pathway. Activation of the p38 pathway might be mediated by increased intracellular levels of reactive oxygen species (ROS) induced by the Ras–Raf–MEK–ERK signaling cascade. Activated components of the p38 pathway phosphorylate multiple residues on p53, including Ser33 and Ser46 (by p38), Ser37 (by PRAK), and possibly others, leading to increased transcriptional activity of p53 and induction of a transcriptional target of p53, p21WAF1. Through an unknown mechanism, active p38 also induces the expression of p16INK4A and p14/p19ARF, which, together with the p53–p21WAF1 cascade, cause premature senescence that serves as a tumor-suppressing defense mechanism both in cell culture and in vivo
Retrieved from Han J., Sun P. (2007) "The pathways to tumor suppression via route p38" Trends in Biochemical Sciences, Vol.32, No.8

Cellular senescence in Vivo

Classically, senescence has been described in somatic cells grown in vitro; however, a question begin to rise if such senescence state is simply an artefact of cell culture or if it really exists in vivo. [64]

Signals which induce senescence in culture cells may have a origin in extrinsic sources, and stems from strains stresses that cells experience when they are explanted into culture (“culture shock”) and so, the progressive loss in proliferative capacity may result from cumulative trauma imposed by tissue culture and all the conditions underlying this process like the lack of heterotypic interactions between cell types, plating of plastic, hyperoxia, the medium-to-cell ratio among others. [65]

However, there is recent evidence that senesce also happens in vivo. The observation of fibroblasts containing telomere-induced foci (TIF). [66]

Moreover, genetic manipulation of the classical senescence pathways (p53/p21; p16) has been shown to impact on organismal ageing [67], [68], [69]. Additionally, there is evidence that senescence has a role in cancer, showing once again, evidence that senesce is a tumor suppressive response in vivo. [70][71]

Cellular senescence and mitochondrial dysfunction

Mitochondria suffer changes with replicative lifespan affecting metabolism and ROS and dividing cells in vivo accumulate mtDNA mutations generating deficient respiratory enzymes. These is correlated to mitochondrial dysfunction which can accelerate telomere shortening by the rise in oxidative stress. [72] [73] [74] [75]. (Fig.9)

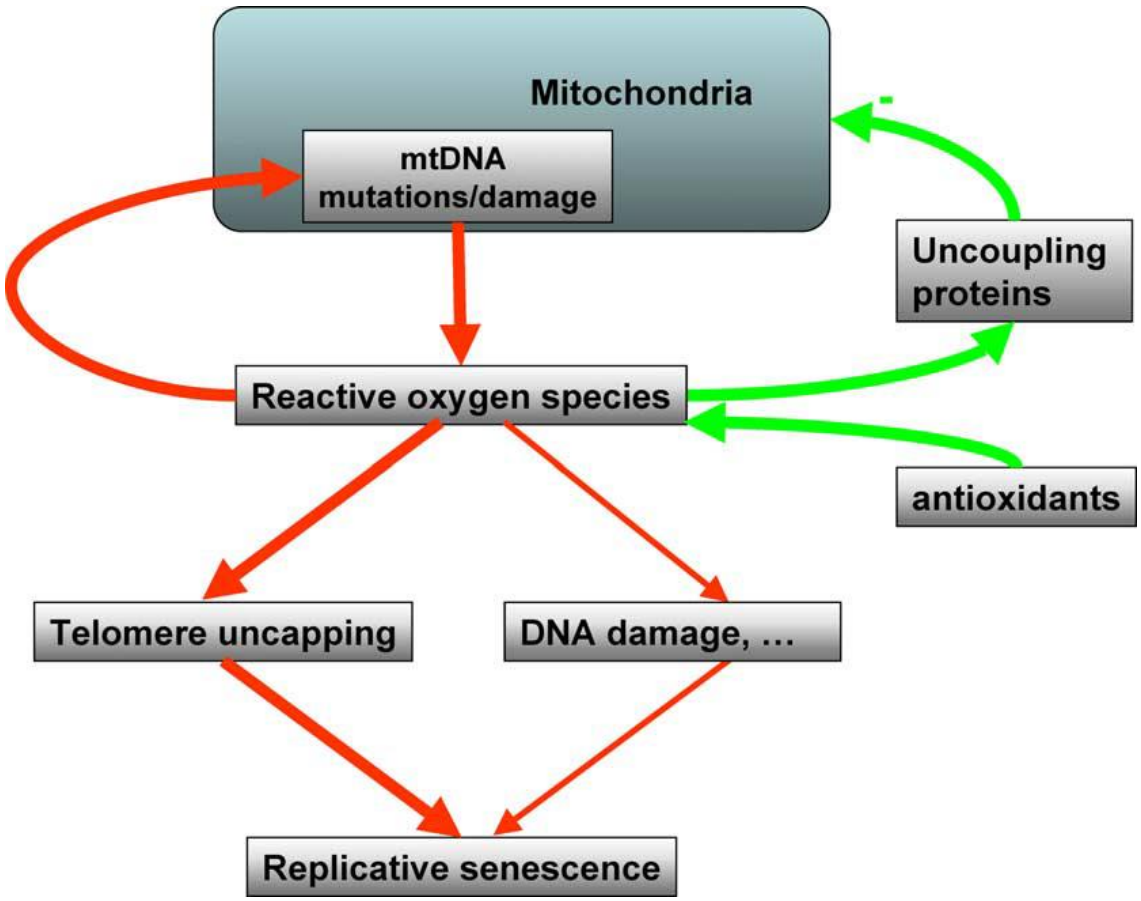


Fig.9 Mitochondrial ROS production contributes to telomere-dependent replicative senescence. ROS are produced as by-product of normal mitochondrial respiration and are partially detoxified by antioxidants. ROS cause mitochondrial DNA damage and mutation, which might enhance further ROS production. ROS accelerate telomere shortening and uncapping and may also result in some unrepaired DNA damage, both of which can activate a permanent DNA damage response that signals and maintains replicative senescence
Retrieved from Passos J.F., von Zglinicki, T "Mitochondria, telomeres and cell senescence" Experimental Gerontology 40 (2005) 466–472

Cellular senescence, ageing and cancer

The primary purpose of senescence is to prevent the propagation of damaged cells and to trigger their demise by the immune system, contributing to rid tissues from damaged and potentially oncogenic cells checkpoint.[1] However this tumor suppressive response, as time pass by, leads to an increase and accumulation of senescent cells, including cells essential for tissue function and regeneration. The depletion of the pool of mitotically competent and functional cells will ultimately lead to a functional decline of various organ systems with increasing age. It would also change the surrounding microenvironment and compromise tissue repair and renewal since senescent cells secrete a number of matrix metalloproteinases and inflammatory cytokines that can alter the surrounding tissue structure and cause local inflammation. [18]

It seems that cellular senescence acts as a mediator in the trade-off between cancer and ageing. Such trade-off can be seen in mice with constitutively hyperactive forms of p53. This mice, despite tumour resistant show multiple signs of accelerated ageing. [67] [76]

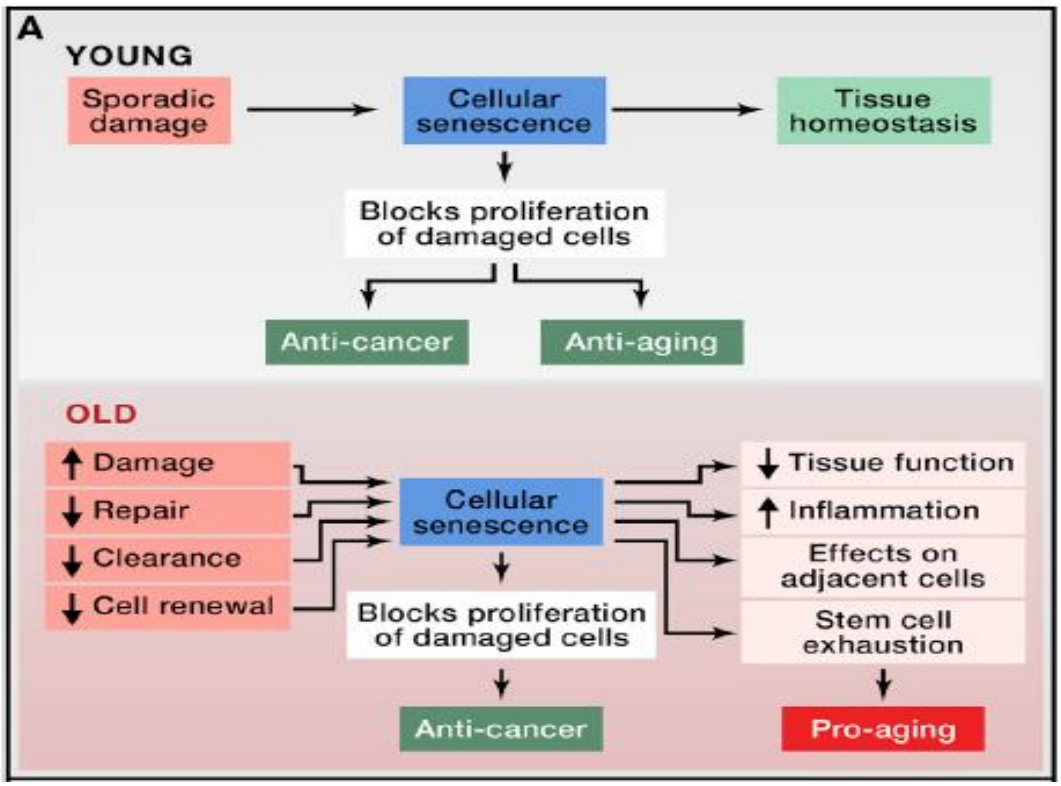


Fig.10 Cellular senescence. In young organisms, cellular senescence prevents the proliferation of damaged cells, thus protecting from cancer and contributing to tissue homeostasis. In old organisms, the pervasive damage and the deficient clearance of senescent cells result in their accumulation, and this has a number of deleterious effects on tissue homeostasis that contribute to aging
Retrieved from Otin C.L., Blasco M.A., "The Hallmarks of Aging" Cell 153, June 6, 2013

Monoamine Oxidases

Monoamine oxidase (MAO, EC 1.4.3.4) is a flavin-adnosine-dinucleotide (FAD) containing enzyme located on the outer mitochondrial membrane. It exists in two functional isozymic forms, termed MAO-A and MAO-B, which have 70% sequence identity as deduced from their cDNA clones. These two forms of the enzyme can be distinguished by differences in substrate preference, inhibitor specificity, tissue distribution, immunological properties, and amino acid sequences. [77]

MAO-A and -B are expressed in most tissues [86]. Even so MAO-A is characteristically abundant in fibroblasts and placenta in contrast to MAO-B isoenzyme which is expressed in platelets and lymphocytes. Both isoenzymes are present in most brain regions; however, certain areas are more prone to express one isoenzyme [88] [89].

