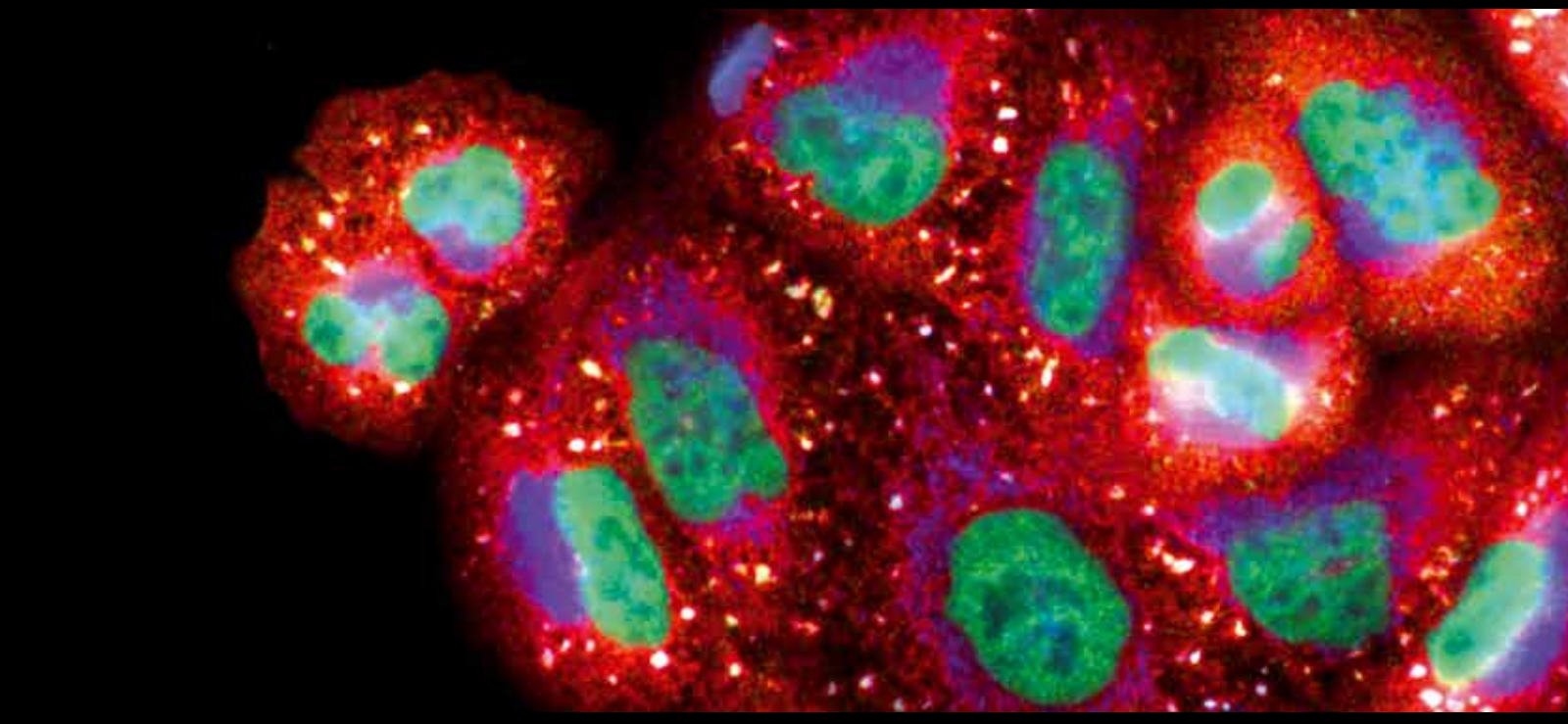
Oxidative Medicine and Cellular Longevity

Cellular Models of Aging

Guest Editors: Paula Ludovico, Heinz D. Osiewacz,
Vitor Costa, and William C. Burhans



Cellular Models of Aging

Oxidative Medicine and Cellular Longevity

Cellular Models of Aging

Guest Editors: Paula Ludovico, Heinz D. Osiewacz, Vitor Costa, and William C. Burhans

Editorial Board

Cellular Models of Aging, Paula Ludovico, Heinz D. Osiewacz, Vitor Costa, and William C. Burhans
Volume 2012, Article ID 616128, 3 pages

DNA Mismatch Repair System: Repercussions in Cellular Homeostasis and Relationship with Aging, Juan Cristóbal Conde-Pérezprina, Miguel Ángel León-Galván, and Mina Konigsberg
Volume 2012, Article ID 728430, 9 pages

Therapeutic Targeting of Redox Signaling in Myofibroblast Differentiation and Age-Related Fibrotic Disease, Natalie Sampson, Peter Berger, and Christoph Zenzmaier
Volume 2012, Article ID 458276, 15 pages

Protein Oxidative Damage at the Crossroads of Cellular Senescence, Aging, and Age-Related Diseases, Martin A. Baraibar, Liang Liu, Emad K. Ahmed, and Bertrand Friguet
Volume 2012, Article ID 919832, 8 pages

Centrosome Aberrations Associated with Cellular Senescence and p53 Localization at Supernumerary Centrosomes, Susumu Ohshima
Volume 2012, Article ID 217594, 9 pages

Nuclear and Mitochondrial DNA Repair in Selected Eukaryotic Aging Model Systems, Ricardo Gredilla, Christian Garm, and Tinna Stevnsner
Volume 2012, Article ID 282438, 12 pages

Pleiotropic Cellular Functions of PARP1 in Longevity and Aging: Genome Maintenance Meets Inflammation, Aswin Mangerich and Alexander Brkle
Volume 2012, Article ID 321653, 19 pages

Persistent Amplification of DNA Damage Signal Involved in Replicative Senescence of Normal Human Diploid Fibroblasts, Masatoshi Suzuki, Keiji Suzuki, Seiji Kodama, Shunichi Yamashita, and Masami Watanabe
Volume 2012, Article ID 310534, 8 pages

Is Physical Activity Able to Modify Oxidative Damage in Cardiovascular Aging?, Graziamaria Corbi, Valeria Conti, Giusy Russomanno, Giuseppe Rengo, Piergiusto Vitulli, Anna Linda Ciccarelli, Amelia Filippelli, and Nicola Ferrara
Volume 2012, Article ID 728547, 6 pages

Overexpression of Fatty-Acid-β-Oxidation-Related Genes Extends the Lifespan of *Drosophila melanogaster*, Shin-Hae Lee, Su-Kyung Lee, Donggi Paik, and Kyung-Jin Min
Volume 2012, Article ID 854502, 8 pages

Nutraceutical Interventions for Promoting Healthy Aging in Invertebrate Models, Yuqing Dong, Sujay Guha, Xiaoping Sun, Min Cao, Xiaoxia Wang, and Sige Zou
Volume 2012, Article ID 718491, 10 pages

Contents

Apple Can Act as Anti-Aging on Yeast Cells, Vanessa Palermo, Fulvio Mattivi, Romano Silvestri, Giuseppe La Regina, Claudio Falcone, and Cristina Mazzoni
Volume 2012, Article ID 491759, 8 pages

ROS in Aging Caenorhabditis elegans: Damage or Signaling?, Patricia Back, Bart P. Braeckman, and Filip Matthijssens
Volume 2012, Article ID 608478, 14 pages

Diet and Aging, Samo Ribarič
Volume 2012, Article ID 741468, 20 pages

Yeast Colonies: A Model for Studies of Aging, Environmental Adaptation, and Longevity, Libuše Váchová, Michal Čáp, and Zdena Palková
Volume 2012, Article ID 601836, 8 pages

Growth Culture Conditions and Nutrient Signaling Modulating Yeast Chronological Longevity, Júlia Santos, Cecília Lea o, and Maria Joã o Sousa
Volume 2012, Article ID 680304, 10 pages

Inhibition of Mitochondrial Cytochrome c Release and Suppression of Caspases by Gamma-Tocotrienol Prevent Apoptosis and Delay Aging in Stress-Induced Premature Senescence of Skin Fibroblasts, Suzana Makpol, Norhazira Abdul Rahim, Chua Kien Hui, and Wan Zurinah Wan Ngah
Volume 2012, Article ID 785743, 13 pages

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 616128, 3 pages
doi:10.1155/2012/616128

Editorial
Cellular Models of Aging

Paula Ludovico,[1,2] Heinz D. Osiewacz,[3] Vitor Costa,[4,5] and William C. Burhans[6]

[1] *Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal*
[2] *ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães, Portugal*
[3] *Department of Molecular Biosciences, Faculty of Biosciences and Cluster of Excellence Macromolecular Complexes, Johann Wolfgang Goethe-Universität Frankfurt, Max-von-Laue-Straße 9, 60438 Frankfurt, Germany*
[4] *Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal*
[5] *Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal*
[6] *Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA*

Correspondence should be addressed to Paula Ludovico, pludovico@ecsaude.uminho.pt

Received 6 December 2012; Accepted 6 December 2012

Biological aging is a complex and multifactorial process driven by genetic, environmental, and stochastic factors that lead to the physiological decline of biological systems. In humans, old age is the most important risk factor for the development of degenerative diseases, some of which are severe (e.g., dementia, cancer). Understanding the basic mechanisms of aging is essential to the development of effective therapeutic interventions against age-related diseases.

Mechanisms of aging have been extensively investigated in various model organisms and systems. Although results obtained from studies with any model system should be viewed with caution when translated to human aging, much of the current understanding of conserved pathways involved in aging and lifespan control was obtained from studies that employed experimentally tractable models. Aging research has now reached a stage where it is possible to move from a reductionist approach studying individual factors towards a more global analysis. With the recent development of technologies that allow for the generation and computational analyses of large data sets, the ultimate goal is to unravel the various interacting molecular pathways governing aging and thus elaborate a holistic understanding of this complex process.

This special issue on was compiled with the following aims: (i) to enhance our understanding of *basic mechanisms* of aging and (ii) to investigate potential *interventions* into aging processes. Both original research articles as well as review articles provide an overview of current research and the status of experimentally accessible aging models.

A number of contributions describe the *molecular pathways* that are part of the network controlling aging and longevity. S.-H. Lee and colleagues analyzed the effect of overexpressing the two main components of fatty-acid β-oxidation and report that this genetic manipulation leads to lifespan extension of the corresponding *Drosophila* transgenic strains. They also show that the extension of lifespan mediated by caloric restriction (CR) becomes reduced in the newly investigated strains. This appears to be related to the activation of the dFOXO transcription factor, a known regulator involved in longevity control.

In an original research article S. Makpol and colleagues address the molecular mechanism of γ-tocotrienol (GTT) in preventing aging, focusing on its antiapoptotic effects in stress-induced premature senescence (SIPS) of human diploid fibroblasts (HDFs). Their results show that SIPS cells exhibit senescent-phenotypic characteristics that include increased expression of senescence-associated β-galactosidase and G0/G1 cell cycle arrest accompanied by telomere shortening and decreased telomerase activity. Their findings suggest that GTT inhibits apoptosis and delays cellular senescence of HDFs by inhibiting the intrinsic mitochondria-mediated apoptotic pathway.

In a review article, R. Gredilla and colleagues discuss the latest research on DNA repair in eukaryotes and its relevance

for aging. Their focus is on research using selected model systems and the role they can play in unraveling conserved pathways relevant to aging of mammals, including normal human aging and diseases arising from impairment of repair functions. In this paper the different pathways involved in repair of different kinds of lesions of both nuclear as well as mitochondrial DNA are covered.

A review article by J. C. Conde-Pérezprina and colleagues continues on the topic of DNA repair with a focus on highly conserved mismatch repair systems that are important for maintaining cellular homeostasis. A research article by S. Ohshima explores the interesting phenomenon of centrosome amplification, which occurs in replicatively aged fibroblasts and is often detected in cancer cells as well. This study describes a previously unknown link between supernumerary centrosomes and the tumor suppressor p53. A paper by M. Suzuki and colleagues addresses questions relevant to the amplification of DNA damage signals recently detected by the authors in irradiated normal human fibroblasts. Their study establishes connections between DNA damage signal amplification, sustained activation of ATM-p53 DNA damage response pathways, and replicative senescence.

The DNA damage response protein PARP1 is an important player in chromatin remodeling, DNA repair, telomere maintenance, resolution of replicative stress, and cell cycle control. A. Mangerich and A. Bürkle review in their paper the molecular mechanisms underlying the role of PARP1 in longevity and aging with special emphasis on cellular studies.

Some papers in this special issue describe new insights obtained from research aimed at understanding the impact of *environmental effects* on aging and longevity. This includes specific *interventions* on aging processes that involve the application of different compounds and environmental manipulations (such as CR) that affect the ability of individuals to survive long periods of environmental stress. The paper by L. Váchová and colleagues reviews the impact of processes occurring in colonies of the unicellular aging model *Saccharomyces cerevisiae*, which in the past was mostly viewed in the context of individual cells and as such, has been investigated as a model for replicative and chronological aging. In their paper the authors discuss the adaptive potential of a yeast population growing as a colony and the cellular processes that allow them to survive long periods of stress.

V. Palermo and colleagues also used the unicellular aging model *S. cerevisiae* to determine the effects of apples and their components, such as flesh, skin, and polyphenolic fractions on aging and oxidative stress. Their data point to a cooperative role of all apple components in promoting yeast chronological lifespan extension.

A review article by Y. Dong and colleagues explores the potential of therapeutic interventions designed to mitigate aging and age-related diseases using nutraceuticals from various plants. Their paper describes how these compounds modulate signaling pathways and can be effective in counteracting the development of various diseases. In their treatise they focus on knowledge gained from investigations with short-lived invertebrate models such as *Caenorhabditis elegans* and *Drosophila* species and discuss the need for more extensive research to unravel the mechanistic basis of the observed beneficial effects. They also emphasize that translational research directed toward demonstrating effects in humans is necessary.

In a review article, S. Ribaric discusses CR, a key environmental manipulation that has been shown to extend lifespan in a variety of aging model systems. The author has focused on adaptive stress responses and increased resistance to stress elicited by CR. These coordinated and adaptive stress responses act at the cellular and whole-organism level by modulating epigenetic mechanisms, signaling pathways regulating cell growth, and aging and cell-to-cell signaling molecules.

J. Santos and colleagues discuss in a review article the role of nutrient/energy signaling pathways in the regulation of yeast chronological lifespan. In particular, the authors focus on extrinsic factors that impact cellular longevity, such as culture medium, products of fermentation, and ammonium. How the modulation of TOR, Sch9, and Ras/protein kinase A nutrient signaling pathways is involved in longevity regulation by CR (glucose or amino acid), ethanol, acetic acid, and ammonium is also covered.

A number of contributions have addressed the role of *reactive oxygen species* (ROS) in the regulation of aging. P. Back and colleagues discuss in a review article recent findings relevant to the role of ROS in aging of the *C. elegans* model organism. The authors report on accumulating evidence that argues against the oxidative stress theory of aging and present an alternative theory, namely the redox signaling theory of aging. This theory proposes that ROS may promote longevity through the modulation of redox-sensitive proteins that control cellular metabolism and stress responses. The progressive prooxidizing shift in the redox state of the cell disrupts redox-regulated signaling mechanisms, leading to aging.

In a review article, N. Sampson and colleagues discuss current evidence highlighting the therapeutic potential of targeting the pro-oxidant shift in redox homeostasis for the treatment of age-related diseases associated with myofibroblast dysregulation. The prooxidant shift in redox homeostasis is mainly due to elevated production of NADPH oxidase 4-(NOX4-)derived hydrogen peroxide and is supported by a concomitant decrease in nitric oxide/cGMP signaling and ROS scavenging enzymes.

For years it has been known that in humans, physical exercise can duplicate the effects of CR, including the activation of oxidative stress defenses that ultimately lead to reduced levels of ROS and oxidative damage. G. Corbi and colleagues review the literature describing the underlying mechanisms, including those in which sirtuins that promote longevity play important roles.

In their original research article, M. Baraibar and colleagues describe an in silico approach to identifying molecular actors and cellular pathways affected by protein damage. A database of proteins modified by carbonylation, glycation, and lipid peroxidation products during aging and in age-related diseases was developed and compared to lists of proteins identified during cellular replicative senescence in vitro. Common cellular pathways that include enzymes

involved in intermediate metabolism were found to be targeted by these modifications.

In the view of the editors, the high quality contributions published in this special issue illuminate many important aspects of aging that have emerged from studies that employed a variety of cellular models in numerous laboratories in recent years. We are grateful to the authors for their contributions and for the opportunity to present their data and ideas in this issue. We hope that readers find these contributions as interesting and informative as we did while preparing this issue for publication.

Paula Ludovico
Heinz D. Osiewacz
Vitor Costa
William C. Burhans

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 728430, 9 pages
doi:10.1155/2012/728430

Review Article

DNA Mismatch Repair System: Repercussions in Cellular Homeostasis and Relationship with Aging

Juan Cristóbal Conde-Pérezprina, Miguel Ángel León-Galván, and Mina Konigsberg

Universidad Autónoma Metropolitana Unidad Iztapalapa, 186 Avenida San Rafael Atlixco, 09340 Mexico City, DF, Mexico

Correspondence should be addressed to Mina Konigsberg, mkf@xanum.uam.mx

Received 31 May 2012; Revised 24 September 2012; Accepted 8 October 2012

Academic Editor: William C. Burhans

The mechanisms that concern DNA repair have been studied in the last years due to their consequences in cellular homeostasis. The diverse and damaging stimuli that affect DNA integrity, such as changes in the genetic sequence and modifications in gene expression, can disrupt the steady state of the cell and have serious repercussions to pathways that regulate apoptosis, senescence, and cancer. These altered pathways not only modify cellular and organism longevity, but quality of life ("health-span"). The DNA mismatch repair system (MMR) is highly conserved between species; its role is paramount in the preservation of DNA integrity, placing it as a necessary focal point in the study of pathways that prolong lifespan, aging, and disease. Here, we review different insights concerning the malfunction or absence of the DNA-MMR and its impact on cellular homeostasis. In particular, we will focus on DNA-MMR mechanisms regulated by known repair proteins MSH2, MSH6, PMS2, and MHL1, among others.

1. Introduction

Throughout their lives, organisms are exposed to many different environmental and internal stimuli that affect or modify their functionality. Aging has been considered an important phenomenon that is promoted or altered by these factors. The aging theory, proposed by Harman [1], establishes that unrepaired oxidative damage to biomolecules caused by free radicals and accumulated during an organism's lifetime, might bring on the aging process. Based on *in vitro* and *in vivo* analysis, it has been established that cellular metabolic pathways, like mitochondrial respiration, generates oxidants that can induce biomolecules oxidation [2]. Prolonged exposure to pollutants, metals, toxic compounds, and ionizing radiation, can also induce degenerative processes associated with premature aging [3, 4]. When oxidative damage occurs, in particular DNA damage, it creates a cascade of events that may contribute to aging and disease [5]. However, cells have different mechanisms to maintain DNA biochemical integrity and stability. One of them is the base excision repair (BER) system, a cellular mechanism that repairs damaged bases in the DNA sequence originating from deamination, oxidation, and alkylation. Nucleotide excision repair (NER) is another DNA repair mechanism which differs from BER. While BER repairs individual damaged bases, the NER system corrects larger portions of DNA damage by removing the strand section that contains a major nucleotide lesion [6–8]. Both BER and NER systems are constantly active in the cell, repairing DNA damage as it is detected. However, there are other mechanisms that are activated during specific cellular stages, such as DNA mismatch repair system (MMR).

2. DNA Mismatch Repair Proteins

DNA MMR is an evolutionarily conserved process that corrects base mismatches generated during DNA replication that have escaped the proofreading process [9]. This is accomplished through the interaction of protein heterodimers with the DNA sequence containing the mismatch. Other dimers (e.g., MutL or MutH) are then recruited and interact with the DNA to initiate the repair signaling. When DNA structural integrity is compromised, specific genes and proteins are activated. One of such groups is

the MutS family of proteins, integrated by MSH2, MSH3, and MSH6. These proteins are highly conserved between species and were originally found in *Escherichia coli* [10]; they have received great attention in recent years due to their relationship with several degenerative and pathologic diseases in humans. MSH proteins recognize errors in the genome sequence during replication, preventing the duplication of the damaged strand and repairing single strand breaks [11, 12]. MSH2 either binds to MSH6 in the presence of base mispairing and other mismatches forming the MutSα heterodimer (MSH2-MSH6), or it binds to MSH3 in the presence of base deletions, generating the MutSβ heterodimer (MSH2-MSH3) [13, 14] (Figures 1(a) and 1(b)). The MutSα heterodimer then binds to the altered region and recruits the MutL family proteins, such as MLH1 and PMS2 (as a MutLα heterodimer). MLH1 and PMS2 in turn engage the enzymes needed for the DNA mismatch repair (Figure 1(c)). The DNA-MMR complex initiates the signaling process to replace the DNA altered region through the action of DNA polymerase δ and DNA ligase I [15, 16] (Figure 1(d)). The mechanism that recruits MMR proteins is ATP dependent [17].

Additionally, the activity of the two MutS dimers at the DNA mismatch site is dependent on interactions with the proliferating cell nuclear antigen (PCNA) [18, 19], which is an important cofactor that participates in both DNA replication and repair mechanisms. PCNA interacts with the MutSα dimer through its MSH6 domain and the MutSβ dimer binds with it at a region near the domain of MSH3 [20] (Figure 1(C)). When the basic human MMR system was reconstituted, the components purified were recombinant MutSα or MutSβ, MutLα, Exonuclease I (EXOI), PCNA, replication factor C (RFC, which loads PCNA onto DNA), the single-strand binding factor replication protein A (RPA), polymerase δ, and DNA ligase I [21, 22]. In this system, the 5′-to-3′ mismatch-directed strand excision required only MutSα, EXOI, and RPA, whereas processing of substrates carrying a 3′ nick required also MutLα, PCNA, and RFC [23].

3. Experimental Models Related with DNA MMR Deficiency

MSH2 deficiency has been associated with frame-shift mutations known as microsatellite regions. These are specific DNA regions of tandem repeated units from one to six nucleotides. Microsatellites are found in centromeres, telomeres, promoters, and transcribed genes segments, causing DNA instability by inserting their repeated units and expanding the genomic sequence. This change in the original DNA sequence due to microsatellites can induce mutations within the genetic sequence, when mismatch bases are not corrected and copied to during DNA replication. Hence, microsatellite instability (MSI) has been widely used as a biochemical marker in clinical and laboratory tests for aging-related diseases and malignancies [24, 25].

MMR pathways have been studied in recent years because of their medical importance in several degenerative and pathological diseases in humans [26]. A positive correlation between MMR proteins deficiency, MSI and the malfunction of different processes, and regulatory mechanisms in cells has been established [27, 28].

4. DNA MMR System and Cell Cycle Arrest Process

Post-replicative DNA-MMR systems are critical during the response against diverse damaging stimuli that affect DNA fidelity, such as oxidative stress, xenobiotics, radiation, and so forth [28]. It has been corroborated that MSH2 participates in cell cycle arrest and apoptosis by different pathways, depending on the extent of DNA damage. Experiments conducted in *msh2*−/− nontumor human lymphoblastoid cell lines have shown that the MMR system promotes G2/M arrest after UV-B induced DNA damage, and that a deficiency in MSH2 leads to a decrease in the induction of G2/M cell cycle checkpoint following this type of radiation. MSH2 proficient human lymphoblastoid cell lines cells also increase cell cycle checkpoint proteins levels such as CHK1, CDC25C, and CDC2, when compared with MSH2-deficient cells, after UV-B radiation treatment [29]. These proteins, when phosphorylated at specific amino acidic residues (Ser345, Ser216, Tyr15, and Thr14), regulate cell cycle control and inhibit cells from dividing until the DNA alterations are corrected, in accordance with MMR system's capacity to induce arrest in proliferative cells [30, 31]. In another study, it was shown that MSH2 deficient nonmalignant mouse fibroblasts were partially resistant to UV-B-induced apoptosis and showed reduced S-Phase accumulation; when *msh2* was modified by a point mutation that affected its expression, it was unable to participate in UV-B-induced apoptosis [32].