The monoamine oxidases catalyse the oxidative deamination of various biogenic amines, such as neurotransmitters, dietary amines and xenobiotics to the corresponding aldehydes as exemplified in fig11. This reaction requires flavin adenine dinucleotide (FAD) as a cofactor [89].

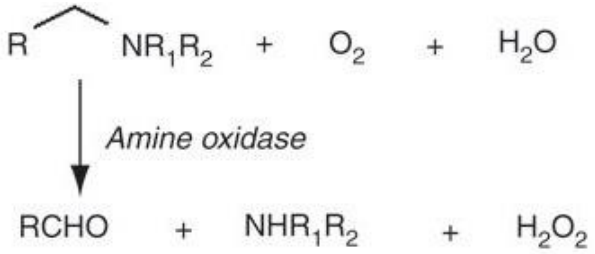


Fig11 Schematic representation of the general reactions of MAO

The preferential substrate of MAO-A is the serotonin (5-HT) and norepinephrine although it can also oxidize tyramine and dopamine [87] and is inhibited by low concentrations of clorgyline [90].

Clorgyline is a well established specific inhibitor of MAO-A which has been used both in research [93] and clinical trials [91] [92]

MAO serves a primary role in the degradation of primary, secondary, and some tertiary xenobiotic amines, which is particularly important to preventing their cardio- and neurotoxicity. [86]

MAO function is highly critical for the regulation the intracellular redox state in neurons and other cells; indeed, one of the by products of MAO-mediated reaction, hydrogen peroxide,

is a potent oxidizer which can trigger the formation of superoxide radicals and other reactive oxygen species, contributing to the progress of neurodegenerative disorders, such as Parkinson as well as sleep and mood disorders. [89]

Aim of the study

Determine the contribution of MAO-A to cellular senescence and possible mechanistic links between them.

Hypothesis: MAO-A and the induction in cellular senescence

MAO-A, the mitochondrial enzyme by catalyzing the oxidative deamination of biogenic and exogenous amines, contributes to the ROS production (H2O2), and so, contributing to senescence induction and ageing. three research articles have already proposed the influence of MAO-A in the ageing heart by induction of cellular senescence through the increase of cardiac oxidative stress and activation of the p53 pathway (fig12). [78], [79], [93], Also it was found in an independent study that MAO-A mRNA expression is increases in human senescent fibroblasts [80]. Furthermore, it is known that ROS production, DNA damage and DDR are all connected in a positive feedback loop despite the precise mechanisms are unknown. [81]

Taking all this into account, further studies to determine the contribution of MAO-A to the link between, ROS production, DNA damage and the permanent DDR to induce and maintain cellular senescence are needed (Fig13)

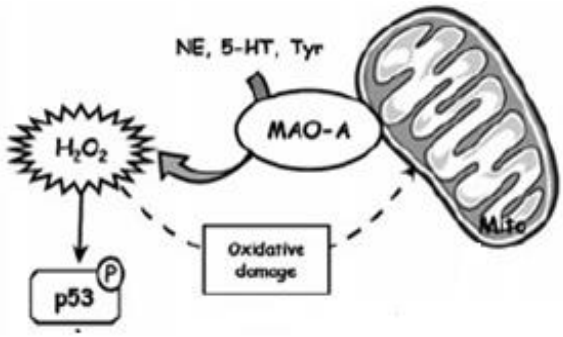


Fig 12 The role of MAO-A in cellular senescence. The p53 activation will ultimately lead to cell senescence
Retrieved from Villeneuve C., Frugier C.G., p53-PGC-1a Pathway Mediates Oxidative Mitochondrial Damage and Cardiomyocyte Necrosis Induced by Monoamine Oxidase-A Upregulation: Role in Chronic Left Ventricular Dysfunction in Mice ANTIOXIDANTS & REDOX SIGNALING Volume 00, Number 00, 2012

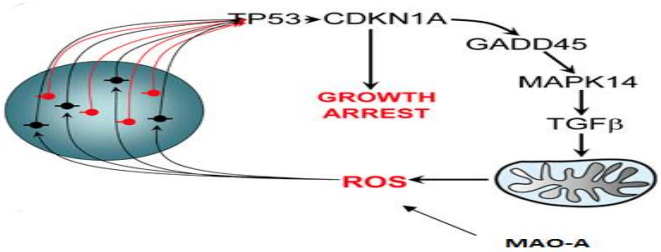


Fig 13 Scheme illustrating the feedback loop model between DDR, mitochondrial dysfunction and ROS generation, Telomere associated foci (TAF) (red), double strand breaks (black) trigger the DDR and signalling through TP53, CDKN1A, CDKN1A, GADD45, MAPK14 and TGFβ leading to mitochondrial dysfunction and an increase in ROS production, ROS causes further DNA damage which leads to a constitutively active DDR causing a permanent growth arrest and stabilisation of the senescent phenotype. MAO-A is hypothesised to contribute to this feedback loop through ROS production

Material and Methods

All chemicals were from Sigma unless indicated otherwise.

Cell lines

Normal human foetal lung fibroblasts were obtained from European Collection of Cell Cultures (ECACC, #05090501) (Salisbury, UK).

Cell culture routine

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, D7596) supplemented with foetal bovine serum (FBS) (10% v/v) (Sigma, 12133C), L-glutamine (2mM) (Sigma, G3126), penicillin (100 units/ml)/streptomycin (100 μg/ml) (Sigma, P4333) and maintained at 37°C, 5% CO_2. Cell culture was carried out using aseptic technique in a class II safety cabinet. Cell passaging included a phosphate buffered saline (PBS) wash, subsequent trypsinisation with pre-warmed Trypsin-EDTA (TE) (0.5% Trypsin, 0.2% EDTA) (Sigma, T3924) at 37°C followed by neutralisation with cell culture medium. Cells were then collected by centrifugation or resuspended in fresh medium at a suitable density and reseeded into tissue culture flasks or dishes. Renew of the medium was done every three days

Cryogenic storage

Exponentially growing adherent cells were trypsinised and centrifuged at 800 g for 5 minutes at room temperature. The supernatant was removed and cells were washed in sterile PBS, recentrifuged and resuspended in FBS containing 5% (v/v) dimethyl sulfoxide (DMSO) (Sigma, D2650) at a density of 1 x 10^6 cells/ml. Aliquots of 1ml cell suspension were immediately transferred to cryo-vials and placed in a Nalgene™ Cryo freezing container filled with isopropanol. Cells were kept for 24 hours at -80°C to allow them to slowly freeze before being stored long-term in liquid nitrogen.

Thawing of frozen cells

Cryo-vials were removed from liquid nitrogen and quickly thawed at 37°C for 1 to 2 minutes. Cells were immediately seeded into a 75 cm^2 with 20 ml pre-warmed medium. Medium was replaced after 24 hours to remove DMSO and cell debris.

Calculation of cell density and population doublings

To determine the concentration of cells within the cell suspension, 20 µl of suspension was added to a Fuchs-Rosenthal 0.2 mm haemocytometer (VWR International UK) and cells manually counted under a standard microscope (DMIL, Leica Microsystems, UK). The average of four counts of 8 squares was taken, which is equivalent to the number of cells x 10^4/ml. Cells within and touching the top and left sides of the square were counted. Total cell number was calculated by multiplying the total volume of cell suspension by the number of cells per ml. With every cell passage, population doubling (PD) was calculated by comparing total cell number with number previously plated, using the following equation:

$$PD = X + (\ln(N1/N2))/\ln 2$$

Where:

PD= population doubling

X= previous PD

N1 = number of cells harvested

N2 = number of cells seeded

Cell treatments

Stress induced senescence in MRC5 fibroblasts was induced by X-ray irradiation with 20 Gy for 8 min. Following treatment, culture medium was refreshed. In the following experiments cells that were stress induced by this method were analysed 3 to 10 days after radiation

To inhibit MAO-A, cells were treated with Clorgyline (Sigma M3778-50MG) dissolved in water. The chemical inhibitor Clorgyline was replaced every 3 days in cell culture at 10 or 100 µM concentration unless in one experiment were it was replaced every day at a concentration of 1 µM. (The goal of this experiment was to see the effect in PD of chronic clorgyline treatment in MRC5 life span called here afterwards long term experiment (LTE)).

FACS

Calibration of the flow cytometer (Partec) was carried out using fluorescent beads to ensure optimum performance and reproducibility. . For each experiment 1 x 10^5 cells were analysed per measurement.

For reactive oxygen species (ROS) production measurements, MRC5 fibroblasts (non-irradiated and irradiated; treated and non-treated with clorgyline) were trypsinised followed by neutralisation using DMEM (Sigma, Dorset, UK) supplemented with 1% penicillin/streptomycin, 2 mM L-Glutamine and 10% heat-inactivated foetal bovine serum. Cells were centrifuged at 1600 rpm for 2 minutes at room temperature, and the pellet was re-suspended in 1 ml of serum-free DMEM containing either dihydroethidium (DHE; 5mM stock; 1µl/ml) (#D-23107, Invitrogen), a blue fluorescent dye used to monitor superoxide production which, when reacts with superoxide anions, DHE forms a red fluorescent product or dihydrorhodamine (DHR123; 5mM stock; 3µl/ml) (#D-23806, Invitrogen) which is a cell-permeable dye used to detect ROS, including peroxynitrite and peroxide. Upon oxidation by ROS, Rhodamine 123 is formed, which is highly fluorescent.

Cells were incubated in the dark for 30 minutes at 37°C. Following centrifugation at 1600 rpm for 2 minutes, the supernatant was discarded and serum free DMEM was added to the pellets, which were re-suspended immediately before analysis by flow cytometry (Partec). The population of live cells is defined in a FSC/SSC dot plot and apoptotic cells and debris are excluded. Fluorescence intensity is determined in FL3 for DHE and FL1 for DHR123, conveniently in a FSC/FL3 or FSC/FL1 dot plot.

Western Blotting

Samples were prepared by adding the appropriate amount of protein lysate to 2X sample buffer containing 50 µl 2-mercaptoethanol, such that a final concentration of 1µg/µl of protein was achieved

MRC5 fibroblasts were lysed using ice-cold RIPA buffer (150 mM NaCl, 1% NP- 40, 0.5% NaDoC, 0.1% SDS, 50 mM tris-HCl, pH 7.4, supplemented with Halt™ complete phosphatase and protease inhibitor cocktail 1%(Thermo)). Cell lysates was then centrifuged at 4^{o}C at 13000 rpm for 10 mins to remove insoluble cellular components. Protein concentration was measured by the Bradford assay (BioRad) using an Omega FLUOstar plate reader. Samples were prepared by adding to them, SDS-Loading buffer (BioRad) in the presence of 5% β-Mercaptoethanol. Final

protein concentration in each sample should be between 30-50µg.

Samples were then heated at 100°C for 5 minutes, and placed on ice until loading.

Protein samples were loaded and run on 15% resolving gel (2.3 ml sterile H_2O, 5 ml 30% acrylamide, 2.5 ml 1.5 M Tris pH 8.8, 100 µl 10% SDS, 100 µl 10% ammonium persulphate and 4 µl TEMED) with running buffer Samples were run for 90 minutes at a voltage of 125 V in the presence of running buffer (25mM Tris, 0.192 mM Glycine) and transferred to Immobilon®-P (Millipore) membrane using Trans-Blot® Semi-Dry transfer (BioRad).
A protein ladder (Precision Plus Protein™ Dual Color Standards) was also loaded.

Following transfer, membranes were incubated in Ponceau solution for 2 minutes to allow protein band visualisation, and excess solution was washed off with water. Afterwards the membrane was blocked in freshly prepared PBS containing 10% non-fat dry milk and 0.2% Tween-20 (PBST-MLK) for 60 minutes at room temperature with shaking.

Membranes were incubated with 10-15 ml primary antibodies diluted in 5% milk PBST (0.2%) sodium azide (20%) overnight at 4° (antibodies used: Rabbit anti-MAO-A 1:100, Santa Cruz (sc-20156); Rabbit anti-p21 1:200, Abcam (ab7960); Rabbit anti-GAPDH, 1:5000, Cell Signalling)

Following 5 washes in MilliQ water for 5 minutes each, the membrane were either incubated with goat anti-mouse or goat anti-rabbit HRP conjugated IgG secondary antibody (1:5000) (Sigma, UK) in PBS containing 5% non-fat milk and 0.2% Tween-20 for 1 hours at room temperature on a shaker. Membranes were then 5 times in MilliQ water, 4 times in PBS with 0.05% Tween for 3 mins and a further 5 times in MilliQ water. Signal was detected by chemiluminescence using FujiFilm LAS-4000 and the Amersham™ ECL™ prime Western blotting kit (GE Healthcare)

Immunofluorescence/Immunocytochemistry

MRC5 fibroblasts were grown in coverslips and fixed with 2% PFA in PBS for 10 minutes at room temperature. PFA was removed and cells were washed two times with PBS.

To permeabilise, cells were incubated with 1 ml PBG-Triton for 45 minutes at room temperature.

Cells were incubated with primary antibody of interest (table 1) at room temperature in a shaker for 2 hours. Subsequently, Cells were washed three times with PBG-Triton for 5

minutes and incubated for 45 minutes to 1 hour with fluorescein-conjugated secondary antibody of interest (table 2) diluted 1:4000 in PBG-Triton away from light. Cells were washed 3 times with PBS for 5 minutes each, and then mounted with Vectashield hardset mounting medium with DAPI. Imaging was done using a Leica DM5500B microscope.

Antibody	Species	Dilution
Ki67 (Abcam, ab15580)	Rabbit polyclonal	1:250
anti-phospho-histone H2A.X (γH2AX) (Ser139) (Millipore, 05-636)	Mouse monoclonal	1:2000
anti-53BP1 antibody (Cell Signalling)	Rabbit	1:250
Rabbit anti-p21 1:200, Abcam (ab7960	Rabbit polyclonal	1:200

Table 1 Primary antibodies used for immunofluorescence on cells

Antibody	Species	Dilution
Anti-rabbit Fluorescein-conjugated secondary antibody AlexaFluor 594 (Invitrogen, A21213)	Goat	1:4000
Anti-mouse Fluorescein-conjugated secondary antibody AlexaFluor 488 (Invitrogen, A21042)	Goat	1:4000

Table 2 Secondary antibodies used for immunofluorescence on cells

Immuno FISH (γH2AX-TeloFISH) staining on fixed cells

MRC5 fibroblasts were grown in coverslips and fixed with 2% paraformaldehyde (PFA) in PBS. Cells were incubated with rabbit monoclonal anti-γH2A.X (Ser139) (1:200) (#9718A, Cell Signalling) and mouse monoclonal anti-TRF2 (1:200) (#05-521, Clone 4A794, Millipore) primary antibodies overnight at 4°C. Subsequently, AlexaFluor 647 goat anti-rabbit IgG (1:1000) (#A21244, Invitrogen) and AlexaFluor 488 goat anti-mouse IgG (1:1000) (#A11001, Invitrogen) secondary antibodies were applied and left to incubate for 1 hour at room temperature. Cells were washed in PBS three times for 5 minutes, and fixed using methanol and acetic acid (3:1) for 30 minutes. Following fixation, cells were dehydrated with 70%, 90% and 100% cold ethanol for 2 minutes each, and then immersed in PBS at 37°C for 5 minutes. After incubation with 4% PFA at 37°C for 2 minutes each, cells were washed with PBS and again dehydrated with 70%, 90%, 100% cold ethanol. Next, 10 µl of hybridisation buffer (70% deionised formamide, 25 mM $MgCl_2$, 1 mM Tris pH 7.2, 5% blocking reagent (Roche)) containing Cy-3-labelled telomere specific (C3TA2)3 peptide nucleic acid (PNA) probe (4 ng μl^{-1}) (Panagene) was applied to each coverslip, followed by denaturation at 80°C for 10 minutes and hybridisation for 2 hours at room temperature. Cells were washed with wash buffer containing 70% formamide and 30% SSC 2% three times for 10 minutes each, and then with TBS-Tween 0.05% three times for 5 minutes each. Following dehydration with 70%, 90% and 100% cold ethanol for 2 minutes each, cells were allowed to air dry and then mounted with Vectashield hardset mounting medium with DAPI. Imaging was done using Leica DM5500B microscope objective.

Senescence-associated β-galactosidase staining

Cells were grown in coverslips and fixed with 2% PFA in PBS. Cells were washed twice with PBS and then incubated overnight at 37° C in senescence-associated β-galactosidade staining solution containing 2 mM magnesium chloride, 150 mM sodium chloride, 40 mM citric acid, 12 mM sodium phosphate dibasic, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 1 mg/l 5-bromo-4-chloro-3-inolyl-β-d-galactoside (X-Gal) at pH 6.0. Next, cells were washed three times with PBS for 5 minutes each, and nuclei were stained by incubating with DAPI for 10 minutes at room temperature. Following three PBS washes for 5 minutes each, coverslips were mounted using Vectashield hardset mounting medium and imaged using a Leica DFC310FX microscope.

Microscopy

Samples for ImmunoFISH were imaged with a Leica DM 5500B Widefield Microscope through an HCX PL APO 100x/1.40-0.70 oil objective using a Leica DFC 360 FX camera. Images were captured as z-stacks separated by 0.247µm using the LAS-Leica software suite. Slides obtained using immunofluorescence procedures were imaged on the same system using an HCX PL APO 40x/1.25 oil objective for 53bp1 and γH2AX markers and an HCX PL APO 20x objective for Ki67 and p21 markers.For the senescence-associated β-galactosidase staining images were obtained using Leica DFC310FX microscope with an HCX PL APO 20x magnification Images were analysed and prepared using the software ImageJ (http://rsb.info.nih.gov/ij/), Adobe Photoshop CS6 and paint.

ELISA (IL8)

Cytokine concentrations in cell culture supernatants were determined using commercially available ELISA kits (Duoset, R&D systems), according to the manufacturer's instructions [94]. All assays were carried out at room temperature.

Results

MAO-A is expressed in proliferative and overexpressed in IR and RS MRC5 fibroblasts

Using MRC5 fibroblasts as a model for studying cellular senesce [82], the first thing to do was to observe differences in expression of MAO-A protein content between proliferative, IR and RS cells, the last two representing 2 different models of cell senescence and check if indeed there is some link between MAO-A and cellular senescence. To test this, a western blot was made with anti MAO-A with the results represented in fig14.

The results show us an increase in the expression of MAO-A in IR cells at the early time points and a huge increase of MAO-A in RS cells, both compared to proliferative cells. Such observations indicate that, somehow, senescent cells, even in different models of cellular senescence, MAO-A protein content are correlated even if it is not possible to determine the casual/consequence of this relationship

It is still possible to observe that MAO-B protein content is much more expressed in IR (all time points) and RS compared to proliferative cells.