It has also been shown that UV-B induced DNA damage increases MSH6 protein levels in a p53 nondependent pathway. But in *msh6*−/− primary mouse embryonic fibroblasts cells repair response to this type of damage was found to be significantly less sensitive to UV-B radiation cytotoxic effects as explained by a reduction in MSH6 protein levels. Therefore, MSH6 deficient cells were significantly less sensitive to the UV-B radiation cytotoxic effects and underwent significantly less apoptosis following irradiation than MSH6 proficient cells, thus indicating that UV-B-induced apoptosis was partially dependent on MSH6 levels [33]. These experiments suggest that MSH2 modulates both cell cycle regulation and apoptosis through independent and uncoupled mechanisms.

MMR proteins are also able to repair DNA through homologous recombination, a mechanism that repairs double-strand breaks using perfectly matched nucleotide sequences between two DNA strands. Both genomic and mitochondrial DNA sequences are exchanged through breaking and rejoining by specific protein complexes. The efficiency of homologous recombination depends on the length of uninterrupted sequence identity, as well as on the percentage of sequence identity within the region of homology [34]. These experiments suggest that MSH2 modulates both cell cycle regulation and apoptosis, through independent and uncoupled mechanisms.

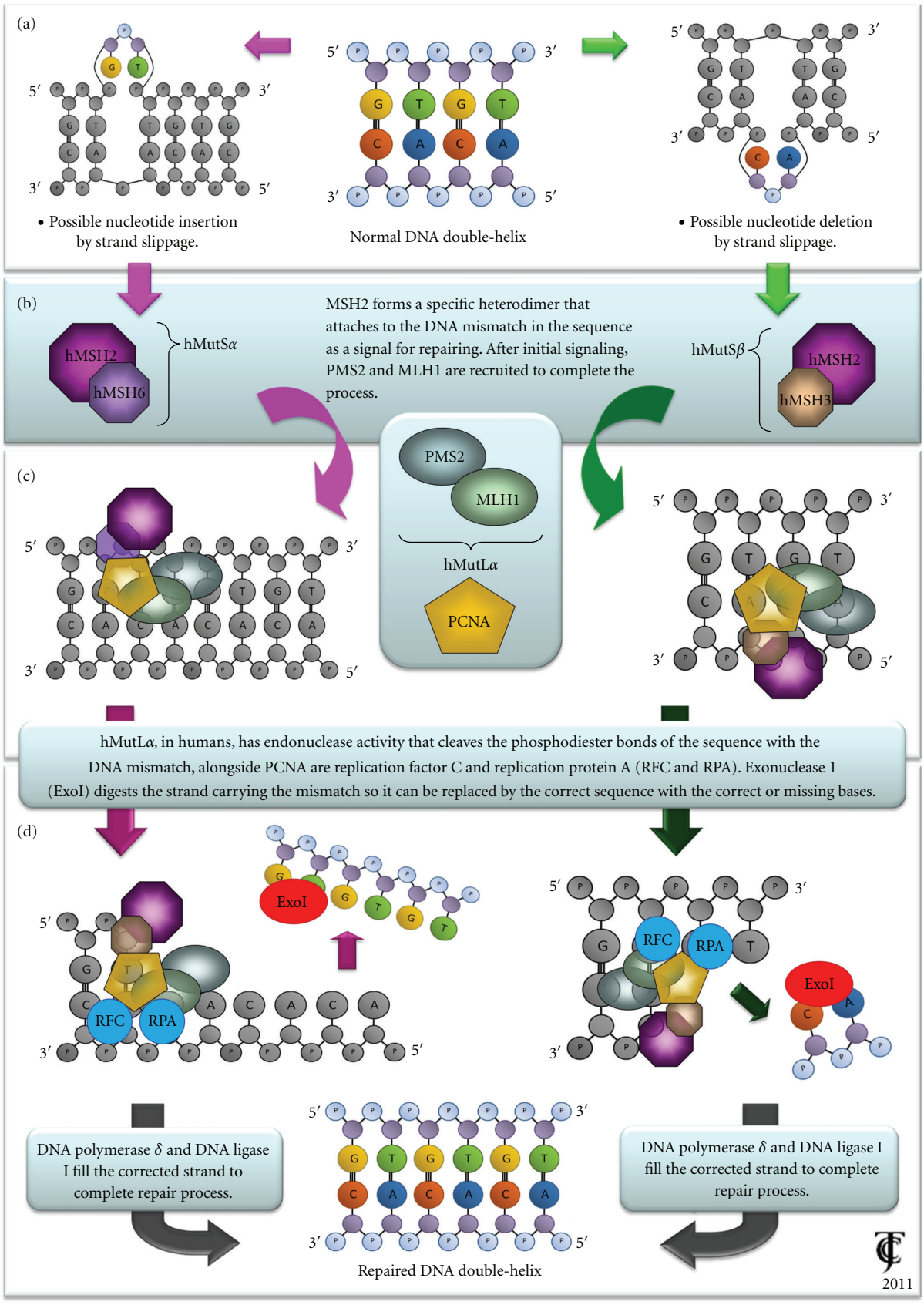


Figure 1: General DNA repair mechanisms mediated through MMR proteins MSH2, MSH6, and MSH3. Depending on the specific type of lesion in the genomic sequence, MSH protein family can initiate repair signaling pathways for maintaining genome integrity and fidelity.

5. MMR Deficiency Associated with Aging and Senescence

When deficient DNA repair pathways, such as MMR system, do not detect altered DNA sequences, cell signaling pathways, as well as cell homeostasis, become unstable because DNA fidelity is compromised. Recent data have established a relationship between damaging stimuli, DNA lesions, and aging, with the absence or decrease in DNA repair systems [35]. It has been determined that MSH2 and MLH1 respond to oxidative DNA damage induced by UV-A radiation [36], and that MSH2 malfunction promotes degenerative conditions that increase with age and affect cell cycle and viability.

MMR efficiency has been studied in the detection of DNA-induced damage through cytotoxic compounds, such as cisplatin, used for chemotherapy in cancer patients, and it has been suggested that MSH2, besides its repair function, is able to induce programmed cell death, reducing abnormal cells in organisms. MSH2's role in signaling DNA damage-induced apoptosis has been recently demonstrated by Negureanu and Salsbury, along with confirmation that MMR defects alone can trigger tumorigenesis [37]. Stage II-III colorectal cancer patients, whose tumors retain DNA MMR activity, have shown benefits and better prognosis when 5-fluorouracil, a cytotoxic compound that induces DNA damage, is used; but patients with tumors that lost MMR activity do not [38]. In another study, MSH2 activity was tested on *Trypanosoma cruzi* and *Trypanosoma brucei msh2*-mutant strains, and it was shown that MMR system can also repair DNA lesions caused by oxidative stress [39]. Mutant NER-deficient XP-A fibroblasts were shown to have an increase in CG > TT transitions in methylated CpG islands as a result of prolonged oxidative stress, compared with control fibroblasts with an active NER system [40]. Estes and collaborators [41] using a *Caenorhabditis elegans msh2* knock-out model demonstrated that both fertility and survival rate were severely compromised; the life expectancy of these mutants also decreased significantly due to irreversible physiological damage. In another animal model, using CD-1 female mice, physiological stress related to breeding, methylation levels increased in *msh2* promoter region associated to age, ending ultimately in the accumulation of DNA damage markers due to MSH2 reduced expression [42]. All the previous evidence supports the idea that proficiency of MMR-system protects the cells from the early tumor occurrence.

Besides apoptosis, premature senescence has been proposed as another way in which cellular homeostasis is maintained. Damaged cells that undergo senescence do not proliferate and therefore the cellular damage can be stopped. However, studies that correlate MMR systems with the phenomenon of senescence are few. Some experiments conducted in young and senescent colonic and human embryonic lung fibroblast cell lines (CCD-18Co and IMR90, respectively) showed that MMR activity was significantly reduced in the senescent phenotype. In particular, MSH2 and MSH6 proteins were markedly downregulated in senescent cells. However, purified MSH2 and MSH6 extracts that were added to senescent cells led to the restoration of MMR activity. Semiquantitative RT-PCR analyses also showed that MSH2 mRNA levels were reduced in senescent cells from the same fibroblast cell lines mentioned before [43]. This evidence suggests that MMR system activity decreases during the senescent state, which could be interesting while studding other processes related to the aging process [44].

6. Telomere Shortening and Senescence Related with MMR Deficiency

Classic replicative senescence involves cell cycle arrest due to telomere shortening. Telomeres are protective structures at the ends of chromosomes that consist of tandem TTAGGG repeats bound to a protein complex known as shelterin [45]. Mouse embryonic fibroblasts (MEFs) deficient in *msh2* expression showed a significant increase in chromosome aneuploidy, centrosome amplification, and defective mitotic spindle organization and unequal chromosome segregation [46]. Studies conducted in several models demonstrate that MMR system can regulate telomere maintenance and, therefore, alter cell senescence. In *msh2* knock-out mouse tissues and primary MEFs there was no apparent change in telomerase activity, telomere length, or recombination at telomeres. However, *msh2* knockout MEFs presented chromosome ending without detectable telomeric DNA which correlated with an increase in chromosome end-to-end fusions [47]. Interestingly, it was reported that mutations in MMR proteins in yeast, particulary MSH2, rescued telomerase-deficient strains survival, apparently by allowing telomere stabilization through an independent telomerase mechanism known as "alternating lengthening of telomeres" (ALT), which uses recombination-based pathways to maintain telomere length [48]. However, when this was studied in a mammalian *in vivo* model, by generating double mutant mice for telomerase and PMS2 (a MutL homolog), the previously proposed role for MMR genes in controlling telomere recombination and telomere length was not observed [49]. Similar results were reported in experiments with human colorectal, endometrial, and ovarian tumors, with or without MMR activity, indicating that in mammals the ALT mechanism is not essential during telomere maintenance [47, 49]; nevertheless MMR may be important in detecting DNA damage induced by radiation, cytotoxic compounds, and oxidative stress, and depending on the damage extent, might be linked to cellular fate determination, leading either to apoptosis or to senescence, and therefore is important during tumorogenesis [50].

7. Pathologies Related to MMR System Failure in Humans

As discussed above, the MMR system is responsible for preserving DNA integrity and stability upon exposure to different damaging stimuli. If the MMR system fails, cell structure and functions will probably be affected, resulting in immortalization, malignant transformation, tumorigenesis, and/or degenerative diseases. One of such diseases is the Muir-Torre syndrome, a rare autosomal dominant disorder,

which is characterized by the predisposition to both sebaceous neoplasms and internal malignancies. This condition is caused by an inherited germ-line mutation in one allele of MMR genes *msh2* and *mlh1*, leading to MMR deficiency. This condition causes MSI which in turn leads to DNA mutations [51–53].

Approximately 15% of colorectal cancers display MSI in one or more MMR genes: *mlh1, msh2, msh6,* or *pms2* [54]. Lynch syndrome, also called non-polyposis colorectal cancer and the most common form of inherited colorectal cancer, is a good example of a malignant disease associated to MSI. It develops in 60% of the patients with MMR gene mutations, commonly before 50 years of age [51, 55]. Muir-Torre, as well as Lynch syndrome, is a condition caused by decreased DNA-MMR activity. Both diseases are characterized by high levels of MSI, supporting the fact that microsatellites are considered excellent markers to detect failures in DNA integrity maintenance [56].

The Ontario Familial Colorectal Cancer Registry is a population-based study of Lynch syndrome occurrence in relation to age, sex, and mutated genes [57]. This study indicated that the cumulative risk for colorectal cancer rose in patients of 70 years old or more, compared with the risk found in patients between 30 to 50 years old. This increase of cumulative risk was correlated with an increase in MSH2 and MLH1 mutations in 70 years patients. The male patients in this particular study presented more mutations in the *mlh1* gene compared with the female patients in all 3 age-dependent groups. However, female patients showed increased mutation levels in the *msh2* gene compared with the male patients, once again in all 3 age-dependent groups. These observations suggest that when genes responsible for maintaining DNA integrity, such as *msh2* and *mlh1*, are mutated, and their capacity to repair DNA decreases, the accumulation of mutations and strand breaks may lead to aging and disease. In other words, when the organisms fail to maintain their homeostasis, they become susceptible to aging and degenerative diseases. Although some of these mutations may be inherited, exposure to toxic and damaging insults like ROS, chemicals, and radiation can abruptly alter or accelerate their formation.

Cell-microorganism interactions can further generate failure of MMR system activity. One example is the mucosa-associated bacteria that significantly contribute to the colonic environment in humans. Enteropathogenic *Escherichia coli* (EPEC) carry the *eae* gene, which encodes the bacterial adhesion protein intimin; this protein enables bacteria to attach to the intestinal epithelium by using a type III secretion system which translocates effector proteins into host cells. These bacteria can influence molecular pathways involved in colorectal tumorigenesis. The ability of EPEC to downregulate DNA-MMR proteins such as MSH2 and MLH1 demonstrates that environmental factors can increase tumorigenesis in colonic epithelial cells [58]. Other tumorigenic processes resulting from MMR system failure are melanoma and breast cancer. MSI and mutations in MMR genes contribute significantly to both conditions in human patients, confirming MMR potential role in carcinoma susceptibility [59, 60]. Another gene associated with MMR defects that has been extensively studied is the tumor-suppressor gene *BRCA1* [61] related to breast cancer. *BRCA1* encodes a protein that repairs damaged DNA and initiates the apoptotic process. Errors in BRCA1 expression result in unrepaired DNA [62]. Such mutations cause uncontrollable cell growth resulting in tumor formation. In experimental trials, the role of the BRCA1 protein in modulating a 6-thioguanine (6-TG) induced MMR damage response was studied, using an isogenic human breast cancer cell line model, including a BRCA1 mutated cell line (HCC1937). The results showed that the tumor cells were more resistant to chemotherapeutic drugs, like 6-thioguanine, as manifested by a reduced G2/M arrest and decreased apoptosis [63]. In this context, *BRCA1*-mutant cells are more resistant to damage by this drug, than *BRCA1*-positive cells. This might suggest a probable mechanism by which the MMR system may regulate cell cycle and apoptosis, in correlation with the functionality of tumor suppressing genes like *BRCA1* [64].

8. The Use of MMR to Study Aging: The Bats Example

As mentioned before, the accumulation of DNA damage is known to induce alterations in the cell's mechanisms and structure that might lead to aging; and since DNA-MMR system is highly conserved between species, our group decided to determine the importance of this system during the aging process in a different animal model. Bats (class: Mammalia, order: Chiroptera) are an interesting model due to their high longevity and particular resistance to adverse environmental factors [65], and therefore, the levels of MMR proteins MSH2 and MLH1, as well as microsatellite markers were quantified, in liver, lung, and brain of young, adult, and old bats, both female and male. Individuals of two different wild bats species were used: *Myotis velifer* and *Desmodus rotundus*. Catalase, glutathione peroxidase, and superoxide dismutase were also analyzed to determine if the antioxidant protection negatively correlated with DNA damage [66]. The first species, the cave *Myotis*, is an insectivorous bat that has an average lifespan of 8 to 12 years (11.3 years in the field) based on mark-recapture studies and an average adult weight of 10.1 g [67] which migrates to higher altitudes to mate and hibernate [68, 69]. On the other hand, the vampire bat *D. rotundus* has an average lifespan of 12 to 20 years [70] and an average adult weight of 32.2 g [67]. Vampire bats feed on the blood of stock animals and do not undergo hibernation [71, 72]. The study conducted determined that *M. velifer* (the short lived species) presented decreased levels of MSH2 and MLH1 with increased MSI (Figure 2(a)) and reduced antioxidant activity as these insectivorous bats became older. However, in the case of *D. rotundus* (the long lived species), there was a reduced MSI in the vampire bats from adult to old age, compared with the young control bats, which correlated with reduced levels of MSH2 and MLH1 (Figure 2(b)) along with improved antioxidant activities in the old bats that matched the activities seen in the adult groups from the other species [66]. An important feature of this study was the demonstration that the analysis of MMR deficiency and

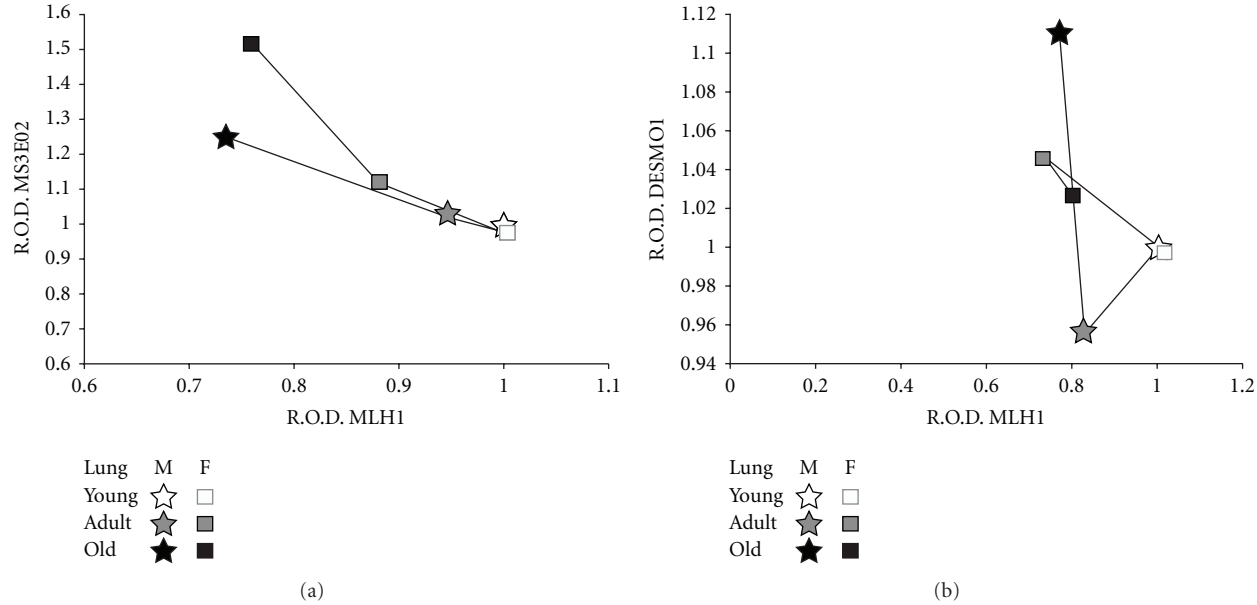


Figure 2: Comparisons between MLH1 levels and microsatellite instability. The figure shows the correlations between MSI and MLH1 protein levels, found in the female (F) and male (M) bat lung samples. The correlations were performed considering the relative optical densities (RODs) obtained from the densitometries of the PCR products from DNA microsatellite sequences (MS3EO2 for *M. velifer* and DESMO1 for *D. rotundus*), and the MLH1 protein levels were determined by Western Blot analysis in both species. Young bats were given the arbitrary value of one, in order to compare them with the other age groups. Each age group consisted of 5 invidious ($n = 5$) either male or female bats. One-way ANOVA tests, followed by Tukey-Kramer variance analyses, were used to compare among groups, by sex and age. A 0.05 level of probability was used as a minimum criterion of significance in all analyses. The figures correspond to: (a) *Myotis velifer* (MS3EO2 versus MLH1) and (b) *Desmodus rotundus* (DESMO1 versus MLH1).

the variations of MSI are an excellent model that can be used in aging studies to correlate Harman's theory of aging with processes related to DNA damage.

9. Final Considerations

Although most of the studies related to the MMR deficiency and genomic alterations are associated with carcinogenic processes, there is a high prospective in using these tools for aging and senescence studies. Studying MMR impairment could also be used to correlate cancer and aging. It is known that cancer predisposition is more frequent when DNA repair is deteriorated in the elderly, which correlates with DNA damage accumulation (e.g., MSI) throughout lifetime, and it might explain the origin of these diseases as proposed by Harman [1, 73]. However, mutations in MMR proteins have been related with other malignancies found not only in elderly patients, but also in children, such as haematological malignancies, brain tumors, Lynch syndrome, and neurofibromatosis [74, 75]. Therefore it would be important to determine which of these malignant conditions are exclusively age-dependent processes or solely DNA damage/mutation-dependent. Another issue to consider is the interaction with other proteins complexes that could assist in the MMR system. It was recently determined in *msh2*-knockout mouse embryonic fibroblasts, HEK, and HeLa cell lines that MSH2 is also in charge of the recruitment of the ATR protein, which is in charge, along with ATM protein, of the DNA damage response signaling, frequently associated with cell death by apoptosis. According to this study ATR is recruited by two different pathways: one of which is mediated by RPA (replication protein A) and relays a signal to the Chk1 protein, whereas the other pathway depends on MSH2 and is responsible for Chk2 activation [76]. Further investigations could be benefited from experiments based on correlations between MMR deficiencies, DNA damage markers in new models such as bats, since they have prolonged longevities that might increase our understanding of MSH2 pathways, revealing new processes that regulate cellular homeostasis by maintaining DNA integrity.

Acknowledgments

The authors would like to acknowledge Dr. Banalata Sen from the National Institute of Environmental Health Sciences and Dr. Carmen Ríos from the Barshop Institute for Aging and Longevity Studies-UTHSCSA for their critical reading and edition of the paper. Mr. Joel Kipi Turok for English editing; Dr. H. González-Márquez, UAM-I, for his help with Figure 1. This work was supported by CONACyT Grant no. CB-2006-1-59659. J. C. Conde-Pérezprina is a CONACyT scholarship holder.

References

[1] D. Harman, "The free radical theory of aging," *Antioxidants and Redox Signaling*, vol. 5, no. 5, pp. 557–561, 2003.

[2] B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, Oxford University Press, New York, NY, USA, 3rd edition, 2004.

[3] A. M. Florea, E. N. Yamoah, and E. Dopp, "Intracellular calcium disturbances induced by arsenic and its methylated derivatives in relation to genomic damage and apoptosis induction," *Environmental Health Perspectives*, vol. 113, no. 6, pp. 659–664, 2005.

[4] S. E. Boley, V. A. Wong, J. E. French, and L. Recio, "p53 heterozygosity alters the mRNA expression of p53 target genes in the bone marrow in response to inhaled benzene," *Toxicological Sciences*, vol. 66, no. 2, pp. 209–215, 2002.

[5] R. Barouki, "Ageing free radicals and cellular stress," *Medecine/Sciences*, vol. 22, no. 3, pp. 266–272, 2006.

[6] E. Moustacchi, "DNA damage and repair: consequences on dose-responses," *Mutation Research*, vol. 464, no. 1, pp. 35–40, 2000.

[7] C. Bernstein, H. Bernstein, C. M. Payne, and H. Garewal, "DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis," *Mutation Research*, vol. 511, no. 2, pp. 145–178, 2002.

[8] T. Nakano, A. Katafuchi, H. Terato, T. Suzuki, B. Van Houten, and H. Ide, "Activity of nucleotide excision repair enzymes for oxanine cross-link lesions," *Nucleic Acids Symposium Series*, no. 49, pp. 293–294, 2005.

[9] T. A. Kunkel and D. A. Erie, "DNA mismatch repair," *Annual Review of Biochemistry*, vol. 74, pp. 681–710, 2005.

[10] J. Jiricny, "MutLα: at the cutting edge of mismatch repair," *Cell*, vol. 126, no. 2, pp. 239–241, 2006.

[11] D. Kültz, "Molecular and evolutionary basis of the cellular stress response," *Annual Review of Physiology*, vol. 67, pp. 225–257, 2005.

[12] D. N. Mullins, E. L. Crawford, S. A. Khuder, D. A. Hernandez, Y. Yoon, and J. C. Willey, "CEBPG transcription factor correlates with antioxidant and DNA repair genes in normal bronchial epithelial cells but not in individuals with bronchogenic carcinoma_aptad 20050829," *BMC Cancer*, vol. 5, article 141, 2005.

[13] P. Peltomäki, "Deficient DNA mismatch repair: a common etiologic factor for colon cancer," *Human Molecular Genetics*, vol. 10, no. 7, pp. 735–740, 2001.

[14] S. Santucci-Darmanin and V. Paquis-Flucklinger, "Homologs of MutS and MutL during mammalian meiosis," *Medecine Sciences*, vol. 19, no. 1, pp. 85–91, 2003.

[15] Q. H. Phung, D. B. Winter, R. Alrefai, and P. J. Gearhart, "Cutting edge: hypermutation in Ig V genes from mice deficient in the MLH1 mismatch repair protein," *Journal of Immunology*, vol. 162, no. 6, pp. 3121–3124, 1999.

[16] H. Saribasak, D. Rajagopal, R. W. Maul, and P. J. Gearhart, "Hijacked DNA repair proteins and unchained DNA polymerases," *Philosophical Transactions of the Royal Society B*, vol. 364, no. 1517, pp. 605–611, 2009.