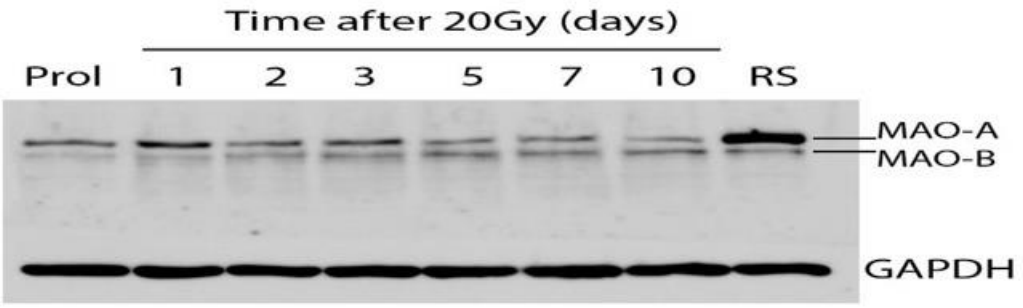


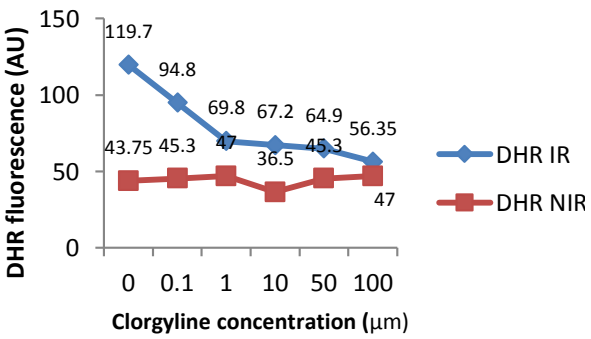
Fig.14 Western blot to determine the expression of MAO-A protein content in proliferative, IR (20 Gy, timepoints 1,2,3,5,7,10 days) and RS MRC5 fibroblasts. Using anti-MAO-A antibody, two bands at distinctive molecular weights appear. The top one corresponds to MAO-A (61 kDa) and the lower one corresponds to MAO-B (59 kDa). GAPDH (37 kDa) was the loading control

Clorgyline treated MRC5 fibroblasts decreases ROS in IR cells but not in proliferative ones

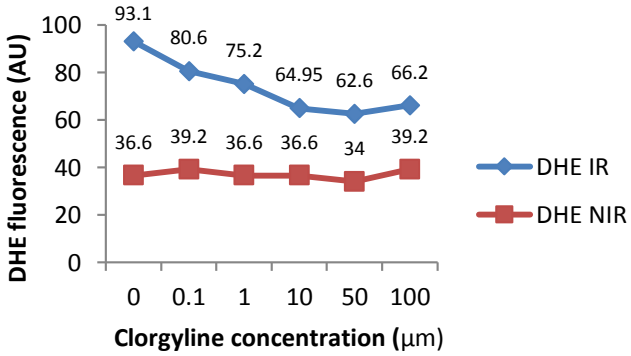
In order to further characterise the role of MAO-A in cellular senescence we analysed ROS measurements in IR and NIR MRC5 fibroblasts treated with clorgyline by FACS. Clorgyline is a specific chemical inhibitor of MAO-A, and so, cells treated with clorgyline should have an impact on the amount of ROS produced and has been shown that ROS are important in the establishment and maintenance of senescence through a feedback loop involving DDR and mitochondrial dysfunction.

The results obtained in fig15 (A) and (B) show us a clarify decrease in DHR and DHE fluorescence intensity (and so in ROS) in IR MRC5 fibroblasts alongside an increasing gradient concentration of Clorgyline but not in NIR cells. Moreover the DHR and DHE value increases slightly in NIR cells. Also, it is to be noted that the decrease in DHR is higher than the one observed for DHE and the rate of decrease of fluorescence in both of them is much lower around 10 µm of clorgyline. (C) and (D) show us the same experiment but with just 10 and 100 µm of clorgyline used in cells (the ones that had greater effect). The results have the same pattern as in (A) and (B).

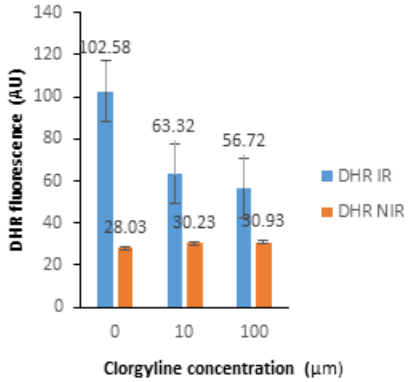
A



B



C



D

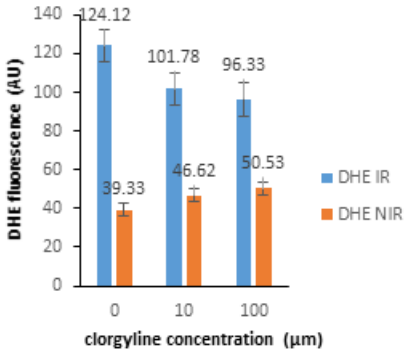


Figure 15 Quantification of ROS in IR and NIR MRC5 fibroblasts with clorgyline treatment, obtained using DHR (A and C) and DHE (B and D) intensity measured by flow cytometry. IR cells values were measured after 3 days of 20Gy X-ray radiation. (A) Change of DHR fluorescence intensity of MRC5 fibroblasts IR and NIR over a gradient concentration of clorgyline. (B) Change of DHE fluorescence intensity of MRC5 fibroblasts IR and NIR over a gradient concentration of clorgyline. (C) Change of DHR fluorescence intensity of MRC5 fibroblasts IR and NIR in controls and clogyline treated (10 µm and µm100) data are mean ± s.e.m of *n*=3(D) Change of DHR fluorescence intensity of MRC5 fibroblasts IR and NIR in controls and clogyline treated (10 µm and µm100). data are mean ± s.e.m of *n*=3

Clorgyline treated MRC5 fibroblasts slightly decreases senescent phenotype in IR compared to control IR

After the observation that clorgyline reduces ROS in MRC5 IR cells (and so, in senescent cells), we decided to proceed to the quantification of senescent marker [8] (ki67 and γH2A.X) by immunocytochemistry in different four populations (control NIR; control IR; treated NIR; treated IR) MRC5 fibroblasts cells. Clorgyline concentrations used, were 10 and 100 µm. MRC5 IR were analysed 3 days after irradiation.

Through fig 16 (A) and respective quantification (C) MRC5 irradiation eradicates proliferation which can be seen by the steep decrease of the proliferative marker ki67 between NIR an IR MRC5, although cells treated with clorgyline show some rescue. The higher the concentration of clorgyline, the higher the rescue.

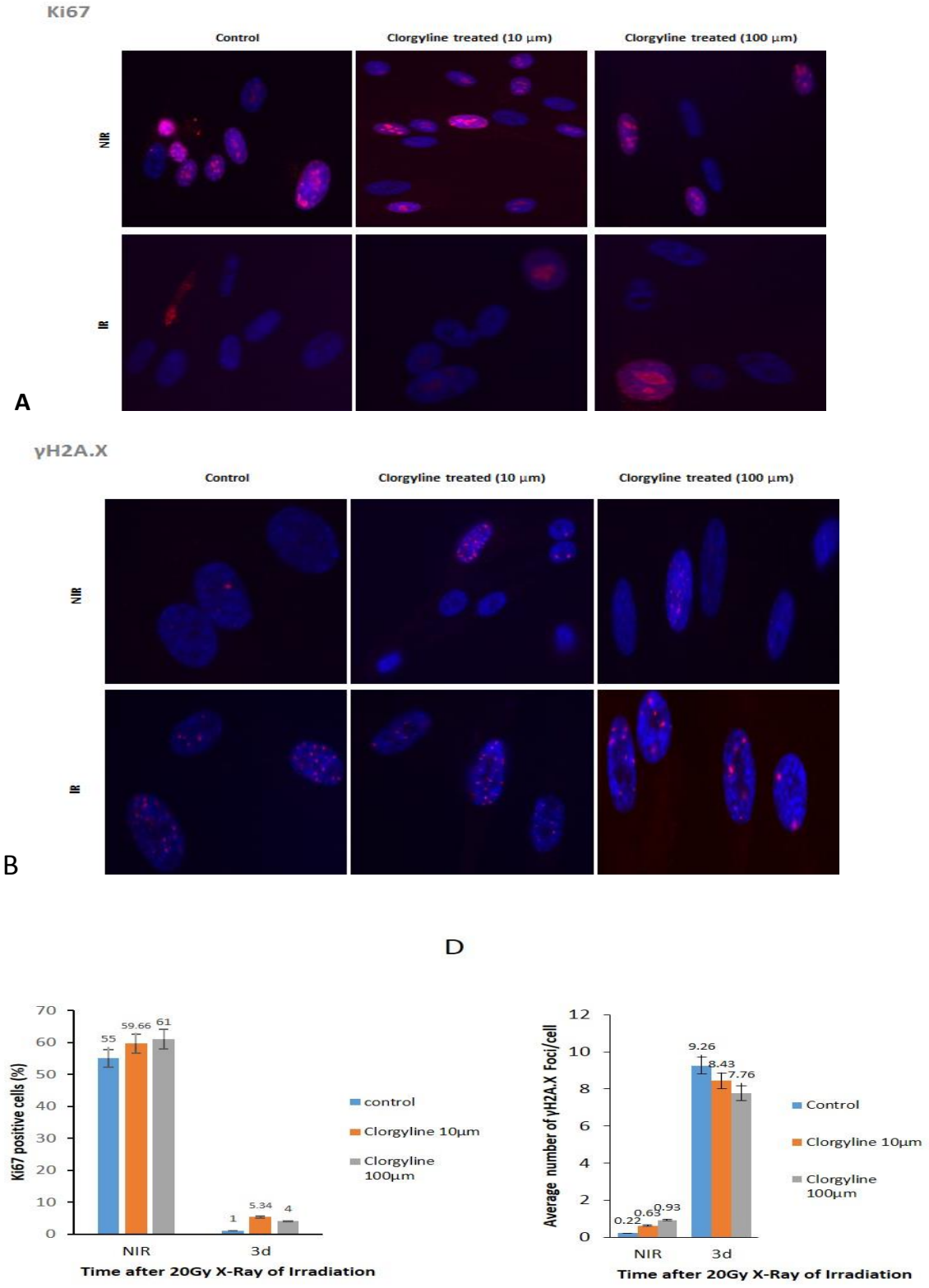


Fig 16 Representative images of *NIR and IR (*20Gy X-Ray irradiation) MRC5 cells treated with different concentrations of clorgyline, immunostained using (A) anti-Ki67 (B) anti- γH2A.X (γH2A.X and Ki67:red, Nucleus: blue) ;(C) Percentage of Ki67-positive cells in NIR and IR 3 day cells treated with MRC5 treated with different concentrations of clorgyline (D) Average number of γH2A.X foci per cell in NIR and IR 3 day cells treated with MRC5 treated with different concentrations of clorgyline. data are mean±s.e.m of n≥30.

The same kind of observation can be made into γH2A.X Foci (and so, DNA damage), by looking into fig 16 (B) and (D). Irradiation induces in average, much more γH2A.X Foci per cell than NIR cells but still, clorgyline treatment reduces the amount of Foci in irradiated cells (the higher the concentration, the higher is the decrease), compared to irradiated non treated cells. On the other way around it seems that in NIR cells, treatment with clorgyline increases the average number of γH2A.X Foci per cell, despite, this increase is in the order of decimal (from 0.22 to 0.93) while in IR MRC5 the decrease in Foci is in units (from 9.26 to 7.76)

Furthermore, the same kind of analyse was made in a similar but independent experiment which cells were analysed by the same senescent markers plus SA-β-Galactosidase with different four populations (control NIR; control IR; treated NIR; treated IR) MRC5 fibroblasts cells, but this time, MRC5 IR were analysed 10 days after irradiation.

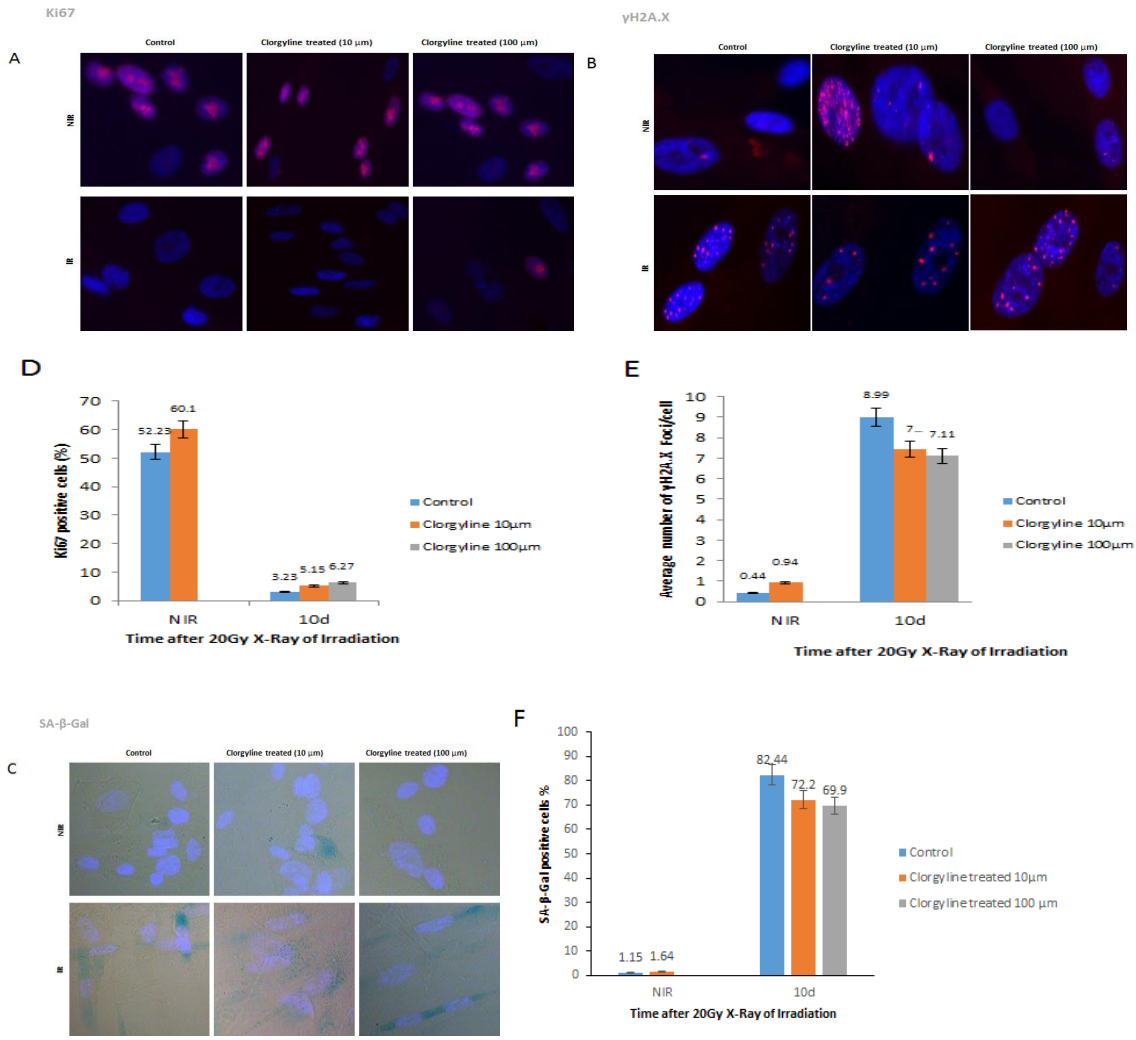


Fig 16 Representative images of *NIR and IR (*20Gy X-Ray irradiation) MRC5 cells treated with different concentrations of clorgyline, immunostained using (A) anti-Ki67 (B) anti- γH2A.X (C) SA-β-Gal (γH2A.X and Ki67:red, SA-β-Gal: green blue shade around nucleus, Nucleus: blue) ;(D) Percentage of Ki67-positive cells in NIR and IR 10 day cells treated with MRC5 treated with different concentrations of clorgyline (E) Average number of γH2A.X foci per cell in NIR and IR 10 day cells treated with MRC5 treated with different concentrations of clorgyline. (F) Percentage of SA-β-G cells in NIR and IR 10 day cells treated with MRC5 treated with different concentrations of clorgyline. Data are mean±s.e.m of n≥30.

Regarding to ki67 and γH2A.X the results can be described as the same in the previous experiment. An increase in proliferation markers in MRC5 treated with clorgyline both NIR and IR; a decrease in DNA damage foci in cells treated with clorgyline in IR cells and a slightly increase in NIR. After 10 days, cells treated with clorgyline 100µM were dead (NIR) in this experiment, probably due to cytotoxicity of the chemical for prolonged exposure to the chemical. In IR clorgyline has a beneficial effect and so, the opposite effect. Concerning SA-β-Gal staining, the results are consistent to the results obtained for other markers. IR MRC5 treated with clorgyline have a decrease (around 10 %) of SA-β-Gal stained compared to non treated. Differences between NIR are not significant.

Clorgyline treatment slightly decreases senescent phenotype from proliferative MRC5 fibroblasts until they reach RS

So far, the model of stress induced senescence through X-ray radiation has been the major model for characterizing MAO-A in cellular senescence. In this experiment the RS model was the one to be characterize.

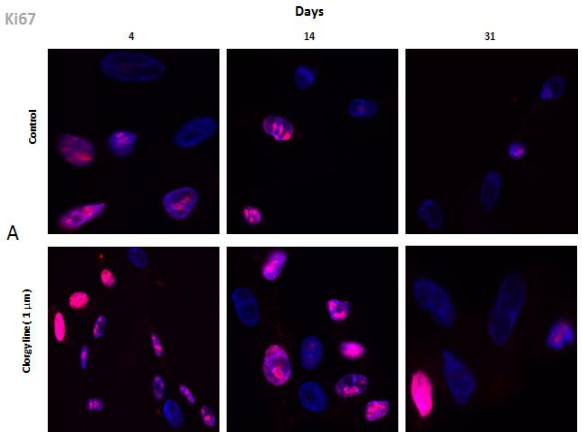
Proliferative MRC5 cells with a PD around 30, started to being treated with clorgyline at a low concentration (1µ) every day. Time points were obtained until they reached senescent phenotype. This experiment lasted around 30 to 40 days (LTE).

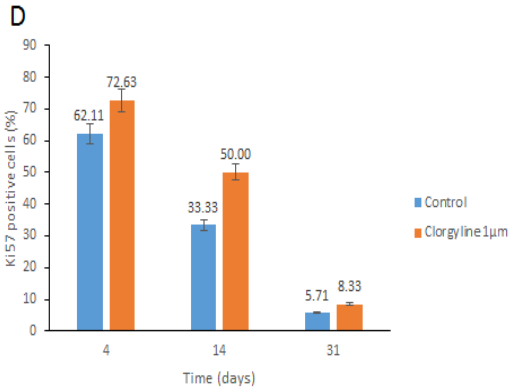
Senescent markers (ki67 and 53bp1, P21), population doubling curve and ROS production were analysed/obtained as depicted in fig.17. 53bp1 like γH2A.X is a marker of DNA damage.

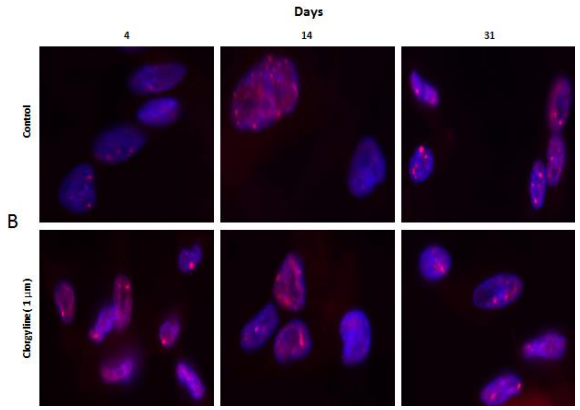
Despite senescent markers intensity increasing over time/(over PD) as they approach RS state, MRC5 treated with clorgyline resulted, alike in the stress induced model through radiation, a reduction in senescent markers compared to the control population.