[17] H. Geng, M. Sakato, V. DeRocco et al., "Biochemical analysis of the human mismatch repair proteins hMutSα MSH2^{G674A}-MSH6 and MSH2-MSH6^{T1219D}," *Journal of Biological Chemistry*, vol. 287, no. 13, pp. 9777–9791, 2012.

[18] Z. Hong, J. Jiang, K. Hashiguchi, M. Hoshi, L. Lan, and A. Yasui, "Recruitment of mismatch repair proteins to the site of DNA damage in human cells," *Journal of Cell Science*, vol. 121, no. 19, pp. 3146–3154, 2008.

[19] S. V. Mudrak, C. Welz-Voegele, and S. Jinks-Robertson, "The polymerase η translesion synthesis DNA polymerase acts independently of the mismatch repair system to limit mutagenesis caused by 7,8-dihydro-8-oxoguanine in yeast," *Molecular and Cellular Biology*, vol. 29, no. 19, pp. 5316–5326, 2009.

[20] F. J. López de Saro, "Regulation of interactions with sliding clamps during DNA replication and repair," *Current Genomics*, vol. 10, no. 3, pp. 206–215, 2009.

[21] Y. Zhang, F. Yuan, S. R. Presnell et al., "Reconstitution of 5′-directed human mismatch repair in a purified system," *Cell*, vol. 122, no. 5, pp. 693–705, 2005.

[22] N. Constantin, L. Dzantiev, F. A. Kadyrov, and P. Modrich, "Human mismatch repair: reconstitution of a nick-directed bidirectional reaction," *Journal of Biological Chemistry*, vol. 280, no. 48, pp. 39752–39761, 2005.

[23] L. Dzantiev, N. Constantin, J. Genschel, R. R. Iyer, P. M. Burgers, and P. Modrich, "A defined human system that supports bidirectional mismatch-provoked excision," *Molecular Cell*, vol. 15, no. 1, pp. 31–41, 2004.

[24] Y. Lin and J. H. Wilson, "Diverse effects of individual mismatch repair components on transcription-induced CAG repeat instability in human cells," *DNA Repair*, vol. 8, no. 8, pp. 878–885, 2009.

[25] H. Alazzouzi, E. Domingo, S. González et al., "Low levels of microsatellite instability characterize MLH1 and MSH2 HNPCC carriers before tumor diagnosis," *Human Molecular Genetics*, vol. 14, no. 2, pp. 235–239, 2005.

[26] P. Modrich and R. Lahue, "Mismatch repair in replication fidelity, genetic recombination, and cancer biology," *Annual Review of Biochemistry*, vol. 65, pp. 101–133, 1996.

[27] A. D. Auerbach and P. C. Verlander, "Disorders of DNA replication and repair," *Current Opinion in Pediatrics*, vol. 9, no. 6, pp. 600–616, 1997.

[28] Z. Yang, R. Lau, J. L. Marcadier, D. Chitayat, and C. E. Pearson, "Replication inhibitors modulate instability of an expanded trinucleotide repeat at the myotonic dystrophy type 1 disease locus in human cells," *American Journal of Human Genetics*, vol. 73, no. 5, pp. 1092–1105, 2003.

[29] K. A. Narine, K. E. Felton, A. A. Parker, V. A. Tron, and S. E. Andrew, "Non-tumor cells from an MSH2-null individual show altered cell cycle effects post-UVB," *Oncology Reports*, vol. 18, no. 6, pp. 1403–1411, 2007.

[30] C. S. Sørensen, L. T. Hansen, J. Dziegielewski et al., "The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair," *Nature Cell Biology*, vol. 7, no. 2, pp. 195–201, 2005.

[31] V. Leung-Pineda, C. E. Ryan, and H. Piwnica-Worms, "Phosphorylation of Chk1 by ATR is antagonized by a chk1-regulated protein phosphatase 2A circuit," *Molecular and Cellular Biology*, vol. 26, no. 20, pp. 7529–7538, 2006.

[32] M. Seifert, S. J. Scherer, W. Edelmann et al., "The DNA-mismatch repair enzyme hMSH2 modulates UV-B-induced cell cycle arrest and apoptosis in melanoma cells," *Journal of Investigative Dermatology*, vol. 128, no. 1, pp. 203–213, 2008.

[33] L. C. Young, K. J. Thulien, M. R. Campbell, V. A. Tron, and S. E. Andrew, "DNA mismatch repair proteins promote apoptosis and suppress tumorigenesis in response to UVB irradiation: an in vivo study," *Carcinogenesis*, vol. 25, no. 10, pp. 1821–1827, 2004.

[34] M. Kappeler, E. Kranz, K. Woolcock, O. Georgiev, and W. Schaffner, "Drosophila bloom helicase maintains genome integrity by inhibiting recombination between divergent DNA sequences," *Nucleic Acids Research*, vol. 36, no. 21, pp. 6907–6917, 2008.

[35] A. M. Skinner and M. S. Turker, "Oxidative mutagenesis, mismatch repair, and aging," *Science of Aging Knowledge Environment*, vol. 2005, no. 9, article re3, 2005.

[36] P. Pitsikas, D. Lee, and A. J. Rainbow, "Reduced host cell reactivation of oxidative DNA damage in human cells deficient in the mismatch repair gene hMSH2," *Mutagenesis*, vol. 22, no. 3, pp. 235–243, 2007.

[37] L. Negureanu and F. R. Salsbury Jr., "The molecular origin of the MMR-dependent apoptosis pathway from dynamics analysis of MutSα-DNA complexes," *Journal of Biomolecular Structure & Dynamics*, vol. 30, no. 3, pp. 347–361, 2012.

[38] M. Iwaizumi, S. Tseng-Rogenski, and J. M. Carethers, "DNA mismatch repair proficiency executing 5-fluorouracil cytotoxicity in colorectal cancer cells," *Cancer Biology and Therapy*, vol. 12, no. 8, pp. 756–764, 2011.

[39] P. C. Campos, V. G. Silva, C. Furtado et al., "Trypanosoma cruzi MSH2: functional analyses on different parasite strains provide evidences for a role on the oxidative stress response," *Molecular and Biochemical Parasitology*, vol. 176, no. 1, pp. 8–16, 2011.

[40] D. H. Lee, T. R. O'Connor, and G. P. Pfeifer, "Oxidative DNA damage induced by copper and hydrogen peroxide promotes CG → TT tandem mutations at methylated CpG dinucleotides in nucleotide excision repair-deficient cells," *Nucleic Acids Research*, vol. 30, no. 16, pp. 3566–3573, 2002.

[41] S. Estes, P. C. Phillips, D. R. Denver, W. K. Thomas, and M. Lynch, "Mutation accumulation in populations of varying size: the distribution of mutational effects for fitness correlates in caenorhabditis elegans," *Genetics*, vol. 166, no. 3, pp. 1269–1279, 2004.

[42] J. C. Conde-Pérezprina, A. Luna-López, N. E. López-Diazguerrero, P. Damián-Matsumura, A. Zentella, and M. Königsberg, "Msh2 promoter region hypermethylation as a marker of aging-related deterioration in old retired female breeder mice," *Biogerontology*, vol. 9, no. 5, pp. 325–334, 2008.

[43] I. Y. Chang, M. Jin, P. Y. Sang et al., "Senescence-dependent MutSα dysfunction attenuates mismatch repair," *Molecular Cancer Research*, vol. 6, no. 6, pp. 978–989, 2008.

[44] L. C. Young, A. C. Peters, T. Maeda et al., "DNA mismatch repair protein msh6 is required for optimal levels of ultraviolet-b-induced apoptosis in primary mouse fibroblasts," *Journal of Investigative Dermatology*, vol. 121, no. 4, pp. 876–880, 2003.

[45] S. W. L. Chan and E. H. Blackburn, "New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin," *Oncogene*, vol. 21, no. 4, pp. 553–563, 2002.

[46] M. R. Campbell, Y. Wang, S. E. Andrew, and Y. Liu, "Msh2 deficiency leads to chromosomal abnormalities, centrosome amplification, and telomere capping defect," *Oncogene*, vol. 25, no. 17, pp. 2531–2536, 2006.

[47] P. Martinez, I. Siegl-Cachedenier, J. M. Flores, and M. A. Blasco, "MSH2 deficiency abolishes the anticancer and pro-aging activity of short telomeres," *Aging Cell*, vol. 8, no. 1, pp. 2–17, 2009.

[48] A. Rizki and V. Lundblad, "Defects in mismatch repair promote telomerase-independent proliferation," *Nature*, vol. 411, no. 6838, pp. 713–716, 2001.

[49] I. Siegl-Cachedenier, P. Muñoz, J. M. Flores, P. Klatt, and M. A. Blasco, "Deficient mismatch repair improves organismal fitness and survival of mice with dysfunctional telomeres," *Genes and Development*, vol. 21, no. 17, pp. 2234–2247, 2007.

[50] I. Ibanez de Caceres, N. Frolova, R. J. Varkonyi et al., "Telomerase is frequently activated in tumors with microsatellite instability," *Cancer Biology and Therapy*, vol. 3, no. 3, pp. 289–292, 2004.

[51] S. Perera, L. Ramyar, A. Mitri et al., "A novel complex mutation in MSH2 contributes to both Muir-Torre and Lynch Syndrome," *Journal of Human Genetics*, vol. 55, no. 1, pp. 37–41, 2010.

[52] A. Morales-Burgos, J. L. Sánchez, L. D. Figueroa et al., "MSH-2 and MLH-1 Protein expression in muir torre syndrome-related and sporadic sebaceous neoplasms," *Puerto Rico Health Sciences Journal*, vol. 27, no. 4, pp. 322–327, 2008.

[53] D. Torre, "Multiple sebaceous tumors," *Archives of Dermatology*, vol. 98, no. 5, pp. 549–551, 1968.

[54] E. Vilar and S. B. Gruber, "Microsatellite instability in colorectal cancerthe stable evidence," *Nature Reviews Clinical Oncology*, vol. 7, no. 3, pp. 153–162, 2010.

[55] F. C. C. da Silva, M. D. Valentin, F. D. O. Ferreira, D. M. Carraro, and B. M. Rossi, "Mismatch repair genes in Lynch syndrome: a review," *Sao Paulo Medical Journal*, vol. 127, no. 1, pp. 46–51, 2009.

[56] P. M. Lynch, "The hMSH2 and hMLH1 genes in hereditary nonpolyposis colorectal cancer," *Surgical Oncology Clinics of North America*, vol. 18, no. 4, pp. 611–624, 2009.

[57] Y. H. Choi, M. Cotterchio, G. McKeown-Eyssen et al., "Penetrance of colorectal cancer among MLH1/MSH2 carriers participating in the colorectal cancer familial registry in Ontario," *Hereditary Cancer in Clinical Practice*, vol. 7, no. 1, article 14, 2009.

[58] O. D. K. Maddocks, A. J. Short, M. S. Donnenberg, S. Bader, and D. J. Harrison, "Attaching and effacing Escherichia coli downregulate DNA mismatch repair protein in vitro and are associated with colorectal adenocarcinomas in humans," *PLoS ONE*, vol. 4, no. 5, Article ID e5517, 2009.

[59] G. Ponti, L. Losi, G. Pellacani et al., "Malignant melanoma in patients with hereditary nonpolyposis colorectal cancer," *British Journal of Dermatology*, vol. 159, no. 1, pp. 162–168, 2008.

[60] S. A. Martin, A. McCarthy, L. J. Barber et al., "Methotrexate induces oxidative DNA damage and is selectively lethal to tumour cells with defects in the DNA mismatch repair gene MSH2," *EMBO Molecular Medicine*, vol. 1, no. 6-7, pp. 323–337, 2009.

[61] F. Fostira, G. Thodi, I. Konstantopoulou, R. Sandaltzopoulos, and D. Yannoukakos, "Hereditary cancer syndromes," *Journal of B.U.ON.*, vol. 12, no. 1, pp. S13–S22, 2007.

[62] A. Agarwal, S. Gupta, and R. K. Sharma, "Role of oxidative stress in female reproduction," *Reproductive Biology and Endocrinology*, vol. 3, article 28, 2005.

[63] K. Yamane, J. E. Schupp, and T. J. Kinsella, "BRCA1 activates a G2-M cell cycle checkpoint following 6-thioguanine-induced DNA mismatch damage," *Cancer Research*, vol. 67, no. 13, pp. 6286–6292, 2007.

[64] R. A. Jensen, M. E. Thompson, T. L. Jetton et al., "BRCA1 is secreted and exhibits properties of a granin," *Nature Genetics*, vol. 12, no. 3, pp. 303–308, 1996.

[65] G. Neuweiler, *The Biology of Bats*, Oxford University Press, New York, NY, USA, 2000.

[66] J. C. Conde-Pérezprina, A. Luna-López, V. Y. González-Puertos, T. Zenteno-Savín, M. A. León-Galván, and M. Königsberg, "DNA MMR systems, microsatellite instability and antioxidant activity variations in two species of wild bats: myotis velifer and Desmodus rotundus, as possible factors associated withlongevity," *Age*. In press.

[67] G. S. Wilkinson and J. M. South, "Life history, ecology and longevity in bats," *Aging Cell*, vol. 1, no. 2, pp. 124–131, 2002.

[68] J. H. Fitch, K. A. Shump, and A. U. Shump, "Myotis velifer," *Mammalian Species*, vol. 149, pp. 1–5, 1981.

[69] K. D. Jürgens and J. Prothero, "Scaling of maximal lifespan in bats," *Comparative Biochemistry and Physiology A*, vol. 88, no. 2, pp. 361–367, 1987.

[70] R. D. Lord, F. Muradali, and L. Lazaro, "Age composition of vampire bats (Desmodus rotundus) in Northern Argentina and Southern Brazil," *Journal of Mammalogy*, vol. 57, pp. 573–575, 1976.

[71] A. Balmori, "El estudio de los quirópteros a través de sus emisiones ultrasónicas," *Galemys Boletín SECEM*, vol. 10, pp. 12–19, 1998.

[72] A. K. Brunet-Rossinni and S. N. Austad, "Ageing studies on bats: a review," *Biogerontology*, vol. 5, no. 4, pp. 211–222, 2004.

[73] T. Arai, M. Sawabe, T. Hosoi, and N. Tanaka, "Role of DNA repair systems in malignant tumor development in the elderly," *Geriatrics and Gerontology International*, vol. 8, no. 2, pp. 65–72, 2008.

[74] K. Wimmer and J. Etzler, "Constitutional mismatch repair-deficiency syndrome: have we so far seen only the tip of an iceberg?" *Human Genetics*, vol. 124, no. 2, pp. 105–122, 2008.

[75] S. Krüger, M. Kinzel, C. Walldorf et al., "Homozygous PMS2 germline mutations in two families with early-onset haematological malignancy, brain tumours, HNPCC-associated tumours, and signs of neurofibromatosis type 1," *European Journal of Human Genetics*, vol. 16, no. 1, pp. 62–72, 2008.

[76] N. Pabla, Z. Ma, M. A. McIlhatton, R. Fishel, and Z. Dong, "hMSH2 recruits ATR to DNA damage sites for activation during DNA damage-induced apoptosis," *Journal of Biological Chemistry*, vol. 286, no. 12, pp. 10411–10418, 2011.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 458276, 15 pages
doi:10.1155/2012/458276

Review Article

Therapeutic Targeting of Redox Signaling in Myofibroblast Differentiation and Age-Related Fibrotic Disease

Natalie Sampson,[1] Peter Berger,[2] and Christoph Zenzmaier[2]

[1] *Department of Experimental Urology, Medical University of Innsbruck, 6020 Innsbruck, Austria*
[2] *Institute for Biomedical Aging Research, Austrian Academy of Sciences, 6020 Innsbruck, Austria*

Correspondence should be addressed to Peter Berger, peter.berger@oeaw.ac.at
and Christoph Zenzmaier, christoph.zenzmaier@oeaw.ac.at

Received 29 June 2012; Accepted 18 September 2012

Academic Editor: Paula Ludovico

Myofibroblast activation plays a central role during normal wound healing. Whereas insufficient myofibroblast activation impairs wound healing, excessive myofibroblast activation promotes fibrosis in diverse tissues (including benign prostatic hyperplasia, BPH) leading to organ dysfunction and also promotes a stromal response that supports tumor progression. The incidence of impaired wound healing, tissue fibrosis, BPH, and certain cancers strongly increases with age. This paper summarizes findings from *in vitro* fibroblast-to-myofibroblast differentiation systems that serve as cellular models to study fibrogenesis of diverse tissues. Supported by substantial *in vivo* data, a large body of evidence indicates that myofibroblast differentiation induced by the profibrotic cytokine transforming growth factor beta is driven by a prooxidant shift in redox homeostasis due to elevated production of NADPH oxidase 4 (NOX4)-derived hydrogen peroxide and supported by concomitant decreases in nitric oxide/cGMP signaling and reactive oxygen species (ROS) scavenging enzymes. Fibroblast-to-myofibroblast differentiation can be inhibited and reversed by restoring redox homeostasis using antioxidants or NOX4 inactivation as well as enhancing nitric oxide/cGMP signaling via activation of soluble guanylyl cyclases or inhibition of phosphodiesterases. Current evidence indicates the therapeutic potential of targeting the prooxidant shift in redox homeostasis for the treatment of age-related diseases associated with myofibroblast dysregulation.

1. Myofibroblast Biology

The myofibroblast is a specialized cell type that combines the extracellular matrix (ECM)-producing characteristics of fibroblasts with the cytoskeletal and contractile properties of smooth muscle cells (SMCs) as reviewed recently [1]. Defined by (i) their *de novo* expression of alpha-smooth muscle cell actin (α-SMA, encoded by the gene *ACTA2*) in stress fibers and (ii) contractile force, myofibroblasts play a critical role during normal wound healing and thereby in maintaining tissue integrity [2]. In addition, myofibroblasts secrete growth factors that attract epithelial cells for subsequent wound closure (reepithelialization) [3–5]. Normal tissue function and architecture is restored upon completion of reepithelialization via massive apoptosis of myofibroblasts and vascular cells followed by their subsequent clearance from the wound site [6]. Whilst relatively poorly understood, induction of cellular senescence via telomere-dependent and -independent mechanisms may also facilitate completion of wound healing [7–10]. For example, upon tissue injury telomerase activation is thought to enable cell proliferation for repair of local tissue damage [9]. However, its subsequent downregulation appears to be required for downstream induction of cellular senescence, cessation of wound healing, and cell clearance by the immune system [7, 10].

Dysregulation of the wound healing response has significant pathological consequences. On the one hand, a major clinical problem in the elderly is impaired wound healing, whereby wound repair is temporally delayed and all phases of wound healing exhibit characteristic changes

[11]. By contrast, excessive and/or persistent myofibroblast activity results in continued synthesis of ECM, dysregulation of growth factor signaling and consequently to tissue fibrosis and organ dysfunction [12]. This paper focuses on growing evidence indicating that redox signaling downstream of dysregulated transforming growth factor beta (TGFβ) promotes myofibroblast differentiation and the development/progression of several age-related fibrotic diseases. Findings from cellular fibrosis models are highlighted, which have improved our understanding of the molecular mechanisms underlying such disorders.

2. Myofibroblast Origin and Induction

Although cell types other than fibroblasts, (e.g., vascular SMCs, pericytes, bone marrow-derived fibrocytes, resident epithelial cells via epithelial-to-mesenchymal transition, and endothelial cells via endothelial-to-mesenchymal transition) have been reported to undergo differentiation into myofibroblasts *in vitro*, the extent of their contribution to the *in vivo* myofibroblast pool remains controversial [12, 13]. Rather, it is widely considered that myofibroblasts predominantly originate from the differentiation of local tissue fibroblasts [13].

Following injury or during chronic inflammation, fibroblast-to-myofibroblast differentiation occurs via a two-step process, initiated by changes in mechanical tension of the ECM that are transmitted to the fibroblast cytoskeleton via RhoA/ROCK signaling [14]. Consequently, fibroblasts adopt an "activated" phenotype (referred to as "proto-myofibroblast") and deposit new ECM components [15]. Soluble factors and cytokines produced by platelets and infiltrating leukocytes play a major role in the differentiation to the α-SMA-expressing myofibroblast phenotype. In particular, the combined action of the splice variant ED-A of cellular fibronectin and TGFβ, especially TGFβ1, that initially is secreted by platelets and phagocytic cells at the wound site [15]. However, proto-myofibroblasts and myofibroblasts also secrete and activate TGFβ1 thereby generating an autocrine feed-forward loop further driving myofibroblast differentiation [3, 16].

TGFβ1 is a key inducer of myofibroblast differentiation in cells of diverse histological origin, including breast, skin, prostate, kidney, heart, lung, and liver [17–23]. TGFβ1 exerts its effects via transcriptional events downstream of Smad2/3 activation and Smad-independent regulation of mitogen-activated protein kinase (MAPK) and PI3 kinase/Akt pathways [1, 3, 24]. Collectively, activation of these pathways results in the deposition of ECM and secretion of paracrine- and autocrine-acting growth factors [3, 25]. Importantly, the ECM can directly bind to and release growth factors, such as heparan sulfate binding to fibroblast growth factor 2 [26]. Such interactions can serve to sequester and protect growth factors from degradation and/or enhance their activity [27]. In addition, indirect interactions are required for signal transduction of some growth factors, for example, integrin binding is necessary for induction of angiogenesis by vascular endothelial cell growth factor [28]. Thus, maintaining ECM homeostasis is critical to regulate not only tissue architecture but also cellular signaling cascades.

3. Aging, Dysregulation of Myofibroblast Differentiation, and Fibrosis

Dysregulation of the wound healing response, particularly in association with chronic inflammation and injury (e.g., in the liver due to viral infection, in the lung from chronic obstructive pulmonary disease, in the heart following myocardial infarction) can result in excessive myofibroblast activation and organ fibrosis [29]. It is perhaps therefore not surprising that the incidence of many fibrosis-associated diseases increases sharply with advancing age, for example, cardiovascular disease, fibrosis of the liver, lung and kidney, and benign prostatic hyperplasia (BPH), a classic age-related fibrotic-like disease characterized by fibroblast to myofibroblast differentiation and ECM deposition [24, 30–35]. Fibrosis is also observed in the stromal response to many tumors, including liver and prostate cancer, both of whose incidence is strongly linked with aging and chronic inflammation [36–39]. Myofibroblasts in the tumor-adjacent stroma (termed "reactive stroma") not only actively promote tumorigenesis and angiogenesis via ECM deposition and mitogen secretion but also support cancer cell invasion and metastasis by producing ECM remodeling enzymes [40–44].

Tissue and organ fibrosis are thought to arise from failure of the wave of myofibroblast apoptosis during wound healing, resulting in persistent myofibroblast activation, excessive ECM deposition, altered growth factor signaling and cellular proliferation [12, 45]. In addition, failure of the timely induction of cellular senescence and dysregulation of telomere biology may also lead to organ fibrosis [7–10]. Consistently, shortened telomeres and telomerase mutations have been observed in familial and idiopathic pulmonary fibrosis [46, 47]. However, cell senescence also ameliorates fibrosis via telomere-independent mechanisms [8] and may partially explain discrepancies observed in rodent fibrosis models, whereby telomere deficiency had no effect on chemical-induced fibrosis [48]. The contribution of cellular senescence and telomere biology to age-related fibrosis pathologies remains to be fully investigated.

Given its potent myofibroblast differentiating effects and ability to promote myofibroblast survival in an autocrine manner, TGFβ1 is considered a key molecule underlying the pathophysiology of fibrotic disease [29, 49, 50]. Interestingly, the TGFβ signaling intermediate Smad3 is a direct repressor of telomerase reverse transcriptase [51, 52], a key enzyme required for telomerase activity, suggesting that autocrine TGFβ signaling by myofibroblasts may override the protective mechanism of cellular senescence further exacerbating tissue fibrosis. Consistent with its central role in age-associated fibrogenic pathologies, elevated TGFβ1 levels and signaling are observed in BPH and preneoplastic prostatic lesions, tumor-associated reactive stroma, cardiovascular remodeling, renal interstitial fibrosis, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis (IPF), and in chronic liver disease [44–52].