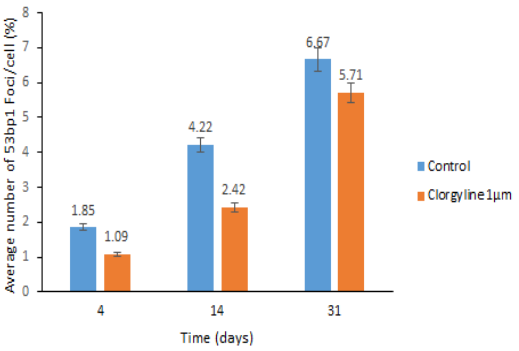
Ki67 (A), (D) presents a difference of 3-20 % positive between treated and non treated MRC5. 53bp1 (B) (E) in comparison to γH2A.X presents less foci per cell, but the ratio of foci between treated and non treated still maintains. MRC5 treated presents 1-2 less foci than non treated. P21 (C) (I) expression, (which is related directly to the induction of senescent phenotype) also show a difference around 20% positive cells between treated and non treated MRC5. ROS production (F) (G) also increase over time in both MRC5 cultures, but again, control MRC5 produce much more than non treated MRC5.

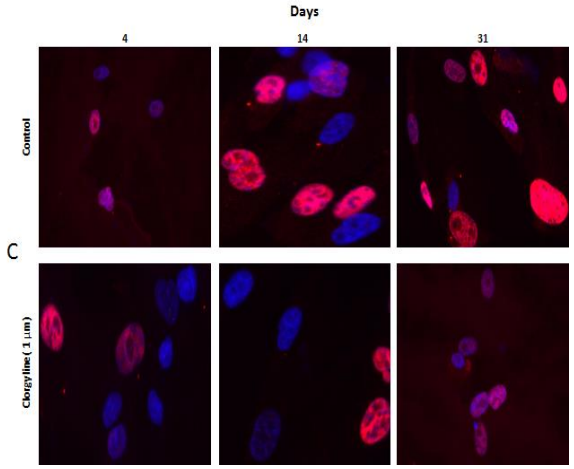
The results in fig17, A B C D E F G probably contribute to the difference of PD between the two populations in H.

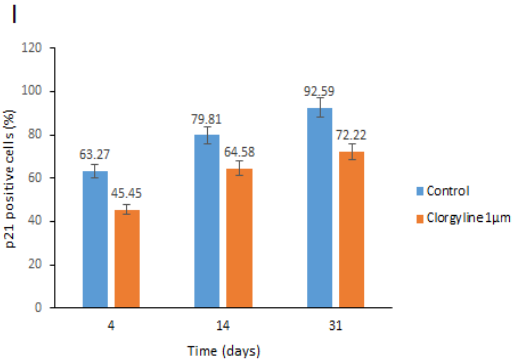

Ki67
Days
4
14
31
Control
Clorgyline (1 µm)
A

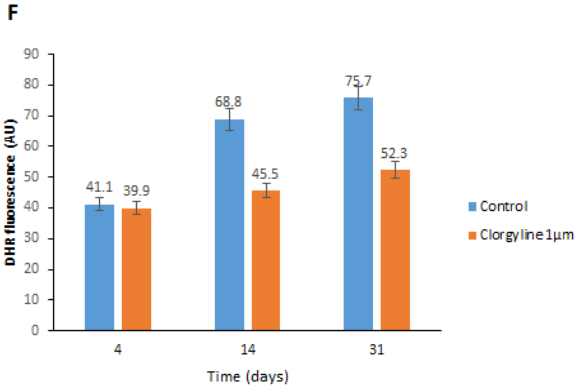

D
Ki67 positive cells (%)
72.63
62.11
50.00
33.33
8.33
5.71
4
14
31
Time (days)
Control
Clorgyline 1µm

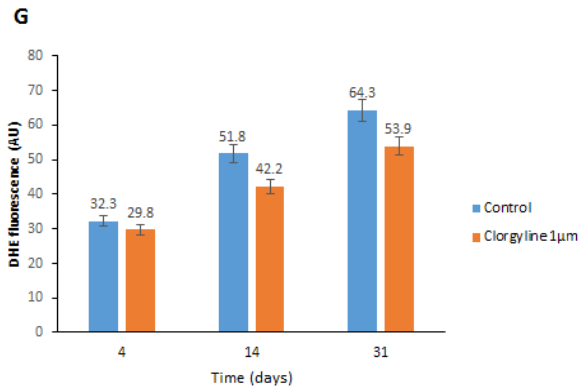

Days
4
14
31
Control
Clorgyline (1 µm)
B


E
Average number of 53bp1 Foci/cell (%)
6.67
5.71
4.22
2.42
1.85
1.09
4
14
31
Time (days)
Control
Clorgyline 1µm


Days
4
14
31
Control
Clorgyline (1 µm)
C


I
p21 positive cells (%)
92.59
79.81
72.22
64.58
63.27
45.45
4
14
31
Time (days)
Control
Clorgyline 1µm


F
DHR fluorescence (AU)
75.7
68.8
52.3
45.5
41.1
39.9
4
14
31
Time (days)
Control
Clorgyline 1µm


G
DHE fluorescence (AU)
64.3
51.8
53.9
42.2
32.3
29.8
4
14
31
Time (days)
Control
Clorgyline 1µm

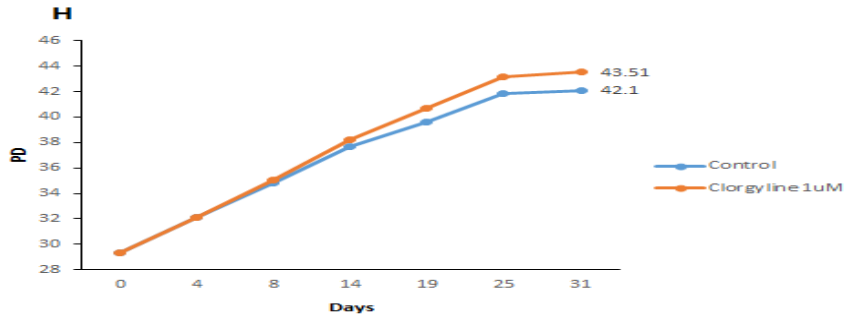


Fig 17Assessment of several markers of senescence in MRC5 fibroblasts from median (PD 29) to senescence (PD 44) Images and ROS data are retrieved from time chosen time points (4, 14 and 31 days after beginning of clorgyline treatment. population doubling Representative images of immunostained using (A) anti-Ki67 (B) anti-53bp1 (C) p21 (53bp1, Ki67 and p21:red, Nucleus: blue) and respective quantifications (D), (E) (I). Data are mean±s.e.m of n≥30. (F) and (G) Change of DHR and DHE fluorescence intensity respectively at the time points previously described. (H) Growth curve showing the difference in PD over time between control (non treated) and treated MRC5

Telomere associated foci (TAF) have been recently associated with the senescent phenotype [51], and were also checked in the described previous experiment.

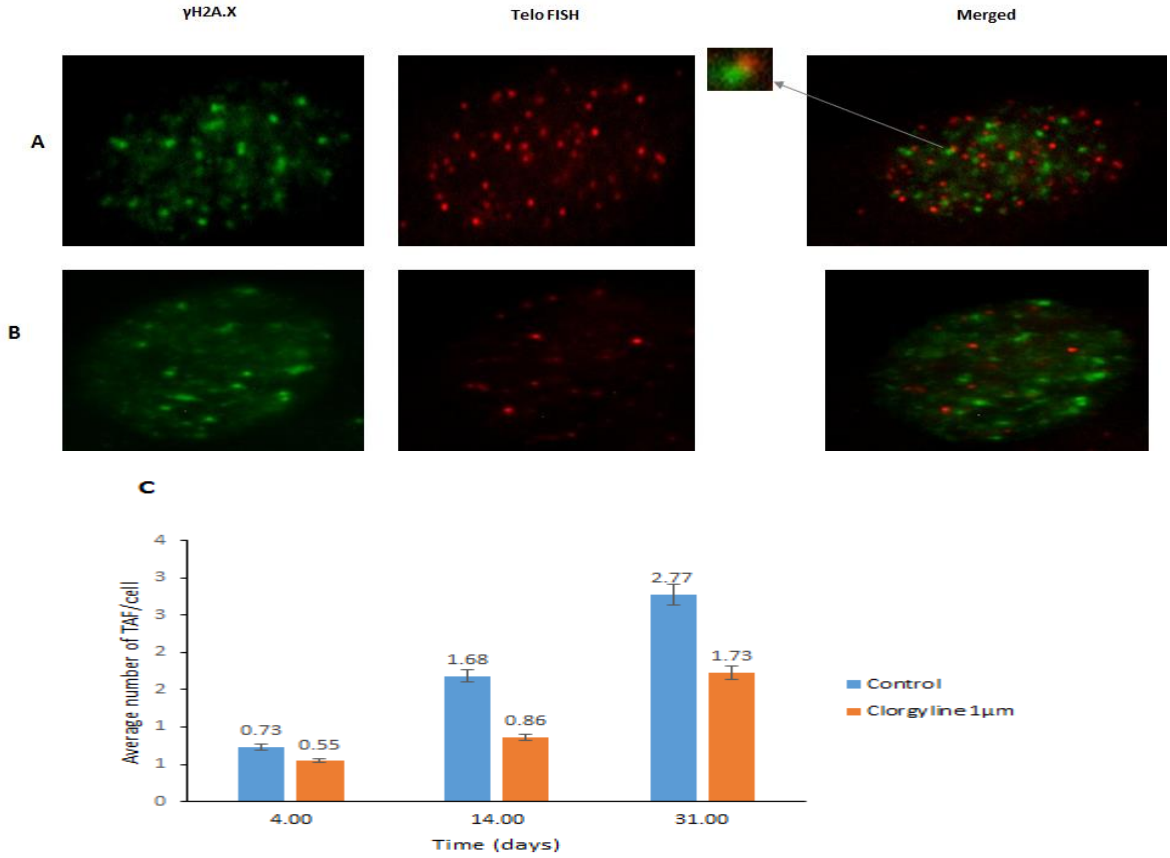


Fig 18 Representative images of γH2A.X immuno-FISH of MRC5 fibroblasts. Both (A) and (B) represent each one, one cell. In (B) there is no TAF while in (A) there is one, amplified and indicated with an arrow. (C) Chart representative of the average number of TAF per cell at the same time points and same populations as previously described.