4. Cellular versus Animal Model Systems for Studying Age-Associated Fibrosis

Several animal models for fibrotic diseases such as systemic sclerosis, pulmonary, liver, cardiac, and renal fibrosis have been established [53–58]. Whilst such models can be valuable tools to understand disease pathology and evaluate novel therapeutic strategies, fibrosis does not normally develop spontaneously in these animals and typically has to be artificially induced, for example by chemical means [53–58]. Moreover, there may be species-specific differences in organ anatomy, physiology, and disease susceptibility. A notable example is the prostate gland [32, 59, 60]. Significant anatomical differences between rodents and humans also have to be considered in other organs for example, lung and skin [61, 62]. In addition, whilst favorable for experimental reproducibility, the use of genetically identical inbred strains does not recapitulate the heterogeneity of human pathologies. Furthermore, animal models are costly and time-consuming and thus not suitable for high throughput drug screening.

Fibrotic diseases are often associated with inflammation, thus it is difficult to dissect if fibrosis suppression in animal models is due to direct antifibrotic effects of a drug or due to indirect anti-inflammatory effects. Cellular *in vitro* model systems to investigate specific cellular or molecular responses can be very useful to elucidate inflammation-independent antifibrotic targets. Moreover, cellular *in vitro* models offer the significant advantage that human-derived cells can be analyzed. In addition, they are less costly, deliver more rapid results than animal models, can be easily genetically manipulated and are amenable for high throughput screening.

Numerous *in vitro* models of fibrosis have been successfully devised. *In vitro* culture of fibroblasts in 3D collagen-gel matrices leads to progressive contraction of the gel over the course of several days mimicking wound contraction [63]. This model has been widely used to investigate the contractility of fibroblasts/myofibroblasts derived from various tissues affected by fibrotic disease including skin, mucosa, lung, cornea, and heart [64–71].

Overwhelming *in vitro* and *in vivo* data demonstrate the central role of TGFβ1 and myofibroblast differentiation in the etiology of diverse fibrotic disorders (see above). Thus, an *in vitro* approach successfully employed by us and others to model fibroblast-to-myofibroblast differentiation in BPH and prostate cancer reactive stroma applies TGFβ1 to primary prostatic fibroblasts, which induces their robust differentiation into myofibroblasts [3, 25, 39, 72, 73] (Figure 1). This approach has also been extensively employed to model myofibroblast differentiation of hepatic stellate cells and of fibroblasts from breast, skin, kidney, heart, and lung [20, 21, 23, 74–77]. Myofibroblast differentiation can be subsequently monitored at the molecular level by the induction of molecular markers such as α-SMA and collagens as well as at the morphological level whereby the thin and elongated phenotype characteristic of fibroblasts changes to the flattened, less light refractive myofibroblast phenotype, which is accompanied by the appearance of contractile actin bundles (Figure 1). The validity of this *in vitro* model system is highlighted by numerous molecular and cellular parallels with animal models and patient specimens that collectively reveal a central role of dysregulated redox signaling by NADPH oxidase 4 (NOX4) and nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) in the development of age-related fibrotic disease.

5. NADPH Oxidase-Derived Reactive Oxygen Species in the Regulation of Myofibroblast Differentiation

Accumulation of nonspecific oxidative damage by high levels of free radicals is thought to be a major contributor to organismal aging [78]. However, when produced in a regulated manner, reactive oxygen species (ROS), NO, and reactive nitrogen species (RNS) can act as biological second messengers in a variety of signal transduction pathways, including myofibroblast differentiation [79].

The NADPH oxidase (NOX) enzyme family, comprising seven members, catalyzes the transfer of electrons across biological membranes from NADPH to oxygen thereby generating superoxide ($O_2^{\bullet -}$) [80]. The NOX family is thereby unique as ROS production is their primary function and not a byproduct as is the case for other ROS-producing enzyme systems, such as xanthine oxidase, mitochondrial respiratory chain, lipid peroxidases, or uncoupled endothelial NO synthase [81].

NOX4 is unique among NOX enzymes in that it is constitutively active with primary regulation occurring at the transcriptional level [82, 83]. Moreover, whilst other NOX enzymes produce superoxide, NOX4 is associated with constitutive hydrogen peroxide (H_2O_2) production, however whether this occurs via superoxide dismutation or direct H_2O_2 production remains controversial [82, 84, 85]. The greater stability but lower reactivity of H_2O_2 compared to superoxide is consistent with a signaling function of NOX4-derived ROS as underscored by observations that sustained and elevated NOX4-derived ROS levels induced by cytokines and growth factors do not induce oxidative damage [3, 86, 87].

Critically, several growth factors, in particular TGFβ1, and stimuli implicated in the pathogenesis of fibrotic disease induce NOX4 expression in diverse cell types as reviewed recently [88]. Consequently, NOX4-derived ROS have been implicated in the pathophysiology of fibrotic disorders, including BPH, IPF, cardiac remodeling, renal, and liver fibrosis, tumorigenesis and the stromal response to prostate, breast and liver cancers [3, 20, 74, 89, 90].

Using *in vitro* model systems, NOX4-derived ROS have been shown to be essential downstream inducers of TGFβ1-mediated myofibroblast differentiation in a myriad of cell types of diverse histological origin. For example, we demonstrated using NOX4 silencing and antioxidants that induction of NOX4-derived ROS in response to TGFβ1 drives myofibroblast differentiation of prostatic fibroblasts [3]. Similarly, TGFβ1-induction of NOX4-derived ROS was required for fibroblast-to-myofibroblast differentiation in *in vitro* models of cardiac, pulmonary, renal, and adventitial fibrosis [20, 21, 74, 75, 91]. Besides inducing differentiation

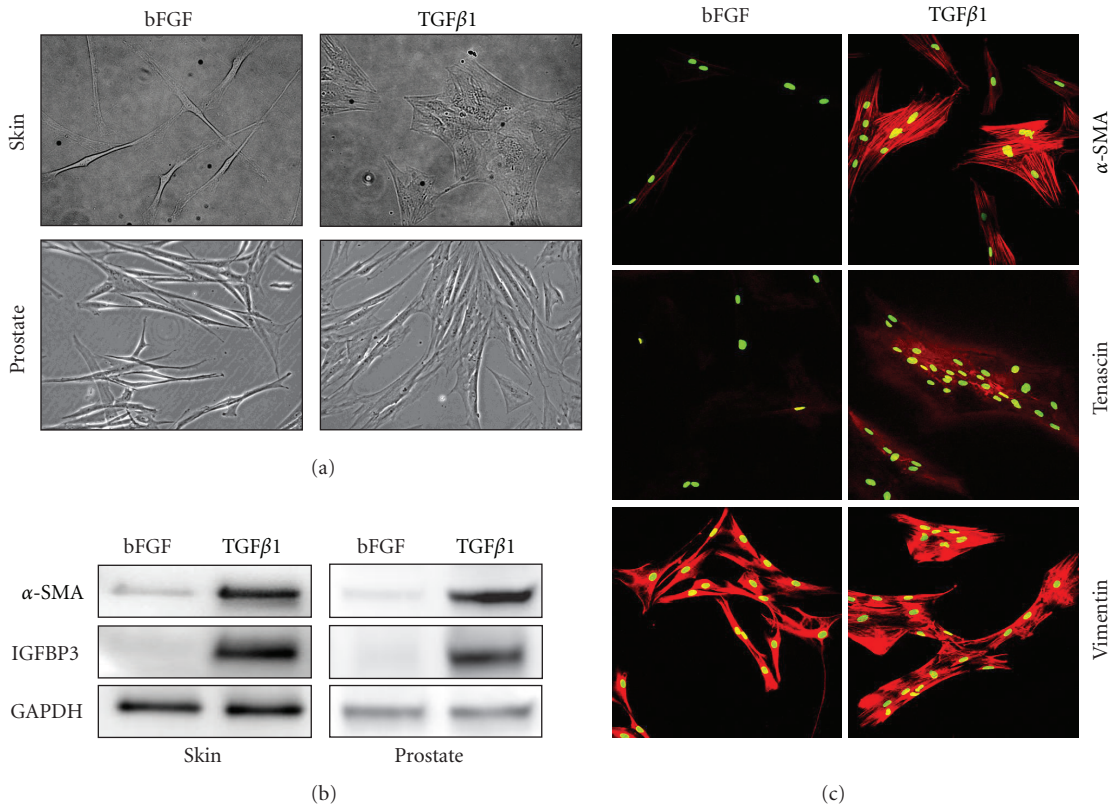


Figure 1: *In vitro* modeling of fibroblast-to-myofibroblast differentiation. Fibroblast-to-myofibroblast differentiation is induced following incubation of fibroblasts for at least 24 hours with 1 ng/mL transforming growth factor beta 1 (TGFβ1) in RPMI-1640 media supplemented with 1% steroid hormone-depleted charcoal-treated bovine calf serum (ctBCS). Steroid hormone depletion is particularly important for fibroblasts derived from endocrine tissues such as the prostate, since androgens attenuate TGFβ signaling and vice versa [32]. Similar to commercial formulations of fibroblast growth media, the fibroblast phenotype of mock control cells is maintained with 1 ng/mL basic fibroblast growth factor (bFGF) and serves to inhibit differentiation-inducing stimuli (e.g., TGFβ) in serum. Myofibroblast differentiation can be monitored at the morphological level by phase contrast microscopy (a) and molecular level by Western blotting (b) or immunofluorescence (c). (a) Primary human dermal fibroblasts *(top left)* and prostatic fibroblasts *(lower left)* exhibit a typical thin, elongated, and light refractive phenotype, whereas upon differentiation with TGFβ1 for 24 hours dermal *(top right)* and prostatic myofibroblasts *(lower right)* display a flattened, less light refractive phenotype. (b) TGFβ1 induces the expression of myofibroblasts markers α-smooth muscle cell actin (α-SMA) and insulin-like growth factor binding protein 3 (IGFBP3) [25] in primary human dermal *(left)* and prostatic *(right)* fibroblasts as determined by Western blotting. GAPDH served as loading control. (c) TGFβ1 treatment of primary human prostatic fibroblasts induces the expression of myofibroblast markers tenascin and filamentous α-SMA as determined by immunofluorescence and confocal laser scanning microscopy. Both myofibroblasts and fibroblasts express the mesenchymal marker vimentin. Nuclei were counterstained with SytoxGreen. (c) Adapted from [25], used with permission.

of fibroblasts, NOX4 was shown to play a role in TGFβ1-mediated cytoskeletal remodeling of vascular endothelial cells and in maintaining the differentiated phenotype of vascular SMCs [92, 93]. Moreover, induction of NOX4 by TGFβ1 is required for hepatic stellate cell activation and their subsequent transdifferentiation into myofibroblasts [22]. Interestingly, NOX4 induction was also required for insulin-induced adipocyte differentiation of preadipocytes [94]. Collectively, these data suggest a broader role of NOX4 in regulating differentiation in response to changes in the cellular environment.

These findings from *in vitro* fibroblast-to-myofibroblast differentiation model systems are supported by several *in vivo* data. We demonstrated that NOX4 mRNA levels specifically correlated with the myofibroblast phenotype in benign prostatic tissue [3]. NOX4 expression was higher in pulmonary fibroblasts from patients with IPF compared with controls and correlated with mRNA levels of the myofibroblast markers α-SMA and procollagen I α1 [75]. Consistently, NOX4 is expressed *in situ* in fibroblastic foci in the lung of IPF patients and two mouse models of pulmonary fibrosis. Moreover, targeting NOX4 via siRNA or a nonspecific NOX inhibitor diphenylene iodonium attenuated lung fibrosis in two murine models of lung injury [74]. NOX4 may also be involved in vascular remodeling associated with IPF [95]. In animal models of diabetic nephropathy, treatment of diabetic rats with NOX4 siRNAs attenuated renal fibrosis strongly implicating a causative role of NOX4-derived ROS in the fibrogenic response to renal injury [96]. Recently, high levels of NOX4 were found in liver biopsy samples from patients with autoimmune hepatitis, which colocalized with α-SMA. Moreover, liver fibrosis could be attenuated in

mouse models via genetic deletion of NOX4 or application of a dual NOX1/NOX4 inhibitor GKT137831 [22], indicating a direct role of NOX4 in the pathogenesis of fibrosis.

NOX4 induction does not appear to contribute to fibrogenesis via direct oxidative stress but rather by chronic dysregulation of downstream signaling pathways. NOX-derived ROS mediate their signaling functions via reversible oxidation of thiol groups of low pKa cysteine residues in target proteins, including transcription factors, MAPKs, protein tyrosine phosphatases (PTPs), and protein tyrosine kinases (PTKs) [87]. Typically, thiol oxidation results in PTP inactivation but kinase activation thus promoting phosphorylation signaling cascades [87]. Only a small proportion of cysteines exhibit the necessary close proximity to basic amino acids to undergo transition to a sulfenic acid, thereby providing a basis for specificity of thiol redox signaling which reversibly regulates biological function by (i) chemically altering active site cysteines, (ii) altering macromolecular interactions, and (iii) modifying allosteric Cys [97].

The precise oxidative target(s) of NOX4-derived ROS that culminate in myofibroblast differentiation in response to TGFβ remain largely unknown. However, in lung fibroblasts TGFβ1 induced NOX4-derived ROS directly oxidatively inactivate MKP1, a nuclear dual-specificity MAPK phosphatase that targets JNK, and p38, leading to sustained activation of JNK and p38 MAPKs [98]. Similarly, in prostatic fibroblasts, NOX4-derived ROS were required for sustained phosphorylation of JNK whose activity in turn was essential for downstream α-SMA induction, and myofibroblast differentiation [3]. By contrast, NOX4 mediates TGFβ-induced myofibroblast differentiation of renal fibroblasts via ERK1/2 [21], whereas angiotensin II-induction of NOX4-derived ROS and fibronectin/ECM deposition in renal mesangial cells occurred via Src activation [99]. Thus, it is likely that the oxidative target(s) of NOX4-derived ROS are tissue-, cell-type, and/or context-specific.

In summary, whilst acute induction of NOX4 may be beneficial in inducing the myofibroblast phenotype for wound repair, the persistence of myofibroblasts together with autocrine TGFβ signaling may result in chronic NOX4 activation and dysregulation of signaling pathways culminating in myofibroblast differentiation, fibrosis, and organ dysfunction.

The cellular redox status and thus signaling potential of NOX4-derived ROS is regulated by antioxidant systems. Concomitant to TGFβ1-mediated induction of NOX4 during prostatic myofibroblast differentiation, a number of ROS-scavenging enzymes were downregulated, including the selenium transporter SEPP1 and selenium (Se)-containing ROS scavenging enzymes such as glutathione peroxidase 3 (GPX3) and thioredoxin reductase 1 (TXNRD1) [3]. The essential trace element Se is an integral component of GPX3 and TXNRD1 enzymes being incorporated as selenocysteine (Sec) at their active site and is critical for correct protein folding/function [100]. Consistent with the role of SEPP1 in delivering Se to peripheral tissues for selenoprotein biosynthesis [101, 102], supplementation of prostatic fibroblasts with exogenous Se restored expression of GPX3 and TXNRD1 as well as TXNRD1 enzyme activity, depleted TGFβ1-induced ROS downstream of NOX4 induction and inhibited myofibroblast differentiation [3].

Given the central role of elevated TGFβ1 in fibrogenesis and that SEPP1 is a direct transcriptionally-suppressed target of TGFβ1 [103, 104], it is plausible that dysregulation of Se-dependent antioxidant systems occurs not only in BPH and the stromal response to prostate cancer but also in other fibrotic disorders. Indeed, the antifibrotic potential of Se is not restricted to prostatic stromal cells since exogenous Se also inhibited TGFβ-mediated myofibroblast transdifferentiation of hepatic stellate cells [105]. Moreover, in a rat model of thyroid fibrosis, Se deficiency promoted thyroid fibrosis in a TGFβ-dependent manner [106], whereas Se supplementation decreased hepatic fibrosis in mice [107]. Consistent with *in vitro* findings, SEPP1 was specifically lost in the periglandular tumor-associated stroma of prostate cancer patients [3]. These findings are consistent with a large body of animal and human clinical data that Se deficiency or supplementation increases or reduces tumor incidence, respectively [108–111]. Moreover, a recent dose-response meta-analysis revealed that overall prostate cancer risk was 15–25% (for advanced PCa: 40–50%) lower in men with plasma/serum Se levels between 135–170 ng/mL compared with 60 ng/mL [112].

Collectively, data from *in vitro* fibroblast-to-myofibroblast differentiation models together with *in vivo* findings indicate that myofibroblast differentiation in fibrotic disorders and tumor-reactive stroma is driven by a prooxidant shift in intracellular redox signaling caused by elevated ROS and/or reduced antioxidative potential. NOX4 appears to be the major source of elevated ROS and central mediator of TGFβ-induced myofibroblast differentiation in diverse tissues. Thus, restoring cellular redox homeostasis, for example by (i) targeting NOX4, (ii) Se supplementation, and/or (iii) application of antioxidants may represent a promising therapeutic strategy for fibrotic disease.

6. Nitric Oxide/cGMP Signaling in the Regulation of Myofibroblast Differentiation

The free radical NO is an important signaling molecule in a variety of biological processes. *In vivo* NO is biosynthesized from L-arginine by nitric oxide synthases (NOS), involving the oxidation of NADPH and the reduction of molecular oxygen. NO activates soluble guanylyl cyclase (sGC), which generates the second messenger cGMP. cGMP exerts multiple effects, for example it regulates cGMP-dependent protein kinases such as protein kinase G (PKG), cyclic nucleotide phosphodiesterases (PDEs), and cation channels and may have other unknown effects [113].

PDEs comprise a superfamily of phosphohydrolases that regulate cellular cGMP and cyclic adenosine monophosphate (cAMP) levels. PDE type 5 (PDE5) specifically hydrolyzes cGMP and is the major therapeutic target in erectile dysfunction (ED), whereby PDE5 inhibitors increase intracellular cGMP levels to enhance NO/cGMP signaling and thereby promote vasodilation [114].

Besides treatment of ED, PDE5 inhibitors are employed in the treatment of pulmonary arterial hypertension and

BPH [115, 116]. Noticeably, patients treated with PDE5 inhibitors for ED exhibited beneficial effects on lower urinary tract symptoms (LUTS) secondary to BPH [117, 118]. These effects were thought to be due to changes in prostatic smooth muscle tone [119–121]. However, our data implicate a direct role of PDE5, which is predominantly expressed in the stromal compartment of the prostate *in vivo*, and NO/cGMP signaling in myofibroblast differentiation [73]. Pharmacological or genetic inhibition of PDE5 significantly attenuated TGFβ1-induced myofibroblast differentiation of prostatic fibroblasts *in vitro*, indicating that enhancing intracellular cGMP levels inhibits myofibroblast differentiation [73]. Consistently, stimulating the generation of intracellular cGMP by the soluble NO donor sodium nitroprusside (SNP) dose-dependently inhibited TGFβ1-induced differentiation and additional blocking of cGMP hydrolysis by the PDE5 inhibitor tadalafil synergistically enhanced this effect [73].

These findings are consistent with numerous studies implicating an inhibitory role of the NO/cGMP pathway in fibroblast-to-myofibroblast differentiation in other tissues. On the one hand, treatment of human dermal fibroblasts with TGFβ1 significantly reduced NOS activity and NO levels, whereas restoring cGMP signaling downstream of NOS using SNP and the cell-permeable cGMP analog 8-bromo-cGMP significantly suppressed TGFβ1-induced collagen production [122]. Furthermore, the NOS inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME) synergistically potentiated TGFβ1-induced collagen production in dermal fibroblasts [122]. In addition, increasing cGMP levels using the PDE5 inhibitor sildenafil alone or in combination with the sGC activator BAY 58-2667 attenuated myofibroblast differentiation of fibroblasts from human Peyronie's disease plaques or lung, respectively [23, 123]. In a similar approach, 8-bromo-cGMP inhibited TGFβ1-induced myofibroblast differentiation of cardiac fibroblasts isolated from wild-type mice [76]. Likewise, the sGC stimulator BAY 41-2272 elevated intracellular cGMP levels and inhibited myofibroblast differentiation in cardiac fibroblasts [77] and dermal fibroblasts from healthy individuals and patients with systemic sclerosis [124].

In addition to the documented improvement of LUTS secondary to BPH by PDE5 inhibitors [117, 118], beneficial effects of enhancing NO/cGMP signaling have also been reported in several fibrosis models *in vivo*. For example, attenuating NO signaling by inhibition of inducible NOS (iNOS) activity in rats with TGFβ1-induced fibrotic plagues of tunica albuginea (a model of Peyronie's disease) resulted in increased myofibroblast abundance and collagen I synthesis in the plaques [125]. Similarly, fibrosis in the penile corpora cavernosa upon streptozotocin-induced diabetes was intensified in iNOS knockout mice compared with wild type [126]. Consistently, stimulating NOS activity in rats via oral administration of the NOS substrate L-arginine resulted in an 80–95% reduction in both plaque size and collagen: fibroblast ratio in PD-like plaques induced by TGFβ1 [123]. Similar effects were observed in parallel groups that received the competitive nonselective PDE inhibitor pentoxifylline or PDE5-selective inhibitor sildenafil [123]. Likewise, promoting NO synthesis via administration of the NOS substrate L-arginine, significantly elevated endothelial NOS expression but decreased TGF-β1 expression and ultimately ameliorated renal interstitial fibrosis, which was markedly aggravated by L-NAME administration in rats with unilateral ureteral obstruction [127].

Alternative approaches to increase cGMP production and NO/cGMP signaling by stimulating sGC activity exhibit similar antifibrotic effects. The sGC stimulator BAY 41-2272 significantly limited progression of anti-Thy-1-induced chronic renal fibrosis in rats [128]. Likewise, BAY 41-2272 reduced the number of myofibroblasts and decreased collagen accumulation in hypertension-induced cardiac fibrosis in rats [77]. Another sGC stimulator riociguat (BAY 63-2521) exerted similar inhibitory effects on cardiac and renal interstitial fibrosis in two rat models of hypertension [129] and attenuated fibrotic tissue remodeling in the myocardium and renal cortex of Dahl salt-sensitive rats [130]. Furthermore, BAY 41-2272 prevented the development of bleomycin-induced dermal fibrosis and skin fibrosis in Tsk-1 mice [124]. Interestingly, NO-independent activation of sGC by BAY 60-2770 also attenuated liver fibrosis in rats [131].

Many studies investigating NO/cGMP signaling in fibrotic disease exploit the specific hydrolytic activity of PDE5 for cGMP to indirectly enhance cGMP levels and thereby promote NO signaling. However, an active role of PDE5 in fibrogenesis via altered enzyme activity has also been reported. Glomerular PDE5 expression was increased during anti-Thy1-induced mesangial proliferative glomerulonephritis in the rat kidney *in vivo*, and PDE5 inhibition by vardenafil increased glomerular cGMP levels leading to subsequent inhibition of mesangial cell proliferation and ECM accumulation [132].

Collectively, findings from *in vitro* and *in vivo* model systems indicate that myofibroblast differentiation is associated with reduced NO/cGMP signaling, suggesting the potential therapeutic benefit of enhancing NO/cGMP signaling (by stimulating sGC activity, and/or preventing cGMP degradation via PDE5 inhibition) in fibrotic disease.

7. Potential Interplay of ROS and NO/cGMP Signaling in Regulating Myofibroblast Differentiation

NOX4-derived ROS play a key role in driving myofibroblast differentiation in response to TGFβ1, whereas increasing NO/cGMP signaling attenuates TGFβ1-induced fibroblast-to-myofibroblast differentiation. This raises an interesting possibility that crosstalk between NO/cGMP and NOX4-derived ROS signaling may coordinately regulate myofibroblast differentiation.

Crosstalk between superoxide and NO signaling has been extensively documented, in particular the ability of superoxide to reduce NO levels by direct chemical scavenging or by NOS uncoupling. For example, superoxide can react with NO generating peroxynitrite ($ONOO^-$), thereby depleting NO levels [133]. In addition, superoxide can oxidize the critical nitric oxide synthase (NOS) cofactor tetrahydrobiopterin (BH_4) leading to NOS uncoupling, which

results in superoxide generation rather than NO production [134]. However, TGFβ-induced fibroblast-to-myofibroblast differentiation is associated with induction of NOX4 and thus presumably generation of H_2O_2 [84, 85], which unlike superoxide does not appear to react directly with NO. Thus, any opposing regulation of myofibroblast differentiation by NO/cGMP signaling and NOX4-derived H_2O_2 presumably occurs via distinct mechanism(s).