In fig18 C the results obtained are consistent with the ones previously described. The number of TAF increases over time in both populations but the number of TAF obtained for each population is different, being the control one, the one with higher mean over the treated one.

In the last result, SASP and more recently IL-8 have been related to the induction and maintenance of the senescent phenotype [84]. IL-8 content was checked according to the sample time points previously described (fig. 19)

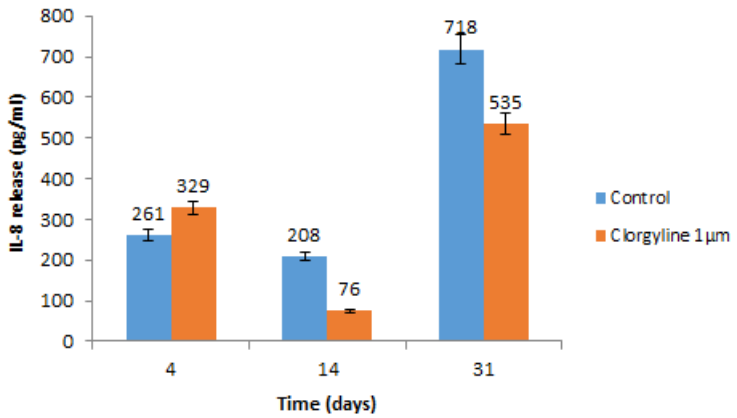


Fig 19 ELISA for IL-8 performed in samples corresponding to the same time points and same populations as previously described.

The result show us that IL-8 secretion by MRC5 over time will increase more in the population control than in the clorgyline treated. These data is consistent with the ones previously described.

Discussion

MAO-A has been vastly described in many tissues and systems but never described its role in cellular senescence. Because MAO-A produces (H2O2) as a byproduct of its catalytic reaction, it is natural that MAO-A has a role in cellular senesce via the positive feedback loop between ROS production, DNA damage and the permanent DDR [81].

Using MRC5 as a cellular model [82], we checked the protein content of MAO-A in this cell line and, as expected, in models of cellular senescence (RS cells or stress induced by X-ray radiation) this enzyme is overexpressed. It is of reference that MAO-B (which also contributes to production of ROS) is also overexpressed in these cellular models and possibly also has a role in cellular senescence.

Also, for all the markers established until now to quantify cellular senescence (proliferation markers, DNA damage Foci; components of SASP; specific assays like Senescence-associated β-galactosidase staining, ROS production), [52] [82] [88], for all of them, MAO-A specific chemical inhibition with clorgyline [90], leaded always to an alleviation of senescent phenotype in models of cellular senescence, although in proliferative cells, some senescent markers tend to be slightly increased, probably due to cytotoxic effects of this inhibitor.

Future perspectives and conclusion

This study shows for the first time a possible link between MAO-A and cellular senescence. Because it is the first of its kind, it is also in the elementary stages of research and future work is required to understand better this link between this enzyme and this cellular state.

First, immunohistochemistry of tissues with different ages, specially brain, skin and lung with senescent markers should be the next steps to provide a better characterization between MAO-A and cellular senescence. Also genomics to see the expression of MAO-A at the mRNA levels in cells and tissues at different PD/age would give important insights how theses process are linked.

Moreover, genetically engineered MRC5 to induce overexpression of MAO-A as also its inhibition through shRNA would give new models to categorize better the effects of MAO-A in cellular senescence.

Also, work should be done regarding to the monoamine oxidase B to check if does or does not follow the same patterns regarding to the link between MAO-A and cellular senescence.

At last but not least, experiments with mice should be an available option. If mice treated with clorgyline had an effect in their longevity, such finding would definitely boost this field of research

Much works still needs to be done but the perspective are enormous.

MAO-A inhibitors including Clorgyline are already in use, not just in research but also in clinical trials for treatments of mood, sleep, aggressiveness disorders. Combine this therapeutically potential with one described as anti-ageing (because fighting senescence is the same as fighting age) is the same to unite the good of two worlds

MAO-A inhibition in MRC5 leads, even in a slightly way, to a decrease of senescent markers, a decrease in ROS, and a somewhat extension of lifespan of the population, alleviating senescent phenotype. This decrease in the senescent phenotype is probably due to the break of feedback positive loop between ROS production, DNA damage and permanent DNA damage response.

Despite just a slightly decrease in senescent phenotype on the model studied, is undeniable that MAO-A has a role in cellular senescence and it is a question of time until MAO-A research is focused on this cellular pathway.

Acknowledgments

I would like to offer my special thanks to Dr Joao Passos for accepting me in and enthusiastic encouragement for this research work. Also I would like to thank all my colleagues from the Passos lab for their advice, training, patience and support during this project.

I would also like to thank the European Union and the University of Coimbra through the Erasmus Programme for providing the mobility and funding and so, allowing me to pursue my dream of studying and doing this thesis abroad in the expertise field that I wanted.

To my friends, who were always with me, thanks for all your support and friendship.

Most importantly, my gratitude to my family. None of this would have been possible without the love and support from them.

Statement of originality

I hereby declare that this submission is my own work and that all the assistance received throughout this project has been acknowledged.

References

1. Otin C.L., Blasco M.A., "The Hallmarks of Aging" Cell 153, June 6, 2013
2. http://www.senescence.info/aging_definition.html
3. Austad, S. N. (1997a). Why We Age: What Science Is Discovering about the Body's Journey through Life
4. Kowald A and Kirkwood TB. A network theory of ageing: the interactions of defective mitochondria, aberrant proteins, free radicals and scavengers in the ageing process. Mutat Res 316: 209–236, 1996
5. [5] HAYFLICK L., "Biological Aging Is No Longer an Unsolved Problem" Ann. N.Y. Acad. Sci. 1100: 1–13 (2007)
6. Mortality patterns suggest lack of senescence in hydra. Exp Gerontol 1998; 33: 217–25
7. F. Boero, B. Aeschbach, V. Schmid (1996). "Reversing the life cycle: medusae transforming into polyps and cell transdifferentiation in Turritopsis nutricula (Cnidaria, Hydrozoa)". Biological Bulletin 190 (3): 302–312
8. Weinert B.T., Timiras P.S.; Invited Review: Theories of aging, J Appl Physiol VOL 95 OCTOBER 2003
9. Teixeira INDO, Guariento ME, Biology of aging: theories, mechanisms, and perspectives, Ciência & Saúde Coletiva, 15(6):2845-2857, 2010
10. Kirkwood T.B.L, Austad S.N., "Why do we age?" Nature, vol. 408, 9 November 2000
11. Medawar PB. An Unsolved Problem in Biology. London: Lewis, 1952.
12. Williams, G. C. (1957). "Pleiotropy, natural selection, and the evolution of senescence." Evolution 11:398-411.
13. Kirkwood, T. B. (1977). "Evolution of ageing." Nature 270(5635):301-304
14. MacArthur, R. H., and Wilson, E. O. (1967). The Theory of Island Biogeography. Princeton University Press, Princeton
15. Robertson OH. Prolongation of the life span of Kokanee salmon (Oncorhynkus nerka kennerlyi) by castration before beginningof gonad development. Proc Natl Acad Sci U S A 1961; 49:517–21.
16. Austad, S. N. (1997b). "Comparative aging and life histories in mammals." Exp Gerontol 32(1-2):23-38
17. Orgel, L. E. (1963). "The maintenance of the accuracy of protein synthesis and its relevance to ageing." Proc Natl Acad Sci U S A 49:517-521. Harman, D. (1956). "Aging: a theory based on free radical and radiation chemistry." J Gerontol 11(3):298-300

18. Campisi, J. & d'Adda di Fagagna, F. Cellular senescence: when bad things happen to good cells. Nat. Rev. Mol. Cell. Biol. 8, 729–740 (2007)
19. Hani Rayess, Marilene B. Wang, Eri S. Srivatsan Cellular senescence and tumor suppressor gene p16 Int J Cancer. 2012 April 15; 130(8): 1715–1725
20. Goldstein, S. (1990). "Replicative senescence: the human fibroblast comes of age." Science 249(4973):1129-1133
21. Hayflick, L., and Moorhead, P. S. (1961). "The serial cultivation of human diploid cell strains." Exp Cell Res 25:585-621
22. Hayflick, L. (1985). "The cell biology of aging." Clin Geriatr Med 1(1):15-27
23. . Olovnikov AM (1971) Principle of marginotomy in template synthesis of polynucleotides. Dokl Akad Nauk SSSR201:1496–1499
24. Sager R. 1991. Senescence as a mode of tumor suppression. Environ. Health Persp. 93:59–62
25. Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, et al. 2003. Reversal of human cellular senescence: roles of the p53 and p16 pathways. EMBO J. 22:4212–22
26. Serrano, M., & Blasco, M. A. (2001). Putting the stress on senescence.
27. d'Adda di Fagagna, F., Teo, S. H. & Jackson, S. P. Functional links between telomeres and proteins of the DNA-damage response. Genes Dev. 18, 1781–1799 (2004).
28. Griffith, J. D. et al. Mammalian telomeres end in a large duplex loop. Cell 97, 503–514 (1999).
29. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, et al. 2003. A DNA damage checkpoint response in telomere-initiated senescence. Nature 426:194–98
30. Rodier F, Kim SH, Nijjar T, Yaswen P, Campisi J. 2005. Cancer and aging: the importance of telomeres in genome maintenance. Int. J. Biochem. Cell Biol. 37:977–90
31. Fumagalli M, Rossiello F, Clerici M, Barozzi S, Cittaro D, et al. 2012. Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. Nat. Cell Biol. 14:355–65
32. Collins, K. & Mitchell, J. R. Telomerase in the human organism. Oncogene 21, 564–579 (2002).
33. Weng NP, Hodes RJ. 2000. The role of telomerase expression and telomere length maintenance in human and mouse. J. Clin. Immunol. 20:257–67
34. Bodnar, A. G. et al. Extension of life span by introduction of telomerase into normal human cells Science 279, 349–352 (1998).
35. Sedelnikova OA, Horikawa I, Zimonjic DB, Popescu NC, Bonner WM, Barrett JC. 2004. Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. Nat. Cell Biol. 6:168–70
36. Chang BD, Swift ME, Shen M, Fang J, Broude EV, Roninson IB. 2002. Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent. Proc. Natl. Acad. Sci. USA 99:389–94