Several examples of opposing interaction between H_2O_2 and NO/cGMP signaling have been reported. For example, incubation of isolated rat hepatocytes with an NO donor prevented H_2O_2-induced cell death, presumably via cGMP-activated downstream signaling since the prosurvival effect of NO was ablated by cycloheximide indicating the requirement of *de novo* protein synthesis [135]. Conversely, H_2O_2 impaired NO production in porcine aortic endothelial cells, possibly via direct oxidative inactivation of eNOS cofactors [136]. Moreover, H_2O_2 eliminates the endothelium-dependent vasodilatory response to acetylcholine, a potent inducer of NO synthesis [137].

There are several potential mechanisms by which NOX4-derived H_2O_2 and NO/cGMP signaling may interact to elicit opposing functions during myofibroblast differentiation. For example, both NOS and NOX require NADPH as an electron donor for enzyme activity. NOX4 induction is an early event during TGFβ1-mediated differentiation [3, 20], whereas enhancing cGMP levels inhibits/reverses prostatic fibroblast-to-myofibroblast differentiation without impairing NOX4 mRNA induction by TGFβ1 (our unpublished observations) [138], indicating that NO/cGMP signaling acts downstream of NOX4-derived H_2O_2 production. Thus, NADPH consumption/depletion due to elevated NOX4 activity may attenuate NO-dependent cGMP production and thereby impair NO/cGMP-mediated inhibition of differentiation.

A further potential mode of opposing interaction may occur via mutually exclusive modification of NOX/NO target proteins. For example, NO upregulates the activity of sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) via *S*-glutathiolation on cysteine 674 [139]. Interestingly, TGFβ1-mediated induction of NOX4 in aortic SMCs resulted in SERCA oxidation of the same thiol group, inhibited NO-mediated *S*-glutathiolation, and attenuated NO inhibition of SMC migration [140, 141]. Similar effects were observed upon exposure of vascular SMCs to H_2O_2 or high glucose, which induced NOX4 levels leading to SERCA oxidation, inhibition of NO-induced *S*-glutathiolation and migration. Moreover, NOX4 knockdown decreased SERCA oxidation and restored the inhibition of SMC migration by NO [142].

NOX4-derived H_2O_2 may also attenuate NO signaling by downregulating sGC expression/activity and consequently NO-dependent cGMP generation. H_2O_2 decreased sGC expression and NO-dependent cGMP generation in pulmonary arterial SMCs from lambs with persistent pulmonary hypertension of the newborn [143]. Moreover, incubation of rat aortic SMCs or freshly isolated vessels with ROS-generating agents (including H_2O_2) significantly decreased sGC expression and reduced SNP-induced cGMP formation in the SMCs [144]. In addition, PTP inhibitors or H_2O_2 promoted tyrosine phosphorylation of the beta 1 subunit of sGC, most likely via Src-like kinases [145]. Subsequent studies revealed that cGMP levels are cross-regulated via a mechanism that involves c-Src-dependent phosphorylation of sGC, which attenuates sGC activity and cGMP formation [146]. These studies suggest that elevated NOX4-derived H_2O_2 during myofibroblast differentiation may oxidatively inactivate protein tyrosine phosphatases and/or activate Src kinase, which in turn promote inhibitory phosphorylation of sGC leading to reduced cGMP signaling.

Interactions between TGFβ and NO signaling have also been reported. For example, the NO donor *S*-nitroso-*N*-acetyl-penicillamine or treatment with 8-bromo-cGMP decreased TGFβ3 mRNA levels in neonatal rat cardiac fibroblasts, whereas TGFβ1 mRNA levels were modestly increased [147]. Furthermore, *S*-nitroso-*N*-acetyl-penicillamine and ROS, most likely H_2O_2, both increased TGFβ1 release from human epithelial alveolar cells [148]. On the other hand, TGFβ1 decreased sGC and PKGI expression in pulmonary artery and aortic SMCs from adult rats and mice and a TGFβ-neutralizing antibody prevented the reduction of sGC and PKGI protein expression in chronic oxygen-induced lung injury in mouse pups [149].

Thus, similar to reciprocal inhibition of superoxide and NO, H_2O_2, and NO also appear to interact in a functionally opposing manner during myofibroblast differentiation. However, rather than direct radical quenching as observed for superoxide and NO, the basis of reciprocal inhibition between H_2O_2 and NO apparently occurs at multiple indirect levels. Cumulatively, such interplay would be expected to lead to downregulation of NO/cGMP signaling upon TGFβ1-mediated induction of NOX4-derived H_2O_2 and thereby promote fibroblast-to-myofibroblast differentiation. Consistent with this hypothesis, TGFβ1 significantly decreased NO production in dermal fibroblasts, whereas increasing NO signaling by stimulating sGC generation and/or inhibiting cGMP degradation counteracted ROS-mediated inactivation of NO signaling and prevented myofibroblast differentiation [122]. Inhibition of differentiation can also be achieved by treatment with 8-bromo-cGMP [122], indicating that suppression of myofibroblast differentiation by enhanced NO signaling is mediated by downstream cGMP-dependent mechanisms and not via the NO radical *per se*.

8. Reversal of Myofibroblast Differentiation and Clinical Implications

Tissue fibrosis is thought to arise from failure of the myofibroblast apoptotic wave during wound healing [12, 45]. Thus, whilst inhibition of myofibroblast differentiation may be suitable to prevent disease progression, curative treatments would also require targeting of preexisting myofibroblasts. Although some promising data for such approaches exist [150], extensive fibroblast heterogeneity and the lack of a consensus "myofibroblast-specific" surface marker mean that tissue- and even disease-specific targeting strategies will be required.

An alternative approach to clear the fibrotic myofibroblast pool envisages inducing their dedifferentiation to the

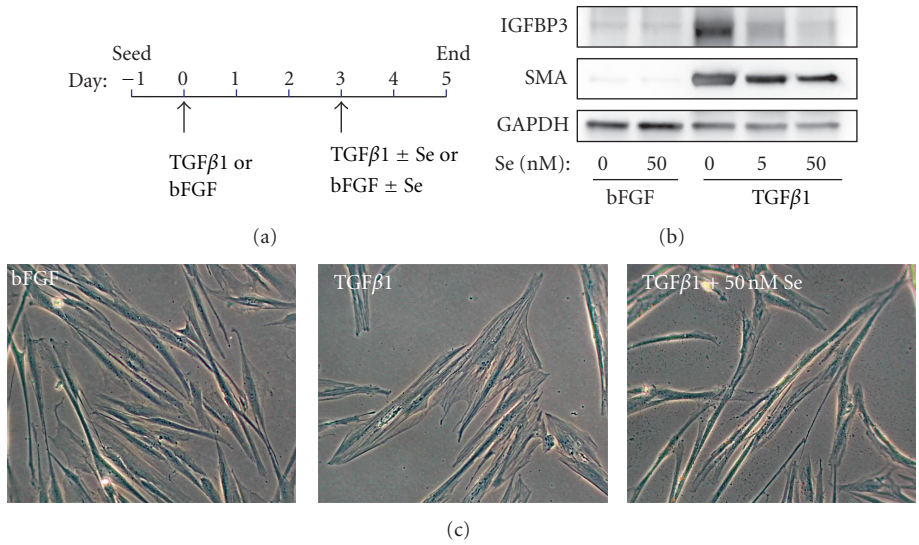


Figure 2: Selenium reverses myofibroblast differentiation of prostatic fibroblasts. Therapeutic targeting of myofibroblast dysregulation in fibrotic disease may be accomplished by promoting myofibroblast dedifferentiation to the nonactivated fibroblast/progenitor phenotype. (a) Methodology outline of selenium (Se)-mediated reversal of fibroblast-to-myofibroblast differentiation in primary human prostatic fibroblasts. Myofibroblast differentiation was induced with 1 ng/mL TGFβ1 or bFGF as mock control. After 72 hours, fresh media was added containing bFGF or TGFβ1 as before but supplemented with selenium (Se) as sodium selenite or vehicle equivalent. Cells were incubated for a further 48 hours before harvesting. Reversal of myofibroblast differentiation of primary human prostatic fibroblasts treated as outlined in (a) was verified by (b) Western blotting for the myofibroblast markers, α-SMA, and IGFBP3 and (c) morphological analysis using phase contrast microscopy. (b) Induction of myofibroblast differentiation by TGFβ1 in the absence of Se (0 nM) is indicated by increased production of myofibroblast markers. However, both α-SMA and IGFBP3 levels are reduced in the presence of Se in a dose-dependent manner. (c) At the morphological level, Se restores the thin, elongated and light refractive phenotype to cells predifferentiated with TGFβ1 *(far right)*, whereas cells treated with TGFβ1 alone *(center panel)* exhibit the typical enlarged and flattened myofibroblast phenotype with visible actin-like filaments. (b-c) Images are representative of three independent experiments using primary cells isolated from different donors.

nonactivated fibroblast/progenitor phenotype. It has long been considered that fibrosis and fibroblast-to-myofibroblast differentiation are irreversible processes. However, recent data from *in vitro* and *in vivo* models indicate that tissue fibrosis and fibroblast-to-myofibroblast differentiation can indeed be reversed. For example, we observe that exogenous Se or PDE5 inhibition restore morphological and molecular characteristics typical of the fibroblast phenotype to *in vitro* differentiated prostatic myofibroblasts even in the continued presence of the TGFβ differentiation-inducing stimulus (Figure 2) [138]. Our data are supported by studies employing myofibroblasts from IPF patients and a three-dimensional coculture model of porcine skin fibrosis that similarly demonstrate the potential utility of ROS scavenging in promoting myofibroblast dedifferentiation [151, 152]. Moreover, treatment of *in vitro* differentiated corneal myofibroblasts with fibroblast growth factor in combination with heparin decreased expression of α-SMA, TGFβ receptors, and cadherins, indicating reversal of myofibroblast differentiation to a fibroblast-like phenotype [153]. Recently, pharmacological inhibition of NOX4 after induction of liver fibrosis in mice was shown to reduce ROS levels and significantly attenuate fibrosis [22].

The principle of enhancing NO/cGMP signaling to induce myofibroblast to fibroblast reversal has also been successfully demonstrated in animal models. Treatment with the PDE5 inhibitor vardenafil reduced myofibroblast numbers and total size of preformed TGFβ1-induced Peyronie's disease plaques in rats [154]. Moreover, BAY 41-2272 reduced established fibrosis in a modified mouse model of bleomycin-induced skin fibrosis and in Tsk-1 mice [124].

Collectively, these findings and those discussed above suggest that signaling via ROS and local growth factors (such as TGFβ and fibroblast growth factor) play key roles not only in driving fibroblast-to-myofibroblast differentiation but also in subsequently maintaining the myofibroblast phenotype. Thus, we hypothesize that high NO/cGMP signaling and low NOX4-derived ROS production coordinately maintain the fibroblast phenotype, whereas differentiation into the myofibroblast phenotype may proceed upon elevated NOX4-derived ROS signaling and/or concomitant inactivation of NO/cGMP signaling (Figure 3).

The observation that fibroblasts and myofibroblasts are interconvertible phenotypes rather than terminally differentiated cell types has significant clinical implications for potential curative therapy of advanced fibrotic disease. In this respect, pharmacological regulation of redox signaling via NOX4 inhibitors, antioxidants, and/or enhancement of NO/cGMP signaling provides a promising option to modulate the fibroblast/myofibroblast ratio at multiple levels in several pathological conditions (Figure 3). For example, local activation of myofibroblast differentiation may provide a means to overcome impaired wound healing in the elderly,

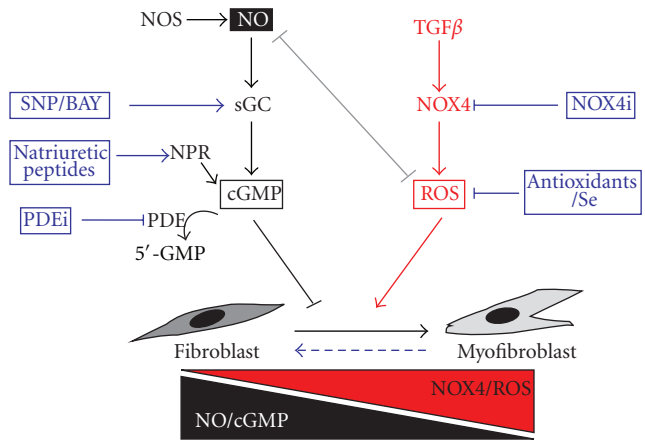


Figure 3: Potential therapeutic targeting of myofibroblast differentiation in the treatment of age-associated fibrotic disease. Myofibroblast differentiation induced by TGFβ1 plays a central role in the etiology of numerous age-related fibrotic disorders. In particular, TGFβ induces a prooxidant shift in intracellular redox homeostasis via the induction of NOX4-derived ROS (in particular H_2O_2), which modulates downstream phosphorylation signaling cascades and transcriptional events that culminate in myofibroblast differentiation. The concomitant downregulation of selenium-dependent ROS scavenging enzymes by TGFβ further potentiates NOX4-derived ROS signaling, which also downregulates NO/cGMP signaling and thereby relieves inhibition of myofibroblast differentiation by NO. Fibroblast-to-myofibroblast differentiation and subsequent tissue fibrosis are reversible processes *(broken horizontal arrow)*. Thus, pharmacological interference of these redox signaling processes to redress redox homeostasis and thereby restore the physiological fibroblast: myofibroblast ratio offers a promising therapeutic strategy for the treatment of age-related pathologies associated with myofibroblast dysregulation. Such pharmacological targeting may succeed at multiple levels. For example, targeting NOX4 directly via NOX4 inhibitors (NOX4i) or indirectly by ROS scavenging with Se or antioxidants would attenuate NOX4-derived ROS signaling and restore the inhibitory effects on differentiation by NO/cGMP signaling. Enhancing NO/cGMP signaling is also sufficient to prevent and reverse fibroblast-to-myofibroblast differentiation and may be achieved by (i) sGC stimulation using the NO donor sodium nitroprusside (SNP) and/or direct sGC stimulators/activators (BAY), (ii) promoting cGMP synthesis via administration of natriuretic peptides that bind to and activate transmembrane guanylyl cyclase natriuretic peptide receptors (NPRs), or (iii) by inhibiting phosphodiesterase (PDE)-mediated cGMP hydrolysis using PDE inhibitors (PDEi).

whereas inhibiting fibroblast-to-myofibroblast differentiation and restoring the fibroblast phenotype may represent a therapeutic strategy for fibrotic disease and also serve as a stromal-targeted chemotherapy approach for solid tumors, such as breast, liver, and prostate cancer.

Despite its critical role in the pathogenesis of fibrosis, TGFβ is not considered a direct clinical target due to its critical function in diverse biological processes and homeostatic maintenance [155]. As central downstream components of TGFβ signaling during fibroblast-to-myofibroblast differentiation, targeting NOX4-derived ROS and NO/cGMP might also be expected to elicit broad and undesirable effects. However, PDE5 inhibitors have a successful history in the treatment of ED, pulmonary arterial hypertension, and BPH [114–116]. Moreover, it should be noted that (i) none of the published Nox4 knockout animals display an obvious basal phenotype, (ii) a dual NOX1/NOX4 inhibitor (at doses that reduced liver fibrosis in mice) does not exert toxic effects in animal models, and (iii) the same inhibitor was also well tolerated in phase I clinical trials [22, 81]. This likely reflects observations that NOX4 does not mediate all of the signaling functions of TGFβ, as illustrated by the delayed temporal induction of NOX4 expression and ROS production (~2 hours following TGFβ1 treatment) compared to the rapid induction of phosphorylation cascades, such as phosphorylation of Smad2/3 and ERK1/2 that occurs within 5 minutes of TGFβ treatment [3, 20, 83]. Thus, selective modulation of NOX4, PDE5, and/or sGC activities may permit continued physiological ROS/NO signaling due to the presence of multiple NOX, PDE, and GC isoforms. Moreover, the fact that these enzymes belong to multimembered families may be clinically exploited to selectively target those isoforms underlying pathology in a given tissue or disease state. For example in the heart, PDE1A appears to play a critical role in cardiac fibrosis and its selective targeting in rats and mice led to regression of cardiac remodeling that is associated with various cardiac diseases [156].

9. Conclusions

Myofibroblast activation and differentiation are central processes of normal wound healing. However, these beneficial effects of myofibroblasts are dysregulated in fibrotic disorders and in the reactive stromal response that promotes tumorigenesis and metastasis. Whilst TGFβ plays a key role in initiating myofibroblast differentiation and ECM deposition during normal wound healing, failure of myofibroblast clearance together with their autocrine production of TGFβ leads to ECM accumulation, fibrosis, and ultimately organ dysfunction. A large body of data from *in vitro* fibroblast-to-myofibroblast differentiation models indicates that TGFβ1-induced myofibroblast differentiation is mediated via induction of NOX4-derived H_2O_2, which

modulates downstream phosphorylation signaling cascades and transcriptional events that culminate in cytoskeletal remodeling and myofibroblast differentiation. In addition, NOX4-derived H_2O_2 appears to downregulate NO/cGMP signaling via multiple mechanisms and thereby relieves inhibition of myofibroblast differentiation by NO. Findings from these *in vitro* cellular models of fibrosis are supported by extensive *in vivo* data, underscoring the value of *in vitro* models in defining molecular mechanisms underlying fibrogenic disease and serving as screening platforms for the discovery of novel therapeutics. Targeting NOX4 directly via NOX4 inhibitors or indirectly by ROS scavenging with Se, antioxidants or enhancing NO/cGMP signaling by sGC stimulation, and/or inhibition of cGMP degradation attenuates TGFβ1-induced differentiation and inhibits myofibroblast activation. Moreover, these agents induce the dedifferentiation/reversal of preexisting myofibroblasts to a quiescent fibroblast phenotype. Thus, pharmacological interference of these redox signaling processes to restore the physiological fibroblast: myofibroblast ratio offers a promising therapeutic strategy not only for the treatment of fibrotic diseases but also for managing tumor invasion and metastasis at the level of stromal remodeling.

Abbreviations

α-SMA:	Alpha smooth muscle cell actin
bFGF:	Basic fibroblast growth factor
BPH:	Benign prostatic hyperplasia
cAMP:	Cyclic adenosine monophosphate
cGMP:	Cyclic guanosine monophosphate
ECM:	Extracellular matrix
ED:	Erectile dysfunction
IPF:	Idiopathic pulmonary fibrosis
L-NAME:	N_ω-nitro-L-arginine methyl ester
LUTS:	Lower urinary tract symptoms
MAPK:	Mitogen-associated protein kinase
NO:	Nitric oxide
NOS:	Nitric oxide synthase
NOX:	NADPH oxidase
PDE:	Phosphodiesterase
PKG:	Protein kinase G
PTK:	Protein tyrosine kinase
PTP:	Protein tyrosine phosphatase
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species
Se:	Selenium
sGC:	Soluble guanylyl cyclase
SMC:	Smooth muscle cell
SNP:	Sodium nitroprusside
TGFβ:	Transforming growth factor beta.

Acknowledgments

N. Sampson is supported by an Elise Richter postdoctoral fellowship from the Austrian Science Fund (FWF; V216-B13). P. Berger is supported by the Austrian Science Fund (FWF; NRN S9307-B05).

References

[1] B. Hinz, S. H. Phan, V. J. Thannickal et al., "Recent developments in myofibroblast biology: paradigms for connective tissue remodeling," *The American Journal of Pathology*, vol. 180, no. 4, pp. 1340–1355, 2012.

[2] G. Gabbiani, "The myofibroblast in wound healing and fibrocontractive diseases," *Journal of Pathology*, vol. 200, no. 4, pp. 500–503, 2003.

[3] N. Sampson, R. Koziel, C. Zenzmaier et al., "ROS signaling by NOX4 drives fibroblast-to-myofibroblast differentiation in the diseased prostatic stroma," *Molecular Endocrinology*, vol. 25, no. 3, pp. 503–515, 2011.

[4] A. T. Grazul-Bilska, M. L. Johnson, J. J. Bilski et al., "Wound healing: the role of growth factors," *Drugs of Today*, vol. 39, no. 10, pp. 787–800, 2003.

[5] I. Haase, R. Evans, R. Pofahl, and F. M. Watt, "Regulation of keratinocyte shape, migration and wound epithelialization by IGF-1 and EGF-dependent signalling pathways," *Journal of Cell Science*, vol. 116, no. 15, pp. 3227–3238, 2003.

[6] A. Desmouliere, M. Redard, I. Darby, and G. Gabbiani, "Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar," *American Journal of Pathology*, vol. 146, no. 1, pp. 56–66, 1995.

[7] J. I. Jun and L. F. Lau, "Cellular senescence controls fibrosis in wound healing," *Aging*, vol. 2, no. 9, pp. 627–631, 2010.

[8] G. N. Pitiyage, P. Slijepcevic, A. Gabrani et al., "Senescent mesenchymal cells accumulate in human fibrosis by a telomere-independent mechanism and ameliorate fibrosis through matrix metalloproteinases," *Journal of Pathology*, vol. 223, no. 5, pp. 604–617, 2011.

[9] S. L. Schissel and M. D. Layne, "Telomerase, myofibroblasts, and pulmonary fibrosis," *American Journal of Respiratory Cell and Molecular Biology*, vol. 34, no. 5, pp. 520–522, 2006.

[10] P. D. Adams, "Healing and hurting: molecular mechanisms, functions, and pathologies of cellular senescence," *Molecular Cell*, vol. 36, no. 1, pp. 2–14, 2009.

[11] A. Gosain and L. A. DiPietro, "Aging and wound healing," *World Journal of Surgery*, vol. 28, no. 3, pp. 321–326, 2004.

[12] K. Kis, X. Liu, and J. S. Hagood, "Myofibroblast differentiation and survival in fibrotic disease," *Expert Reviews in Molecular Medicine*, vol. 13, article e27, 2011.

[13] S. Ueha, F. H. Shand, and K. Matsushima, "Cellular and molecular mechanisms of chronic inflammation-associated organ fibrosis," *Frontiers in Immunology*, vol. 3, p. 71, 2012.

[14] M. Amano, M. Nakayama, and K. Kaibuchi, "Rho-kinase/ROCK: a key regulator of the cytoskeleton and cell polarity," *Cytoskeleton*, vol. 67, no. 9, pp. 545–554, 2010.

[15] J. J. Tomasek, G. Gabbiani, B. Hinz, C. Chaponnier, and R. A. Brown, "Myofibroblasts and mechano: regulation of connective tissue remodelling," *Nature Reviews Molecular Cell Biology*, vol. 3, no. 5, pp. 349–363, 2002.

[16] Y. Kojima, A. Acar, E. N. Eaton et al., "Autocrine TGF-β and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 46, pp. 20009–20014, 2010.

[17] L. Ronnov-Jessen and O. W. Petersen, "Induction of α-smooth muscle actin by transforming growth factor-β1 in quiescent human breast gland fibroblasts," *Laboratory Investigation*, vol. 68, no. 6, pp. 696–707, 1993.

[18] A. Desmouliere, A. Geinoz, F. Gabbiani et al., "Transforming growth factor-β1 induces α-smooth muscle actin expression

in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts," *Journal of Cell Biology*, vol. 122, no. 1, pp. 103–111, 1993.

[19] D. M. Peehl and R. G. Sellers, "Induction of smooth muscle cell phenotype in cultured human prostatic stromal cells," *Experimental Cell Research*, vol. 232, no. 2, pp. 208–215, 1997.

[20] I. Cucoranu, R. Clempus, A. Dikalova et al., "NAD(P)H oxidase 4 mediates transforming growth factor-β1-induced differentiation of cardiac fibroblasts into myofibroblasts," *Circulation Research*, vol. 97, no. 9, pp. 900–907, 2005.

[21] C. D. Bondi, N. Manickam, D. Y. Lee et al., "NAD(P)H oxidase mediates TGF-β1-induced activation of kidney myofibroblasts," *Journal of the American Society of Nephrology*, vol. 21, no. 1, pp. 93–102, 2010.

[22] J. X. Jiang, X. Chen, N. Serizawa et al., "Liver fibrosis and hepatocyte apoptosis are attenuated by GKT137831, a novel NOX4/NOX1 inhibitor in vivo," *Free Radical Biology & Medicine*, vol. 53, no. 2, pp. 289–296, 2012.

[23] T. R. Dunkern, D. Feurstein, G. A. Rossi, F. Sabatini, and A. Hatzelmann, "Inhibition of TGF-β induced lung fibroblast to myofibroblast conversion by phosphodiesterase inhibiting drugs and activators of soluble guanylyl cyclase," *European Journal of Pharmacology*, vol. 572, no. 1, pp. 12–22, 2007.

[24] A. Biernacka, M. Dobaczewski, and N. G. Frangogiannis, "TGF-beta signaling in fibrosis," *Growth Factors*, vol. 29, no. 5, pp. 196–202, 2011.

[25] G. Untergasser, R. Gander, C. Lilg, G. Lepperdinger, E. Plas, and P. Berger, "Profiling molecular targets of TGF-β1 in prostate fibroblast-to- myofibroblast transdifferentiation," *Mechanisms of Ageing and Development*, vol. 126, no. 1, pp. 59–69, 2005.

[26] J. Folkman, M. Klagsbrun, J. Sasse, M. Wadzinski, D. Ingber, and I. Vlodavsky, "A heparin-binding angiogenic protein-basic fibroblast growth factor-is stored within basement membrane," *American Journal of Pathology*, vol. 130, no. 2, pp. 393–400, 1988.

[27] G. S. Schultz and A. Wysocki, "Interactions between extracellular matrix and growth factors in wound healing," *Wound Repair and Regeneration*, vol. 17, no. 2, pp. 153–162, 2009.

[28] D. R. Senger, K. P. Claffey, J. E. Benes, C. A. Perruzzi, A. P. Sergiou, and M. Detmar, "Angiogenesis promoted by vascular endothelial growth factor: regulation through $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 25, pp. 13612–13617, 1997.

[29] J. Rosenbloom, S. V. Castro, and S. A. Jimenez, "Narrative review: fibrotic diseases: cellular and molecular mechanisms and novel therapies," *Annals of Internal Medicine*, vol. 152, no. 3, pp. 159–166, 2010.

[30] K. M. Mak, A. J. Kwong, E. Chu et al., "Hepatic steatosis, fibrosis, and cancer in elderly cadavers," *The Anatomical Record (Hoboken)*, vol. 295, no. 1, pp. 40–50, 2012.

[31] J. T. Isaacs, "Etiology of benign prostatic hyperplasia," *European Urology*, vol. 25, no. 1, pp. 6–9, 1994.

[32] N. Sampson, G. Untergasser, E. Plas, and P. Berger, "The ageing male reproductive tract," *Journal of Pathology*, vol. 211, no. 2, pp. 206–218, 2007.

[33] G. Pannarale, R. Carbone, G. Del Mastro et al., "The aging kidney: structural changes," *Journal of Nephrology*, vol. 23, no. 15, pp. S37–S40, 2010.

[34] M. Volkova, Y. Zhang, A. C. Shaw et al., "The role of Toll-like receptors in age-associated lung diseases," *The Journals of Gerontology A*, vol. 67, no. 3, pp. 247–253, 2012.

[35] D. F. Dai, T. Chen, S. C. Johnson et al., "Cardiac aging: from molecular mechanisms to significance in human health and disease," *Antioxidants & Redox Signaling*, vol. 16, no. 12, pp. 1492–1526, 2012.

[36] H. Nakagawa and S. Maeda, "Molecular mechanisms of liver injury and hepatocarcinogenesis: focusing on the role of stress-activated MAPK," *Pathology Research International*, vol. 2012, Article ID 172894, 2012.

[37] K. S. Sfanos and A. M. De Marzo, "Prostate cancer and inflammation: the evidence," *Histopathology*, vol. 60, no. 1, pp. 199–215, 2012.

[38] M. Otranto, V. Sarrazy, F. Bonte et al., "The role of the myofibroblast in tumor stroma remodeling," *Cell Adhesion & Migration*, vol. 6, no. 3, 2012.

[39] J. A. Tuxhorn, G. E. Ayala, M. J. Smith, V. C. Smith, T. D. Dang, and D. R. Rowley, "Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling," *Clinical Cancer Research*, vol. 8, no. 9, pp. 2912–2923, 2002.

[40] M. Ao, O. E. Franco, D. Park, D. Raman, K. Williams, and S. W. Hayward, "Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium," *Cancer Research*, vol. 67, no. 9, pp. 4244–4253, 2007.

[41] F. Yang, J. A. Tuxhorn, S. J. Ressler, S. J. McAlhany, T. D. Dang, and D. R. Rowley, "Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis," *Cancer Research*, vol. 65, no. 19, pp. 8887–8895, 2005.

[42] J. A. Tuxhorn, S. J. McAlhany, F. Yang, T. D. Dang, and D. R. Rowley, "Inhibition of transforming growth factor-β activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model," *Cancer Research*, vol. 62, no. 21, pp. 6021–6025, 2002.

[43] P. Lu, K. Takai, V. M. Weaver et al., "Extracellular matrix degradation and remodeling in development and disease," *Cold Spring Harbor Perspectives in Biology*, vol. 3, no. 12, 2011.

[44] H. Ikushima and K. Miyazono, "TGFβ 2 signalling: a complex web in cancer progression," *Nature Reviews Cancer*, vol. 10, no. 6, pp. 415–424, 2010.

[45] S. Chabaud, M. P. Corriveau, T. Grodzicky et al., "Decreased secretion of MMP by non-lesional late-stage scleroderma fibroblasts after selection via activation of the apoptotic fas-pathway," *Journal of Cellular Physiology*, vol. 226, no. 7, pp. 1907–1914, 2011.

[46] C. K. Garcia, "Idiopathic pulmonary fibrosis: update on genetic discoveries," *Proceedings of the American Thoracic Society*, vol. 8, no. 2, pp. 158–162, 2011.

[47] J. T. Cronkhite, C. Xing, G. Raghu et al., "Telomere shortening in familial and sporadic pulmonary fibrosis," *American Journal of Respiratory and Critical Care Medicine*, vol. 178, no. 7, pp. 729–737, 2008.

[48] A. L. Degryse, X. C. Xu, J. L. Newman et al., "Telomerase deficiency does not alter bleomycin-induced fibrosis in mice," *Experimental Lung Research*, vol. 38, no. 3, pp. 124–134, 2012.

[49] J. C. Horowitz, D. S. Rogers, V. Sharma et al., "Combinatorial activation of FAK and AKT by transforming growth factor-β1 confers an anoikis-resistant phenotype to myofibroblasts," *Cellular Signalling*, vol. 19, no. 4, pp. 761–771, 2007.

[50] P. Kulasekaran, C. A. Scavone, D. S. Rogers, D. A. Arenberg, V. J. Thannickal, and J. C. Horowitz, "Endothelin-1 and transforming growth factor-β1 independently induce fibroblast resistance to apoptosis via AKT activation," *American Journal*

of Respiratory Cell and Molecular Biology, vol. 41, no. 4, pp. 484–493, 2009.

[51] B. Hu, D. C. Tack, T. Liu, Z. Wu, M. R. Ullenbruch, and S. H. Phan, "Role of Smad3 in the regulation of rat telomerase reverse transcriptase by TGFβ," *Oncogene*, vol. 25, no. 7, pp. 1030–1041, 2006.

[52] H. Li, D. Xu, J. Li, M. C. Berndt, and J. P. Liu, "Transforming growth factor β suppresses human telomerase reverse transcriptase (hTERT) by Smad3 interactions with c-Myc and the hTERT gene," *Journal of Biological Chemistry*, vol. 281, no. 35, pp. 25588–25600, 2006.

[53] C. Beyer, G. Schett, O. Distler, and J. H. W. Distler, "Animal models of systemic sclerosis: prospects and limitations," *Arthritis and Rheumatism*, vol. 62, no. 10, pp. 2831–2844, 2010.

[54] J. Gauldie and M. Kolb, "Animal models of pulmonary fibrosis: how far from effective reality?" *American Journal of Physiology*, vol. 294, no. 2, p. L151, 2008.

[55] B. B. Moore and C. M. Hogaboam, "Murine models of pulmonary fibrosis," *American Journal of Physiology*, vol. 294, no. 2, pp. L152–L160, 2008.

[56] Y. Sun and K. T. Weber, "Animal models of cardiac fibrosis," *Methods in Molecular Medicine*, vol. 117, pp. 273–290, 2005.

[57] C. Weiler-Normann, J. Herkel, and A. W. Lohse, "Mouse models of liver fibrosis," *Zeitschrift fur Gastroenterologie*, vol. 45, no. 1, pp. 43–50, 2007.

[58] M. Zeisberg, M. A. Soubasakos, and R. Kalluri, "Animal models of renal fibrosis," *Methods in Molecular Medicine*, vol. 117, pp. 261–272, 2005.

[59] P. Roy-Burman, H. Wu, W. C. Powell, J. Hagenkord, and M. B. Cohen, "Genetically defined mouse models that mimic natural aspects of human prostate cancer development," *Endocrine-Related Cancer*, vol. 11, no. 2, pp. 225–254, 2004.

[60] P. Sharma and N. Schreiber-Agus, "Mouse models of prostate cancer," *Oncogene*, vol. 18, no. 38, pp. 5349–5355, 1999.

[61] J. P. Mizgerd and S. J. Skerrett, "Animal models of human pneumonia," *American Journal of Physiology*, vol. 294, no. 3, pp. L387–L398, 2008.

[62] G. Rowden, "Macrophages and dendritic cells in the skin. The skin immune system (SIS)," in *Cutaneous Immunology and Clinical Immunodermatology*, J. D. Bos, Ed., pp. 104–146, CRC Press, New York, NY, USA, 1997.

[63] E. Bell, B. Ivarsson, and C. Merrill, "Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 76, no. 3, pp. 1274–1278, 1979.

[64] R. H. Cunnington, B. Wang, S. Ghavami, K. L. Bathe, S. G. Rattan, and I. M. C. Dixon, "Antifibrotic properties of c-Ski and its regulation of cardiac myofibroblast phenotype and contractility," *American Journal of Physiology*, vol. 300, no. 1, pp. C176–C186, 2011.

[65] T. Kohyama, X. Liu, F. Q. Wen et al., "PDE4 inhibitors attenuate fibroblast chemotaxis and contraction of native collagen gels," *American Journal of Respiratory Cell and Molecular Biology*, vol. 26, no. 6, pp. 694–701, 2002.

[66] H. Pan, J. Chen, J. Xu, M. Chen, and R. Ma, "Antifibrotic effect by activation of peroxisome proliferator-activated receptor-gamma in corneal fibroblasts.," *Molecular Vision*, vol. 15, pp. 2279–2286, 2009.

[67] Q. Shi, X. Liu, Y. Bai et al., "In vitro effects of pirfenidone on cardiac fibroblasts: proliferation, myofibroblast differentiation, migration and cytokine secretion," *PLoS ONE*, vol. 6, no. 11, article e28134, 2011.

[68] C. Y. Tsai, K. I. Hata, S. Torii, M. Matsuyama, and M. Ueda, "Contraction potency of hypertrophic scar-derived fibroblasts in a connective tissue model: in vitro analysis of wound contraction," *Annals of Plastic Surgery*, vol. 35, no. 6, pp. 638–646, 1995.

[69] F. Q. Wen, C. M. Sköld, X. D. Liu et al., "Glucocorticoids and TGF-β1 synergize in augmenting fibroblast mediated contraction of collagen gels," *Inflammation*, vol. 25, no. 2, pp. 109–117, 2001.

[70] M. Yokozeki, K. Moriyama, H. Shimokawa, and T. Kuroda, "Transforming growth factor-β1 modulates myofibroblastic phenotype of rat palatal fibroblasts in Vitro," *Experimental Cell Research*, vol. 231, no. 2, pp. 328–336, 1997.

[71] H. Y. Zhang, M. Gharaee-Kermani, K. Zhang, S. Karmiol, and S. H. Phan, "Lung fibroblast α-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis," *American Journal of Pathology*, vol. 148, no. 2, pp. 527–537, 1996.

[72] H. Rumpold, K. Mascher, G. Untergasser, E. Plas, M. Hermann, and P. Berger, "Trans-differentiation of prostatic stromal cells leads to decreased glycoprotein hormone α production," *Journal of Clinical Endocrinology and Metabolism*, vol. 87, no. 11, pp. 5297–5303, 2002.

[73] C. Zenzmaier, N. Sampson, D. Pernkopf, E. Plas, G. Untergasser, and P. Berger, "Attenuated proliferation and trans-differentiation of prostatic stromal cells indicate suitability of phosphodiesterase type 5 inhibitors for prevention and treatment of benign prostatic hyperplasia," *Endocrinology*, vol. 151, no. 8, pp. 3975–3984, 2010.

[74] L. Hecker, R. Vittal, T. Jones et al., "NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury," *Nature Medicine*, vol. 15, no. 9, pp. 1077–1081, 2009.

[75] N. Amara, D. Goven, F. Prost, R. Muloway, B. Crestani, and J. Boczkowski, "NOX4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGFβ1-induced fibroblast differentiation into myofibroblasts," *Thorax*, vol. 65, no. 8, pp. 733–738, 2010.

[76] P. Li, D. Wang, J. Lucas et al., "Atrial natriuretic peptide inhibits transforming growth factor β-induced Smad signaling and myofibroblast transformation in mouse cardiac fibroblasts," *Circulation Research*, vol. 102, no. 2, pp. 185–192, 2008.

[77] H. Masuyama, T. Tsuruda, Y. Sekita et al., "Pressure-independent effects of pharmacological stimulation of soluble guanylate cyclase on fibrosis in pressure-overloaded rat heart," *Hypertension Research*, vol. 32, no. 7, pp. 597–603, 2009.

[78] D. Harman, "Aging: a theory based on free radical and radiation chemistry," *Journal of Gerontology*, vol. 11, no. 3, pp. 298–300, 1956.

[79] H. J. Forman, J. M. Fukuto, T. Miller, H. Zhang, A. Rinna, and S. Levy, "The chemistry of cell signaling by reactive oxygen and nitrogen species and 4-hydroxynonenal," *Archives of Biochemistry and Biophysics*, vol. 477, no. 2, pp. 183–195, 2008.

[80] F. Jiang, Y. Zhang, and G. J. Dusting, "NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair," *Pharmacological Reviews*, vol. 63, no. 1, pp. 218–242, 2011.

[81] S. Altenhofer, P. W. Kleikers, K. A. Radermacher et al., "The NOX toolbox: validating the role of NADPH oxidases in physiology and disease," *Cellular and Molecular Life Sciences*, vol. 69, no. 14, pp. 2327–2343, 2012.

[82] K. D. Martyn, L. M. Frederick, K. Von Loehneysen, M. C. Dinauer, and U. G. Knaus, "Functional analysis of NOX4 reveals unique characteristics compared to other NADPH oxidases," *Cellular Signalling*, vol. 18, no. 1, pp. 69–82, 2006.

[83] L. Serrander, L. Cartier, K. Bedard et al., "NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation," *Biochemical Journal*, vol. 406, no. 1, pp. 105–114, 2007.

[84] S. I. Dikalov, A. E. Dikalova, A. T. Bikineyeva, H. H. H. W. Schmidt, D. G. Harrison, and K. K. Griendling, "Distinct roles of NOX1 and NOX4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production," *Free Radical Biology and Medicine*, vol. 45, no. 9, pp. 1340–1351, 2008.

[85] I. Takac, K. Schröder, L. Zhang et al., "The E-loop is involved in hydrogen peroxide formation by the NADPH oxidase NOX4," *Journal of Biological Chemistry*, vol. 286, no. 15, pp. 13304–13313, 2011.

[86] K. Bedard and K. H. Krause, "The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology," *Physiological Reviews*, vol. 87, no. 1, pp. 245–313, 2007.

[87] D. Trachootham, W. Lu, M. A. Ogasawara, N. R. D. Valle, and P. Huang, "Redox regulation of cell survival," *Antioxidants and Redox Signaling*, vol. 10, no. 8, pp. 1343–1374, 2008.

[88] J. L. Barnes and Y. Gorin, "Myofibroblast differentiation during fibrosis: role of NAD(P)H oxidases," *Kidney International*, vol. 79, no. 9, pp. 944–956, 2011.

[89] K. A. Graham, M. Kulawiec, K. M. Owens et al., "NADPH oxidase 4 is an oncoprotein localized to mitochondria," *Cancer Biology and Therapy*, vol. 10, no. 3, pp. 223–231, 2010.

[90] J. Kuroda, T. Ago, S. Matsushima, P. Zhai, M. D. Schneider, and J. Sadoshima, "NADPH oxidase 4 (NOX4) is a major source of oxidative stress in the failing heart," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 35, pp. 15565–15570, 2010.

[91] S. J. Guo, L. Y. Wu, W. L. Shen et al., "Gene profile for differentiation of vascular adventitial myofibroblasts," *Sheng Li Xue Bao*, vol. 58, no. 4, pp. 337–344, 2006.

[92] T. Hu, S. P. Ramachandrarao, S. Siva et al., "Reactive oxygen species production via NADPH oxidase mediates TGF-β-induced cytoskeletal alterations in endothelial cells," *American Journal of Physiology*, vol. 289, no. 4, pp. F816–F825, 2005.

[93] R. E. Clempus, D. Sorescu, A. E. Dikalova et al., "NOX4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 1, pp. 42–48, 2007.

[94] K. Schröder, K. Wandzioch, I. Helmcke, and R. P. Brandes, "NOX4 acts as a switch between differentiation and proliferation in preadipocytes," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, no. 2, pp. 239–245, 2009.

[95] J. C. Pache, S. Carnesecchi, C. Deffert et al., "NOX-4 is expressed in thickened pulmonary arteries in idiopathic pulmonary fibrosis," *Nature Medicine*, vol. 17, no. 1, pp. 31–33, 2011.

[96] Y. Gorin, K. Block, J. Hernandez et al., "NOX4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney," *Journal of Biological Chemistry*, vol. 280, no. 47, pp. 39616–39626, 2005.

[97] D. P. Jones, "Radical-free biology of oxidative stress," *American Journal of Physiology*, vol. 295, no. 4, pp. C849–C868, 2008.

[98] R. M. Liu, J. Choi, J. H. Wu et al., "Oxidative modification of nuclear mitogen-activated protein kinase phosphatase 1 is involved in transforming growth factor β1-induced expression of plasminogen activator inhibitor 1 in fibroblasts," *Journal of Biological Chemistry*, vol. 285, no. 21, pp. 16239–16247, 2010.

[99] K. Block, A. Eid, K. K. Griendling, D. Y. Lee, Y. Wittrant, and Y. Gorin, "NOX4 NAD(P)H oxidase mediates Src-dependent tyrosine phosphorylation of PDK-1 in response to angiotensin II: role in mesangial cell hypertrophy and fibronectin expression," *Journal of Biological Chemistry*, vol. 283, no. 35, pp. 24061–24076, 2008.

[100] F. P. Bellinger, A. V. Raman, M. A. Reeves, and M. J. Berry, "Regulation and function of selenoproteins in human disease," *Biochemical Journal*, vol. 422, no. 1, pp. 11–22, 2009.

[101] S. C. Low, E. Grundner-Culemann, J. W. Harney, and M. J. Berry, "SECIS-SBP2 interactions dictate selenocysteine incorporation efficiency and selenoprotein hierarchy," *EMBO Journal*, vol. 19, no. 24, pp. 6882–6890, 2000.

[102] M. S. Crane, A. F. Howie, J. R. Arthur, F. Nicol, L. K. Crosley, and G. J. Beckett, "Modulation of thioredoxin reductase-2 expression in EAhy926 cells: implications for endothelial selenoprotein hierarchy," *Biochimica et Biophysica Acta*, vol. 1790, no. 10, pp. 1191–1197, 2009.

[103] V. Mostert, I. Dreher, J. Köhrle, S. Wolff, and J. Abel, "Modulation of selenoprotein P expression by TGF-β1 is mediated by Smad proteins," *BioFactors*, vol. 14, no. 1–4, pp. 135–142, 2001.

[104] V. Mostert, S. Wolff, I. Dreher, J. Köhrle, and J. Abel, "Identification of an element within the promoter of human selenoprotein P responsive to transforming growth factor-β," *European Journal of Biochemistry*, vol. 268, no. 23, pp. 6176–6181, 2001.

[105] E. Mezey, X. Liu, and J. J. Potter, "The combination of selenium and vitamin e inhibits type i collagen formation in cultured hepatic stellate cells," *Biological Trace Element Research*, vol. 140, no. 1, pp. 82–94, 2011.

[106] B. Contempre, O. Le Moine, J. E. Dumont, J. F. Denef, and M. C. Many, "Selenium deficiency and thyroid fibrosis. A key role for macrophages and transforming growth factor β (TGF-β)," *Molecular and Cellular Endocrinology*, vol. 124, no. 1-2, pp. 7–15, 1996.

[107] M. Ding, J. J. Potter, X. Liu, M. S. Torbenson, and E. Mezey, "Selenium supplementation decreases hepatic fibrosis in mice after chronic carbon tetrachloride administration," *Biological Trace Element Research*, vol. 133, no. 1, pp. 83–97, 2010.

[108] B. C. Pence, E. Delver, and D. M. Dunn, "Effects of dietary selenium on UVB-induced skin carcinogenesis and epidermal antioxidant status," *Journal of Investigative Dermatology*, vol. 102, no. 5, pp. 759–761, 1994.

[109] V. Diwadkar-Navsariwala, G. S. Prins, S. M. Swanson et al., "Selenoprotein deficiency accelerates prostate carcinogenesis in a transgenic model," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 21, pp. 8179–8184, 2006.

[110] M. Selenius, A. K. Rundlöf, E. Olm, A. P. Fernandes, and M. Björnstedt, "Selenium and the selenoprotein thioredoxin reductase in the prevention, treatment and diagnostics of cancer," *Antioxidants and Redox Signaling*, vol. 12, no. 7, pp. 867–880, 2010.

[111] A. J. Duffield-Lillico, B. L. Dalkin, M. E. Reid et al., "Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial," *BJU International*, vol. 91, no. 7, pp. 608–612, 2003.

[112] R. Hurst, L. Hooper, T. Norat et al., "Selenium and prostate cancer: systematic review and meta-analysis," *The American Journal of Clinical Nutrition*, vol. 96, no. 1, pp. 111–122, 2012.

[113] F. Murad, "Nitric oxide and cyclic GMP in cell signaling and drug development," *The New England Journal of Medicine*, vol. 355, no. 19, pp. 2003–2011, 2006.

[114] R. C. Rosen and J. B. Kostis, "Overview of phosphodiesterase 5 inhibition in erectile dysfunction," *American Journal of Cardiology*, vol. 92, no. 9, pp. 9M–18M, 2003.

[115] J. Houtchens, D. Martin, and J. R. Klinger, "Diagnosis and management of pulmonary arterial hypertension," *Pulmonary Medicine*, vol. 2011, Article ID 845864, 2011.

[116] M. Oelke, F. Giuliano, V. Mirone et al., "Monotherapy with tadalafil or tamsulosin similarly improved lower urinary tract symptoms suggestive of benign prostatic hyperplasia in an international, randomised, parallel, placebo-controlled clinical trial," *European Urology*, vol. 61, no. 5, pp. 917–925, 2012.

[117] J. I. Martínez-Salamanca, J. Carballido, I. Eardley et al., "Phosphodiesterase type 5 inhibitors in the management of non-neurogenic male lower urinary tract symptoms: Critical analysis of current evidence," *European Urology*, vol. 60, no. 3, pp. 527–535, 2011.

[118] M. Gacci, I. Eardley, F. Giuliano et al., "Critical analysis of the relationship between sexual dysfunctions and lower urinary tract symptoms due to benign prostatic hyperplasia," *European Urology*, vol. 60, no. 4, pp. 809–825, 2011.

[119] S. J. S. Grimsley, M. H. Khan, and G. E. Jones, "Mechanism of phosphodiesterase 5 inhibitor relief of prostatitis symptoms," *Medical Hypotheses*, vol. 69, no. 1, pp. 25–26, 2007.

[120] M. Takeda, "Effects of phosphodiesterase type 5 inhibitor on the contractility of prostate tissues and urethral pressure responses in a rat model of benign prostate hyperplasia: commentary," *International Journal of Urology*, vol. 14, no. 10, p. 951, 2007.