37. Blagosklonny MV. 2003. Cell senescence and hypermitogenic arrest. EMBO Rep. 4:358–62
38. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88:593–602
39. Lin, A. W. et al. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/ MAPK mitogenic signaling. Genes Dev. 12, 3008–3019 (1998).
40. Freund A, Patil PK, Campisi J. 2011. p38MAPK is a novelDNAdamage response-independent regulator of the senescence-associated secretory phenotype. EMBO J. 30:1536–48
41. Narita M, Nunez S, Heard E, Narita M, Lin AW, et al. 2003. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 113:703–16
42. Bakkenist CJ, Kastan MB. 2003. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 421:499–506
43. Chen, Q., Fischer, A., Reagan, J. D., Yan, L. J., & Ames, B. N. (1995). OxidativeDNAdamage and senescence of human diploid fibroblast cells. Proceedings of the National Academic Science USA, 92, 4337–4341.
44. Blander, G., Machado De Oliveira, R., Conboy, C. M., Haigis, M., & Guarente, L. (2003). SOD1 knock down induces senescence in human fibroblasts. Journal of Biological Chemistry, 18, 18
45. von Zglinicki, T., Pilger, R., & Sitte, N. (2000). Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. Free Radical Biology and Medicine, 28 64–74.
46. von Zglinicki, T., Saretzki, G., Docke,W., & Lotze, C. (1995). Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts A model for senescence? Experimental Cell Research, 220, 186–193.
47. Rebbaa, A., Zheng, X., Chou, P. M. & Mirkin, B. L. Caspase inhibition switches doxorubicin-induced apoptosis to senescence. Oncogene 22, 2805–2811 (2003)
48. Trougakos, I. P., Saridaki, A., Panayotou, G. & Gonos, E. S. Identification of differentially expressed proteins in senescent human embryonic fibroblasts. Mech. Ageing Dev127, 88–92 (2006).
49. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O et al (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 92:9363–9367
50. Collado, M. & Serrano, M. The power and the promise of oncogene-induced senescence markers. Nature Rev. Cancer 6, 472–476 (2006).
51. Hewitt, G. Jurk D. (2012) Telomeres are favored targets of a persistent DNA damage response in ageing and stress-induced senescence nature communications 3:708
52. Sherr CJ, McCormick F (2002) The RB and p53 pathways in cancer. Cancer Cell 2, 103-112.

53. McConnell BB, Starborg M, Brookes S, Peters G (1998) Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. Curr Biol 8, 351-354.
54. Bond J, Jones C, Haughton M, DeMicco C, Kipling D, Wynford- Thomas D (2004) Direct evidence from siRNA-directed "knock down" that p16(INK4a) is required for human fibroblast senescence and for limiting ras-induced epithelial cell proliferation. Exp Cell Res 292, 151-156.
55. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 73, 39-85.
56. Harvey DM, Levine AJ (1991) p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. Genes Dev 5, 2375-2385
57. Stein GH, Beeson M, Gordon L (1990) Failure to phosphorylate the retinoblastoma gene product in senescent human fibroblasts. Science 249, 666-669
58. Macip S, Igarashi M, Berggren P, Yu J, Lee SW, Aaronson SA (2003) Influence of induced reactive oxygen species in p53 mediated cell fate decisions. Mol Cell Biol 23, 8576 -8585.
59. Macip S, Igarashi M, Fang L, Chen A, Pan ZQ, Lee SW, Aaronson SA (2002) Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. Embo J 21, 2180-2188.
60. Han J., Sun P. (2007) "The pathways to tumor suppression via route p38" Trends in Biochemical Sciences, Vol.32, No.8
61. Bulavin, D.V. et al. (2004) Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK-mediated activation of the p16(Ink4a)-p19(Arf) pathway. Nat. Genet. 36, 343–350
62. Sun, P. et al. (2007) PRAK is essential for ras-induced senescence and tumor suppression. Cell 128, 295–308
63. Colavitti, R. and Finkel, T. (2005) Reactive oxygen species as mediators of cellular senescence. IUBMB Life 57, 277–281
64. Passos J.F., Simillion C., "Cellular senescence: unravelling complexity" AGE (2009) 31:353–363
65. Sherr C.J. DePinho R.A Cellular Senescence: Mitotic Clock or Culture Shock? Cell, Vol. 102, 407–410, August 18, 2000
66. Jeyapalan JC, Ferreira M, Sedivy JM, Herbig U. Accumulation of senescent cells in mitotic tissue of aging primates. Mech Ageing Dev. 2007 Jan;128(1):36-44
67. Tyner SD, Venkatachalam S, Choi J, Jones S, Donehower LA (2002) p53 mutant mice that display early ageing associated phenotypes. Nature 415:45–53
68. Choudhury AR, Ju Z, Djojosubroto MW, Rudolph KL (2007) Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. Nat Genet 39:99–105

69. Molofsky AV, Krishnamurthy J, Sharpless NE, Morrison SJ (2006) Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. Nature 443:448–452
70. Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M et al. Tumour biology: senescence in premalignant tumours. Nature 2005; 436: 642
71. Braig M, Lee S, Loddenkemper C et al. Oncogene-induced senescence as an initial barrier in lymphoma development. Nature 2005; 436: 660–5
72. Hutter, E., Unterluggauer, H., Uberall, F., Schramek, H., Jansen-Durr, P.,2002. Replicative senescence of human fibroblasts: the role of Rasdependent signaling and oxidative stress. Exp. Gerontol. 37, 1165–1174
73. Taylor, R.W., Barron, M.J., Kirkwood, T., Turnbull, D.M., 2003. Mitochondrial DNA mutations in human colonic crypt stem cells J.Clin, Invest. 112, 1351–1360.
74. Liu, L., Trimarchi, J.R., Smith, P.J., Keefe, D.L., 2002. Mitochondrial dysfunction leads to telomere attrition and genomic instability. Aging Cell. 1, 40–46
75. von Zglinicki, T., 2002. Oxidative stress shortens telomeres. Trends Biochem Sci. 27, 339–344
76. Maier, B. et al. Modulation of mammalian life span by the sho)rt isoform of p53. Genes Dev. 18, 306–319 (2004)
77. Jian Yu "Synthesis and mechanistic studies on the monoamine oxidase (MAO) catalyzed oxidation of 1,4-disubstituted-1,2,3,6- tetrahydropyridines" August 10, 1998 Blacksburg, Virginia
78. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative, and aging. Free Radic Biol Med. 2000 Aug;29(3-4):222-30
79. Villeneuve C., Frugier C.G., p53-PGC-1a Pathway Mediates Oxidative Mitochondrial Damage and Cardiomyocyte Necrosis Induced by Monoamine Oxidase-A Upregulation: Role in Chronic Left Ventricular Dysfunction in Mice ANTIOXIDANTS & REDOX SIGNALING Volume 00, Number 00, 2012
80. Passos J.F., Saretzki G., von Zglinicki, T "Mitochondrial Dysfunction Accounts for the Stochastic Heterogeneity in Telomere-Dependent Senescence" PLoS Biology, May 2007, Volume 5, Issue 5, e110
81. Passos J.F, Nelson G., von Zglinicki, T. "Feedback between p21 and reactive oxygen production is necessary for cell senescence" Mol Syst Biol. 2010;6:347
82. Lawless C., Wang C., Passos J.F “Quantitative assessment of markers for cell senescence” Exp Gerontol. 2010 Oct;45(10):772-8
83. Acosta JC, O'Loghlen A Gil J: Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell 2008, 133:1006–1018.

84. Correia-Melo C, Hewitt G, Passos JF “Telomeres, oxidative stress and inflammatory factors: partners in cellular senescence?” Longev Healthspan. 2014 Jan 16;3(1):1
85. de Lange, T., Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev, 2005. 19(18): p. 2100-10.
86. Grimsby J, Lan NC, Neve R, Chen K, Shih JC. Tissue distribution of human monoamine oxidase A and B mRNA. J Neurochem 1990;55:1166–9
87. Chen K. Organization of MAO A and MAO B promoters and regulation of gene expression. Neurotoxicology. 2004 Jan;25(1-2):31-6
88. Edelstein SB, Breakefield XO. Monoamine oxidases A and B are differentially regulated by glucocorticoids and "aging" in human skin fibroblasts Cell Mol Neurobiol. 1986 Jun;6(2):121-50
89. Bortolato M[1], Shih JC. Behavioral outcomes of monoamine oxidase deficiency: preclinical and clinical evidence. Int Rev Neurobiol. 2011;100:13-42. doi: 10.1016/B978-0-12-386467-3.00002-9
90. Johnston JP. Some observations upon a new inhibitor of monoamine oxidase in brain tissue. Biochem Pharmacol. 1968 Jul;17(7):1285-97
91. Du L, Bakish D, Ravindran A, Hrdina PD (September 2004). "MAOA gene polymorphisms are associated with major depression and sleep disturbance in males". NeuroReport 15 (13): 2097–101
92. Meyer JH, Ginovart N, Boovariwala A, Sagrati S, Hussey D, Garcia A, Young T, Praschak-Rieder N, Wilson AA, Houle S (November 2006). "Elevated monoamine oxidase a levels in the brain: an explanation for the monoamine imbalance of major depression". Arch. Gen. Psychiatry 63 (11): 1209–16.
93. Maurel A, Hernandez C, Kunduzova O, Bompart G, Cambon C, Parini A, Francés B. Age-dependent increase in hydrogen peroxide production by cardiac monoamine oxidase A in rats Am J Physiol Heart Circ Physiol. 2003 Apr;284(4)
94. http://www.rndsystems.com/pdf/dy206.pdf