[121] S. Ückert, M. Sormes, G. Kedia et al., "Effects of phosphodiesterase inhibitors on tension induced by norepinephrine and accumulation of cyclic nucleotides in isolated human prostatic tissue," *Urology*, vol. 71, no. 3, pp. 526–530, 2008.

[122] A. J. Chu and J. K. Prasad, "Up-regulation by human recombinant transforming growth factor β-1 of collagen production in cultured dermal fibroblasts is mediated by the inhibition of nitric oxide signaling," *Journal of the American College of Surgeons*, vol. 188, no. 3, pp. 271–280, 1999.

[123] E. G. A. Valente, D. Vernet, M. G. Ferrini, A. Qian, J. Rajfer, and N. F. Gonzalez-Cadavid, "L-Arginine and phosphodiesterase (PDE) inhibitors counteract fibrosis in the Peyronie's fibrotic plaque and related fibroblast cultures," *Nitric Oxide—Biology and Chemistry*, vol. 9, no. 4, pp. 229–244, 2003.

[124] C. Beyer, N. Reich, S. C. Schindler et al., "Stimulation of soluble guanylate cyclase reduces experimental dermal fibrosis," *Annals of the Rheumatic Diseases*, vol. 71, no. 6, pp. 1019–1026, 2012.

[125] D. Vernet, M. G. Ferrini, E. G. Valente et al., "Effect of nitric oxide on the differentiation of fibroblasts into myofibroblasts in the Peyronie's fibrotic plaque and in its rat model," *Nitric Oxide—Biology and Chemistry*, vol. 7, no. 4, pp. 262–276, 2002.

[126] M. G. Ferrini, S. Rivera, J. Moon, D. Vernet, J. Rajfer, and N. F. Gonzalez-Cadavid, "The genetic inactivation of inducible nitric oxide synthase (iNOS) intensifies fibrosis and oxidative stress in the penile corpora cavernosa in type 1 diabetes," *Journal of Sexual Medicine*, vol. 7, no. 9, pp. 3033–3044, 2010.

[127] D. Sun, Y. Wang, C. Liu et al., "Effects of nitric oxide on renal interstitial fibrosis in rats with unilateral ureteral obstruction," *Life Sciences*, vol. 90, no. 23-24, pp. 900–909, 2012.

[128] Y. Wang, S. Krämer, T. Loof et al., "Enhancing cGMP in experimental progressive renal fibrosis: Soluble guanylate cyclase stimulation vs. phosphodiesterase inhibition," *American Journal of Physiology*, vol. 290, no. 1, pp. F167–F176, 2006.

[129] Y. Sharkovska, P. Kalk, B. Lawrenz et al., "Nitric oxide-independent stimulation of soluble guanylate cyclase reduces organ damage in experimental low-renin and high-renin models," *Journal of Hypertension*, vol. 28, no. 8, pp. 1666–1675, 2010.

[130] S. Geschka, A. Kretschmer, Y. Sharkovska et al., "Soluble guanylate cyclase stimulation prevents fibrotic tissue remodeling and improves survival in salt-sensitive dahl rats," *PLoS ONE*, vol. 6, no. 7, Article ID e21853, 2011.

[131] A. Knorr, C. Hirth-Dietrich, C. Alonso-Alija et al., "Nitric oxide-independent activation of soluble guanylate cyclase by BAY 60-2770 in experimental liver fibrosis," *Arzneimittel-Forschung/Drug Research*, vol. 58, no. 2, pp. 71–80, 2008.

[132] B. Hohenstein, C. Daniel, S. Wittmann, and C. Hugo, "PDE-5 inhibition impedes TSP-1 expression, TGF-β activation and matrix accumulation in experimental glomerulonephritis," *Nephrology Dialysis Transplantation*, vol. 23, no. 11, pp. 3427–3436, 2008.

[133] G. M. Rubanyi and P. M. Vanhoutte, "Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor," *The American Journal of Physiology*, vol. 250, no. 5, part 2, pp. H822–H827, 1986.

[134] N. D. Roe and J. Ren, "Nitric oxide synthase uncoupling: a therapeutic target in cardiovascular diseases," *Vascular Pharmacology*, vol. 57, no. 5-6, pp. 168–172, 2012.

[135] Y. M. Kim, H. Bergonia, and J. R. Lancaster Jr., "Nitrogen oxide-induced autoprotection in isolated rat hepatocytes," *FEBS Letters*, vol. 374, no. 2, pp. 228–232, 1995.

[136] E. A. Jaimes, C. Sweeney, and L. Raij, "Effects of the reactive oxygen species hydrogen peroxide and hypochlorite on endothelial nitric oxide production," *Hypertension*, vol. 38, no. 4, pp. 877–883, 2001.

[137] E. P. Wei and H. A. Kontos, "H_2O_2 and endothelium-dependent cerebral arteriolar dilation. Implications for the identity of endothelium-derived relaxing factor generated by acetylcholine," *Hypertension*, vol. 16, no. 2, pp. 162–169, 1990.

[138] C. Zenzmaier, J. Kern, N. Sampson et al., "Phosphodiesterase type 5 inhibition reverts prostate fibroblast-to-myofibroblast trans-differentiation," *Endocrinology*, 2012. In press.

[139] J. Ying, X. Tong, D. R. Pimentel et al., "Cysteine-674 of the sarco/endoplasmic reticulum calcium ATPase is required for the inhibition of cell migration by nitric oxide," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 4, pp. 783–790, 2007.

[140] T. Adachi, R. Matsui, R. M. Weisbrod, S. Najibi, and R. A. Cohen, "Reduced sarco/endoplasmic reticulum Ca^{2+} uptake activity can account for the reduced response to NO, but not sodium nitroprusside, in hypercholesterolemic rabbit aorta," *Circulation*, vol. 104, no. 9, pp. 1040–1045, 2001.

[141] X. Tong, X. Hou, D. Jourd'Heuil, R. M. Weisbrod, and R. A. Cohen, "Upregulation of NOX4 by TGFβ1 oxidizes SERCA and inhibits NO in arterial smooth muscle of the prediabetic zucker rat," *Circulation Research*, vol. 107, no. 8, pp. 975–983, 2010.

[142] X. Tong and K. Schröder, "NADPH oxidases are responsible for the failure of nitric oxide to inhibit migration of smooth muscle cells exposed to high glucose," *Free Radical Biology and Medicine*, vol. 47, no. 11, pp. 1578–1583, 2009.

[143] S. Wedgwood, R. H. Steinhorn, M. Bunderson et al., "Increased hydrogen peroxide downregulates soluble guanylate cyclase in the lungs of lambs with persistent pulmonary hypertension of the newborn," *American Journal of Physiology*, vol. 289, no. 4, pp. L660–L666, 2005.

[144] C. Gerassimou, A. Kotanidou, Z. Zhou, D. D. C. Simoes, C. Roussos, and A. Papapetropoulos, "Regulation of the expression of soluble guanylyl cyclase by reactive oxygen species," *British Journal of Pharmacology*, vol. 150, no. 8, pp. 1084–1091, 2007.

[145] S. Meurer, S. Pioch, S. Gross, and W. Müller-Esterl, "Reactive oxygen species induce tyrosine phosphorylation of and Src kinase recruitment to NO-sensitive guanylyl cyclase," *Journal of Biological Chemistry*, vol. 280, no. 39, pp. 33149–33156, 2005.

[146] K. S. Murthy, "Inhibitory phosphorylation of soluble guanylyl cyclase by muscarinic m2 receptors via G$\beta\gamma$-dependent activation of c-Src kinase," *Journal of Pharmacology and Experimental Therapeutics*, vol. 325, no. 1, pp. 183–189, 2008.

[147] N. Abdelaziz, F. Colombo, I. Mercier, and A. Calderone, "Nitric oxide attenuates the expression of transforming growth factor-β3 mRNA in rat cardiac fibroblasts via destabilization," *Hypertension*, vol. 38, no. 2, pp. 261–266, 2001.

[148] A. Bellocq, E. Azoulay, S. Marullo et al., "Reactive oxygen and nitrogen intermediates increase transforming growth factor-β1 release from human epithelial alveolar cells through two different mechanisms," *American Journal of Respiratory Cell and Molecular Biology*, vol. 21, no. 1, pp. 128–136, 1999.

[149] P. R. Bachiller, H. Nakanishi, and J. D. Roberts, "Transforming growth factor-β modulates the expression of nitric oxide signaling enzymes in the injured developing lung and in vascular smooth muscle cells," *American Journal of Physiology*, vol. 298, no. 3, pp. L324–L334, 2010.

[150] A. Douglass, K. Wallace, R. Parr et al., "Antibody-targeted myofibroblast apoptosis reduces fibrosis during sustained liver injury," *Journal of Hepatology*, vol. 49, no. 1, pp. 88–98, 2008.

[151] M. Bocchino, S. Agnese, E. Fagone et al., "Reactive oxygen species are required for maintenance and differentiation of primary lung fibroblasts in idiopathic pulmonary fibrosis," *PLoS ONE*, vol. 5, no. 11, Article ID e14003, 2010.

[152] M. C. Vozenin-Brotons, V. Sivan, N. Gault et al., "Antifibrotic action of Cu/Zn SOD is mediated by TGF-β1 repression and phenotypic reversion of myofibroblasts," *Free Radical Biology and Medicine*, vol. 30, no. 1, pp. 30–42, 2001.

[153] O. Maltseva, P. Folger, D. Zekaria, S. Petridou, and S. K. Masur, "Fibroblast growth factor reversal of the corneal myofibroblast phenotype," *Investigative Ophthalmology and Visual Science*, vol. 42, no. 11, pp. 2490–2495, 2001.

[154] M. G. Ferrini, I. Kovanecz, G. Nolazco, J. Rajfer, and N. F. Gonzalez-Cadavid, "Effects of long-term vardenafil treatment on the development of fibrotic plaques in a rat model of Peyronie's disease," *BJU International*, vol. 97, no. 3, pp. 625–633, 2006.

[155] A. Leask, "Potential therapeutic targets for cardiac fibrosis: TGFβ, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation," *Circulation Research*, vol. 106, no. 11, pp. 1675–1680, 2010.

[156] C. L. Miller, Y. Cai, M. Oikawa et al., "Cyclic nucleotide phosphodiesterase 1A: a key regulator of cardiac fibroblast activation and extracellular matrix remodeling in the heart," *Basic Research in Cardiology*, vol. 106, no. 6, pp. 1023–1039, 2011.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 919832, 8 pages
doi:10.1155/2012/919832

Research Article

Protein Oxidative Damage at the Crossroads of Cellular Senescence, Aging, and Age-Related Diseases

Martin A. Baraibar, Liang Liu, Emad K. Ahmed, and Bertrand Friguet

Laboratoire de Biologie Cellulaire du Vieillissement, UR4-IFR83, Université Pierre et Marie Curie-Paris 6, 4 Place Jussieu, 75252 Paris Cedex 05, France

Correspondence should be addressed to Bertrand Friguet, bertrand.friguet@snv.jussieu.fr

Received 30 July 2012; Accepted 14 September 2012

Academic Editor: Paula Ludovico

Protein damage mediated by oxidation, protein adducts formation with advanced glycated end products and with products of lipid peroxidation, has been implicated during aging and age-related diseases, such as neurodegenerative diseases. Increased protein modification has also been described upon replicative senescence of human fibroblasts, a valid model for studying aging *in vitro*. However, the mechanisms by which these modified proteins could impact on the development of the senescent phenotype and the pathogenesis of age-related diseases remain elusive. In this study, we performed *in silico* approaches to evidence molecular actors and cellular pathways affected by these damaged proteins. A database of proteins modified by carbonylation, glycation, and lipid peroxidation products during aging and age-related diseases was built and compared to those proteins identified during cellular replicative senescence *in vitro*. Common cellular pathways evidenced by enzymes involved in intermediate metabolism were found to be targeted by these modifications, although different tissues have been examined. These results underscore the potential effect of protein modification in the impairment of cellular metabolism during aging and age-related diseases.

1. Introduction

Hayflick and Moorhead established the concept that primary cells isolated from mammalian tissues undergo only a finite number of divisions when grown in culture, without spontaneous transformation [1, 2]. On reaching their replicative limit, such cells were termed "senescent" and were viewed as aged cells. Since then, replicative senescence has been considered as a cellular model of aging that may provide insight into organismal aging [3–5]. More recently, different studies have shown the occurrence of cellular senescence *in vivo* [6, 7]. These senescent cells are causally implicated in generating age-related phenotypes, and their removal can prevent or delay tissue dysfunction and extend healthspan [8]. Indeed, whether and how cellular senescence is related to organismal aging, age-related diseases, frailty, and dysfunction is one of the major open questions in the biology of aging field.

A hallmark of aging both at the cellular and organismal level is the accumulation of damaged macromolecules due to increased oxidative stress and failure of protein repair and maintenance systems [9, 10]. Reactive oxygen species (ROS) are routinely produced as a byproduct of aerobic metabolism and oxidative phosphorylation. In addition, ROS production and accumulation are usually increased during disease pathogenesis (i.e., in particular age-related diseases) [11]. Low concentrations or transient exposure to ROS induce cell proliferation and regulate the activation of several signaling pathways [12]. However, unneutralized ROS cause oxidative damage to lipids, proteins, and nucleic acids, thus leading to aberrant molecular activities [13, 14]. Protein oxidation is particularly detrimental as the resulting damages and/or induced conformational changes to protein structures can render oxidized proteins inactive and lead to cellular functional abnormalities [15, 16].

Various types of protein oxidative modifications are induced directly by ROS or indirectly by reactions with secondary products of oxidative stress [17]. Cysteine and methionine residues are particularly prone to oxidative modifications, but they might not be directly linked to

protein damage, since they also participate in cellular signaling events [18]. On the other hand, irreversible oxidation products of other residues are most frequently hydroxylated and carbonylated amino acid side chain derivatives. The exponential rate accumulation of carbonylated proteins during life span both at the cellular and organismal level and their particular increase in organs affected by age-related diseases, imply that this "Oxi-proteome" (i.e., the restricted set of proteins targeted by oxidation) may be a potential molecular substratum for many of the cellular dysfunctions described. Protein carbonylation includes aldehydes and ketones formed via different mechanisms: (i) direct oxidation of the polypeptide backbone leading to truncated peptides; (ii) side chains oxidation of lysine, arginine, proline, and threonine; (iii) reaction of histidine, cysteine, and lysine amino acid residues with aldehydes, for example, produced by lipid peroxidation; and (iv) glycation (nonenzymatic glycosylation) of lysine residues forming Amadori and Heyns rearrangements products (advanced glycated end products: AGE) [19–21]. Carbonylated proteins are generally less active, less thermostable and are exposing hydrophobic amino acids at their surface. Since oxidative modifications that give rise to carbonyl groups generally cause loss of catalytic or structural function in the affected proteins, it has been proposed that the increased level of oxidatively modified proteins observed during aging and age-related disease could have deleterious effects on cellular and organ function.

Increased levels of protein carbonyls have been observed in age-related diseases, such as neurodegenerative diseases (amyotrophic lateral sclerosis, Alzheimer's, Parkinson's, and Huntington's diseases), cataractogenesis, systemic amyloidosis, muscular dystrophy, progeria, Werner's syndrome, rheumatoid arthritis, and respiratory distress syndrome [22–24]. Elevated levels of proteins modified by lipid oxidation products (4-hydroxy-2-nonenal: HNE, malondialdehyde) are associated with neurodegenerative diseases, iron-induced renal carcinogenesis, cardiovascular disease, as well as elevated levels of protein glycation/glycoxidation end products (AGE) are associated with diabetes mellitus, neurodegenerative diseases, atherosclerosis, and Down's syndrome. Significant advances in the past recent years have been made towards the identification of proteins targeted by these modifications, although their possible causative role in the pathogenesis of these diseases has not yet been determined.

Previous studies have addressed the identification of accumulated carbonylated proteins, as well as proteins modified by glycation and conjugation with the lipid peroxidation product HNE in senescent human WI-38 embryonic fibroblasts [25, 26]. Herein, by using *in silico* approaches, we have extended our analyses of modified proteins to those reported during *in vivo* aging and age-related diseases. Over than 180 proteins have been reported in the literature as increasingly modified, that are involved in key cellular pathways such as inflammatory response, energy metabolism, protein folding, and free radical scavenging. We found that several proteins (35%) identified in senescent fibroblasts were also found during organ aging and age-related diseases and when grouped into cellular function and canonical pathways they showed a close relationship. Taken together, these results indicate the particular susceptibility of certain proteins to detrimental posttranslational modifications, suggesting that protein modification is not a random process and is likely to be implicated in the molecular bases of aging and age-related diseases.

2. Material and Methods

Functional and canonical pathways grouping of the identified modified proteins as well as network analyses were generated using the Ingenuity pathway analysis (IPA) software (version 2.0, Ingenuity Systems, Mountain view, CA, USA).

2.1. Functional Analysis of the Entire Data Set. The functional analysis identified the biological functions and/or diseases that were most significant to the data set. Molecules from the data set that were associated with biological functions in the Ingenuity Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a P-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

2.2. Canonical Pathways Analysis. Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. Molecules from the data set that were associated with a canonical pathway in the Ingenuity Knowledge Base were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured in two ways. (1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. (2) Fisher's exact test was used to calculate a P value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

2.3. Network Generation. A data set containing gene (or chemical) identifiers and corresponding expression values was uploaded into the application. Each identifier was mapped to its corresponding object in the Ingenuity Knowledge Base. These molecules, called Network Eligible molecules, were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Knowledge Base. Human, mouse, and rat orthologs of a gene are stored as separate objects in the Ingenuity Knowledge Base but are represented as a single node in the network.

3. Results and Discussion

3.1. Characterization of Carbonylated Proteins in WI-38 Fibroblasts upon Replicative Senescence. The identified modified proteins in senescent fibroblasts reported previously [26] were classified by cellular localization and grouped by functional correlations, canonical pathways, and potential interactions (Figure 1). The subcellular location of the modified proteins indicated that the modified proteins are found in two major fractions: mitochondria (44%) and cytosol—including cytoskeletal proteins—(28%), underlying the high level of protein damage within mitochondria in senescent fibroblasts, especially when taking into account that proteins from this organelle represent only 5–10% of total cellular protein lysates (Figure 1(a)).

Data mining on molecular relationships was performed with the aim of detecting the cellular pathways potentially perturbed upon replicative senescence. Major categories include key cellular processes such as mitochondrial dysfunction; citrate cycle; butanoate; carbohydrate; lipids and pyruvate metabolism; oxidative phosphorylation; arginine and proline metabolism (Figure 1(b)). At the functional level, a decrease in the mitochondrial capability of ATP regeneration has been described during aging [27]. Notably, mitochondrial proteins were particularly targeted by oxidation during replicative senescence in WI-38 fibroblasts, such as the iron-sulfur subunit of complex I and subunit alpha of ATP synthase. In addition, HNE has been previously found to inhibit the activity of many respiratory chain complexes *in vitro* [28], and mitochondrial respiration is also affected by incubation of mitochondria with the AGE inducer methylglyoxal [29]. Enzymes like malate dehydrogenase, 2-oxoglutarate dehydrogenase E1 component, glycerol-3-phosphate dehydrogenase, glycerol kinase, and glutaminase were found increasingly modified upon replicative senescence. These findings suggest that modification of proteins responsible for energy metabolism may participate in the impairment of mitochondrial function observed in senescent cells.

To obtain further insights into the cellular pathways potentially affected during the development of the senescent phenotype, a protein interaction analysis was performed (Figure 1(c)) using the Ingenuity Pathways Analysis software. This analysis showed interrelationships that involve the modified proteins within a defined network. The obtained network shows that peroxisome proliferator-activated receptor gamma (PPARγ), tumor necrosis factor (TNF), and insulin growth factor (IGF1) are central nodes to this network. Interestingly, all three proteins have been previously implicated in cellular senescence. For instance, PPARγ is a member of the nuclear receptor superfamily of ligand-activated transcription factors and regulates gene expression of key proteins involved in glucose/lipid metabolism, vascular inflammation, and proliferation. Previous studies have shown that PPARγ activation promotes cellular senescence in WI-38 fibroblasts through the induction of p16 expression [30]. Furthermore, protein modifications may also result in the alteration of the interactome of targeted proteins, hence, affecting the normal function of other nonmodified proteins. For example, four proteins that are increasingly modified during replicative senescence, are interacting with the sterol regulatory element-binding transcription factor 1 (SREBF1), also involved in cellular senescence [31], making it a likely target for impaired connections with these proteins.

3.2. In Silico Analysis of Carbonylated Proteins Identified during In Vivo Aging and Age-Related Diseases. We next addressed whether the proteins identified as increasingly modified during *in vitro* aging of WI-38 fibroblasts were also identified in either human or mammalian animal models during aging and age-related diseases (neurodegenerative diseases, cancer, and diabetes), where increased protein carbonylation has been consistently evidenced. For that purpose, a specific search was performed for proteins identified as increasingly carbonylated or modified by AGE or HNE in articles published in peer-reviewed journals. Modified proteins identified in humans, but also in mammalian models of diseases (rodents), were selected. A total of 183 proteins were listed (supplementary Table 1, see Supplementary Materials available online at doi:10.1155/2012/919832). Modified proteins were identified in brain, cerebellum, spinal cord, skeletal muscle, liver, eyes, and cerebrospinal and bronchoalveolar fluids. Of note is that most of the proteins identified as increasingly modified belong from the brain due to the high number of studies addressing the importance of protein carbonylation in the pathogenesis of several neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Alzheimer's, Parkinson's and Huntington's diseases. However, several proteins have been identified consistently modified in different organs systems such as central nervous system, liver, and eyes, indicating that the spectrum of proteins targeted by these modifications is conserved. Conditions of acute oxidative stress have not been included in this study, but we have recently shown in human myoblasts that even under high concentrations of hydrogen peroxide, only a restricted set of proteins are targeted by oxidation [32]. Interestingly, these carbonylated proteins are not necessarily the most abundant and differ largely with those proteins that exhibit changes at the expression level in the total cellular proteome [32].

Annotation of subcellular location of referenced proteins was performed based on information deposited in the human protein reference database (HPRD). Cytoplasmic proteins were predominant, followed by proteins from mitochondria, nucleus, endoplasmic reticulum and plasma membrane, respectively (Figure 2(a)). However, it is important to note that most of the studies were performed in total tissue soluble extracts, where mitochondrial and membrane proteins are clearly underrepresented when compared to total cellular proteins.

Functional annotation was also performed using Ingenuity Knowledge Base. Proteins were mainly distributed by biological processes such as inflammatory response, cellular metabolism, free radical scavenging, and protein synthesis and folding (Table 1). In addition, some proteins belonged to different functional families, hence their participation in other crucial cellular processes cannot be ignored.

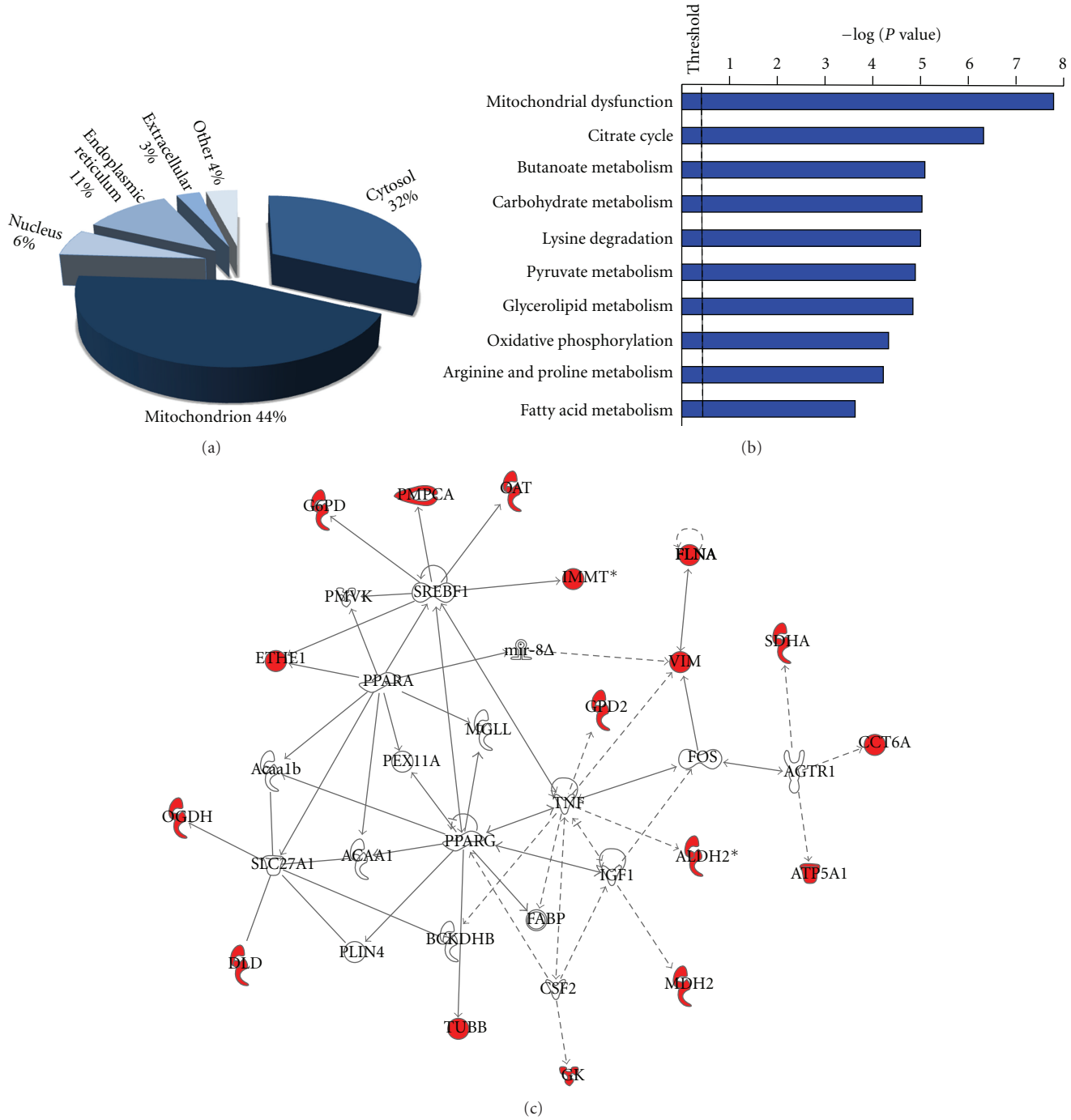


FIGURE 1: Cellular pathways and network analysis of proteins reported to be increasingly modified in senescent WI-38 human fibroblasts. (a) Primary subcellular location of modified proteins previously reported in senescent fibroblasts. Rare primary localization terms were grouped as *Other* representing less than 6% of total identifications. (b) Proteins were grouped by canonical pathways through the use of Ingenuity Pathways Analysis (Ingenuity Systems, http://www.ingenuity.com/). The bars represent the canonical pathways identified, named in the x-axis. The y-axis shows the $-\log$ of the P value calculated based on Fisher's exact test. The dotted line represents the threshold above which there are statistically significantly more genes in a biological function than expected by chance. (c) Protein networks were obtained using Ingenuity Pathway. Proteins in red correspond to some of the proteins identified as increasingly modified in senescent fibroblasts. White open nodes indicate proteins not identified in this analysis, but associated with the regulation of some of the proteins identified. Information about the analysis of biological functions and pathways as well as network interactions is available at the Ingenuity Pathway Analysis website. A line denotes binding of proteins, whereas a line with an arrow denotes "acts on." A dotted line denotes an indirect interaction.

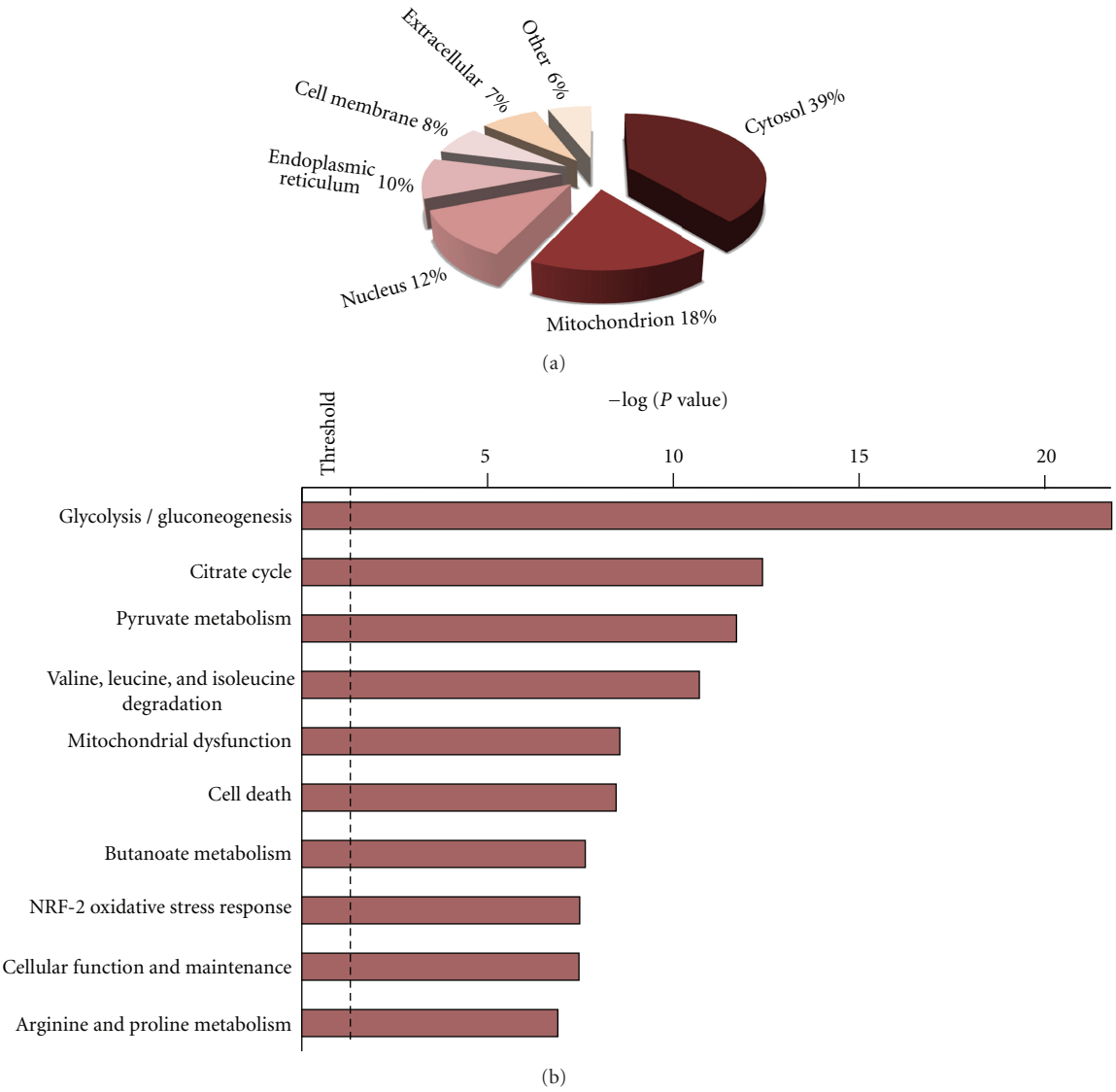


Figure 2: Cellular localization and functional grouping of modified proteins identified during aging and age-related diseases. (a) Primary subcellular location of the identified modified proteins. (b) Functional grouping of modified proteins through the use of Ingenuity Pathways Analysis. The bars represent the biological functions identified, named in the x-axis. The dotted line represents the threshold above which there are statistically significantly more genes in a biological function than expected by chance.

Concerning proteins involved in the inflammatory response, it is important to note that physiological aging is associated with a chronic subclinical systemic inflammatory response, also referred as to "inflamm-aging", characterized by elevated levels of serum proinflammatory cytokines such as interleukin 6 (IL-6), TNFα and acute phase proteins such as C-reactive protein (CRP) [33]. Inflammation is now accepted as a key pathogenic factor in the development of several age-related pathologies including cardiovascular disease, type 2 diabetes and neurodegenerative diseases [33]. Importantly, the inflammatory environment is highly oxidative, and increased protein oxidation has been described, generating a positive feedback process. Proteins involved in energy metabolism were also evidenced in the modified proteins referenced. The most significant canonical pathways across the entire dataset included: *glycolysis/gluconeogenesis*, *citrate cycle*, *pyruvate metabolism, amino acids degradation, mitochondrial dysfunction, cell death, butanoate metabolism, NRF-2 oxidative stress response, and cellular function and maintenance* (Figure 2(b)).

3.3. Comparison of Carbonylated Proteins in Senescent Fibroblasts with those Identified during In Vivo Aging and Age-Related Diseases. Twelve proteins (heat shock cognate 71 KDa protein, vimentin, actin, tubulin, glyceraldehyde-3-phosphate dehydrogenase, ATP synthase, proteasome subunit 11, elongation factor Tu, cytochrome b-c1 complex, annexin A5, proteasome subunit alpha type-2, and malate

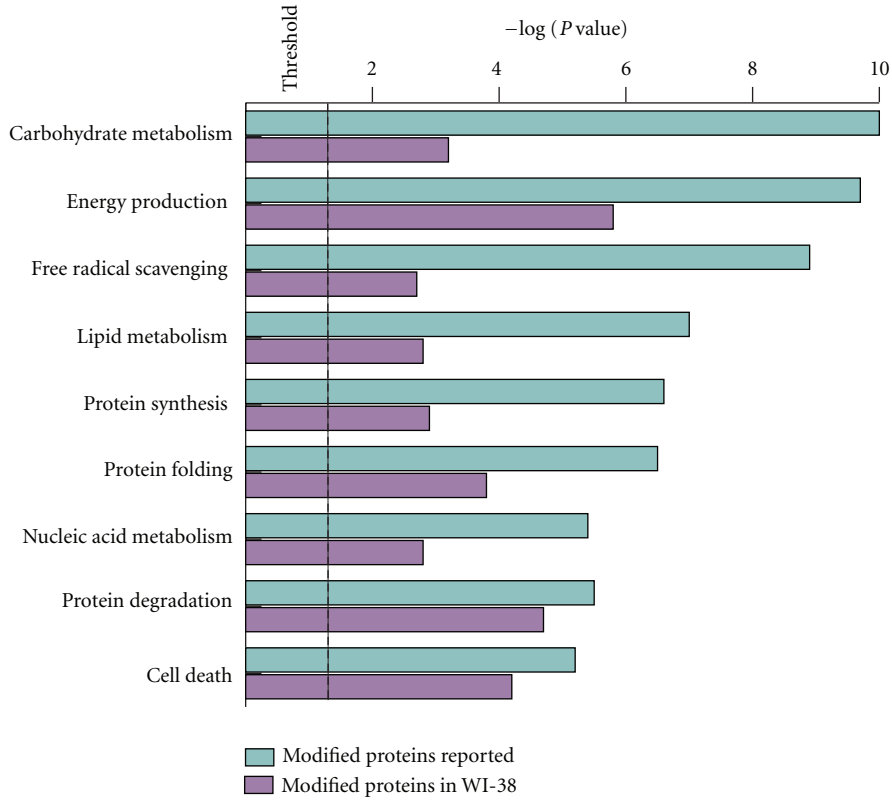


Figure 3: Common cellular functions across the two data sets after comparison analysis. The bars represent the biological functions identified, named in the x-axis. The y-axis shows the $-\log$ of the P value calculated based on Fisher's exact test. The dotted line represents the threshold above which there are statistically significantly more genes in a biological function than expected by chance. The differences observed in the P-values for each function in between the two groups are due to differences in the number of proteins assigned and reference data set.

Table 1: Functional categories of proteins identified as increasingly modified in aging and age-related diseases. Fisher exact test was used and the P value refers to the probability that each biological function assigned is due to chance alone.

Function	P value	Modified proteins
Inflammatory response	$3.7E-28$	ACO2, ACTB, ALDH2, ALDOA, ARG1, ENO1, GAPDH, HSPA5, P4HB, PDIA3, PRDX1, PRDX6, SELENBP1, TKT, TPI1
Carbohydrate metabolism	$6.0E-11$	ALDOA, ENO1, ENO2, FBP1, GAPDH, PGAM1, PGK1, PKLR, PKM2, TPI1, ACADM
Nucleic acid metabolism	$1.4E-10$	ACOT8, ALDH2, ATP5A1, ATP5B, CKM, DPYSL2, GNB1, SCP2, SUCLA2, MDH2
Cell death	$1.0E-9$	ACAT1, ANXA2, ARG1, ATP5A1, G6PD, G5TM5, HSPD1, SLC1A2, PIN1, PRDX2, PARK7, TUBA1A
Lipid metabolism	$6.9E-7$	MDH1, MDH2, SDHA, SDHB, OGDH, SUCLA2, ACOT8, FABP4, GSTP1, SCP2
Free radical scavenging	$1.1E-7$	ALB, SOD1, SOD2, PRDX1, PRDX2, PRDX6, VDAC1, ALDH2, GPX1
Protein folding	$6.9E-6$	HSP90AA1, HSPA1A, ERP29, HSPD1, HSPA8, HSP90AB1
Amino acid metabolism	$5.4E-6$	ARG1, DDAH1, IVD, OAT, PAH, CKM, PLOD3
Protein synthesis	$5.8E-5$	EEF2, GNB2L1, RPS6KB1, TUFM, WARS, CCDC92
Cell migration	$8.0E-4$	AP2M1, FSCN1, GNB1, TPM1, VIM, ACTB, TAGLN2

dehydrogenase), which correspond to about one third of the proteins identified during replicative senescence of WI38 fibroblasts *in vitro,* have been also identified as increasingly modified in other models of aging and age-related diseases.

Comparison of these two data sets indicates a similarity in the proteins targeted by these modifications to some extent. It is important to note that the majority of the studies has been achieved on brain, where the protein expression profile could differ significantly from the one of fibroblasts cultured *in vitro*. Further studies should address this issue by using senescent cells from different tissues. Of particular

interest would be human senescent stem cells since they develop both chronological and replicative aging.

IPA comparison analysis and functional grouping of proteins identified in senescent fibroblasts and those reported in the literature were performed. The analysis showed several common molecular and cellular functions involved in both datasets (Figure 3). Important cellular functions, like energy production, carbohydrate metabolism, protein synthesis, folding, and degradation may be implicated since they are directly targeted by modification of proteins implicated in those cellular pathways. In both cases, underlying common mechanisms are involved, although these *in silico* analyses are rather predictive and should be confirmed by experimental data.

Common processes are suggested to be causally involved or at least contribute to aging and age-related diseases, including increased oxidative stress, accumulation of protein damage, and general metabolic dysfunction. However, these processes have been mostly seen as independent events. An important outcome of the present study is that several enzymes that catalyze intermediate metabolism, such as glycolysis, gluconeogenesis, the citrate cycle, and fatty acid metabolism have been found to be modified. These results indicate a potential effect of protein modification on the impairment of cellular energy metabolism. Future studies should address this important issue by combining metabolomics and targeted proteomic analysis during cellular and organismal aging.

Acknowledgments

The authors are very thankful to the EU-funded projects MYOAGE (no. 223576) and PROTEOMAGE (no. 518230), under FP7 and FP6, as well as to the COST Action CM1001. In addition, they are very grateful to Dr. Adelina Rogowska-Wrzesinska for her advice on cellular pathway analyses.

References

[1] L. Hayflick and P. S. Moorhead, "The serial cultivation of human diploid cell strains," *Experimental Cell Research*, vol. 25, no. 3, pp. 585–621, 1961.

[2] L. Hayflick, "The limited in vitro lifetime of human diploid cell strains," *Experimental Cell Research*, vol. 37, no. 3, pp. 614–636, 1965.

[3] S. N. Garcia and O. Pereira-Smith, "MRGing chromatin dynamics and cellular senescence," *Cell Biochemistry and Biophysics*, vol. 50, no. 3, pp. 133–141, 2008.

[4] D. Röhme, "Evidence for a relationship between longevity of mammalian species and life spans of normal fibroblasts in vitro and erythrocytes in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, no. 8 I, pp. 5009–5013, 1981.

[5] R. Wadhwa, C. C. Deocaris, N. Widodo, K. Taira, and S. C. Kaul, "Imminent approaches towards molecular interventions in ageing," *Mechanisms of Ageing and Development*, vol. 126, no. 4, pp. 481–490, 2005.

[6] J. D. Erusalimsky and D. J. Kurz, "Cellular senescence in vivo: its relevance in ageing and cardiovascular disease," *Experimental Gerontology*, vol. 40, no. 8-9, pp. 634–642, 2005.

[7] J. C. Jeyapalan and J. M. Sedivy, "Cellular senescence and organismal aging," *Mechanisms of Ageing and Development*, vol. 129, no. 7-8, pp. 467–474, 2008.

[8] D. J. Baker, T. Wijshake, T. Tchkonia et al., "Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders," *Nature*, vol. 479, pp. 232–236, 2012.

[9] E. R. Stadtman, "Protein oxidation and aging," *Free Radical Research*, vol. 40, no. 12, pp. 1250–1258, 2006.

[10] N. Ugarte, I. Petropoulos, and B. Friguet, "Oxidized mitochondrial protein degradation and repair in aging and oxidative stress," *Antioxidants and Redox Signaling*, vol. 13, no. 4, pp. 539–549, 2010.

[11] K. C. Kregel and H. J. Zhang, "An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations," *American Journal of Physiology*, vol. 292, no. 1, pp. R18–R36, 2007.

[12] K. Apel and H. Hirt, "Reactive oxygen species: metabolism, oxidative stress, and signal transduction," *Annual Review of Plant Biology*, vol. 55, pp. 373–399, 2004.

[13] P. Mecocci, G. Fanó, S. Fulle et al., "Age-dependent increases in oxidative damage to DNA, lipids, and proteins in human skeletal muscle," *Free Radical Biology and Medicine*, vol. 26, no. 3-4, pp. 303–308, 1999.

[14] S. V. Avery, "Molecular targets of oxidative stress," *Biochemical Journal*, vol. 434, no. 2, pp. 201–210, 2011.

[15] M. A. Baraibar, A. G. Barbeito, B. B. Muhoberac, and R. Vidal, "A mutant light-chain ferritin that causes neurodegeneration has enhanced propensity toward oxidative damage," *Free Radical Biology and Medicine*, vol. 52, no. 9, pp. 1692–1697, 2012.

[16] C. R. Picot, M. Moreau, M. Juan et al., "Impairment of methionine sulfoxide reductase during UV irradiation and photoaging," *Experimental Gerontology*, vol. 42, no. 9, pp. 859–863, 2007.

[17] E. R. Stadtman and R. L. Levine, "Free radical-mediated oxidation of free amino acids and amino acid residues in proteins," *Amino Acids*, vol. 25, no. 3-4, pp. 207–218, 2003.

[18] J. Chiu, C. M. Tactacan, S. X. Tan, R. C. Y. Lin, M. A. Wouters, and I. W. Dawes, "Cell cycle sensing of oxidative stress in *Saccharomyces cerevisiae* by oxidation of a specific cysteine residue in the transcription factor Swi6p," *The Journal of Biological Chemistry*, vol. 286, no. 7, pp. 5204–5214, 2011.

[19] M. Kästle and T. Grune, "Protein oxidative modification in the aging organism and the role of the ubiquitin proteasomal system," *Current Pharmaceutical Design*, vol. 17, no. 36, pp. 4007–4022, 2011.

[20] K. Uchida, "4-Hydroxy-2-nonenal: a product and mediator of oxidative stress," *Progress in Lipid Research*, vol. 42, no. 4, pp. 318–343, 2003.

[21] N. Rabbani and P. J. Thornalley, "Dicarbonyls linked to damage in the powerhouse: glycation of mitochondrial proteins and oxidative stress," *Biochemical Society Transactions*, vol. 36, no. 5, pp. 1045–1050, 2008.

[22] B. S. Berlett and E. R. Stadtman, "Protein oxidation in aging, disease, and oxidative stress," *The Journal of Biological Chemistry*, vol. 272, no. 33, pp. 20313–20316, 1997.

[23] I. Dalle-Donne, D. Giustarini, R. Colombo, R. Rossi, and A. Milzani, "Protein carbonylation in human diseases," *Trends in Molecular Medicine*, vol. 9, no. 4, pp. 169–176, 2003.

[24] A. Martínez, M. Portero-Otin, R. Pamplona, and I. Ferrer, "Protein targets of oxidative damage in human neurodegenerative diseases with abnormal protein aggregates," *Brain Pathology*, vol. 20, no. 2, pp. 281–297, 2010.

[25] E. K. Ahmed, C. R. Picot, A. L. Bulteau, and B. Friguet, "Protein oxidative modifications and replicative senescence of

WI-38 human embryonic fibroblasts," *Annals of the New York Academy of Sciences*, vol. 1119, no. 1, pp. 88–96, 2007.

[26] E. K. Ahmed, A. Rogowska-Wrzesinska, P. Roepstorff, A. L. Bulteau, and B. Friguet, "Protein modification and replicative senescence of WI-38 human embryonic fibroblasts," *Aging Cell*, vol. 9, no. 2, pp. 252–272, 2010.

[27] Y. C. Jang and H. V. Remmen, "The mitochondrial theory of aging: insight from transgenic and knockout mouse models," *Experimental Gerontology*, vol. 44, no. 4, pp. 256–260, 2009.

[28] O. M. Lashin, P. A. Szweda, L. I. Szweda, and A. M. P. Romani, "Decreased complex II respiration and HNE-modified SDH subunit in diabetic heart," *Free Radical Biology and Medicine*, vol. 40, no. 5, pp. 886–896, 2006.

[29] M. G. Rosca, V. M. Monnier, L. I. Szweda, and M. F. Weiss, "Alterations in renal mitochondrial respiration in response to the reactive oxoaldehyde methylglyoxal," *American Journal of Physiology*, vol. 283, no. 1, pp. F52–F59, 2002.

[30] Q. Gan, J. Huang, R. Zhou et al., "PPARγ accelerates cellular senescence by inducing p16INK4α expression in human diploid fibroblasts," *Journal of Cell Science*, vol. 121, no. 13, pp. 2235–2245, 2008.

[31] Y. M. Kim, H. T. Shin, Y. H. Seo et al., "Sterol regulatory element-binding protein (SREBP)-1-mediated lipogenesis is involved in cell senescence," *The Journal of Biological Chemistry*, vol. 285, no. 38, pp. 29069–29077, 2010.

[32] M. A. Baraibar, J. Hyzewicz, A. Rogowska-Wrzesinska et al., "Oxidative stress-induced proteome alterations target different cellular pathways in human myoblasts," *Free Radical Biology and Medicine*, vol. 51, no. 8, pp. 1522–1532, 2011.

[33] C. Franceschi, M. Bonafè, S. Valensin et al., "Inflamm-aging. An evolutionary perspective on immunosenescence," *Annals of the New York Academy of Sciences*, vol. 908, pp. 244–254, 2000.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 217594, 9 pages
doi:10.1155/2012/217594

Research Article

Centrosome Aberrations Associated with Cellular Senescence and p53 Localization at Supernumerary Centrosomes

Susumu Ohshima

Division of Morphological Science, Biomedical Research Center, Saitama Medical University, 38 Morohongo, Moroyama, Iruma, Saitama 350-0495, Japan

Correspondence should be addressed to Susumu Ohshima, sohshima@saitama-med.ac.jp

Received 28 May 2012; Revised 27 August 2012; Accepted 11 September 2012

Academic Editor: William C. Burhans

Centrosome overduplication or amplification has been observed in many human cancers and in premalignant tissue, but the mechanisms leading to such centrosome aberrations are not fully understood. We previously showed that abnormal mitotic cells with supernumerary centrosomes increase with replicative senescence in human fibroblasts, especially in a polyploid subpopulation. This study examines localization of p53 protein at centrosomes in mitotic cells, which is often observed in association with DNA damage response, to investigate a possible association between p53 localization and numerical centrosome aberrations induced by cellular senescence. Cultures at later passages or the 4th day after exposure to H_2O_2 showed increased frequencies of mitotic cells with supernumerary centrosomes, especially in a polyploid subpopulation. Immunohistochemical analysis frequently showed p53-positive foci in mitotic cells, and some were localized at centrosomes. The number of p53-positive foci in mitotic cells and its localization to centrosomes increased with replicative and premature senescence. Supernumerary centrosomes showed higher frequencies of p53 localization compared to normally duplicated centrosomes. Centrosome-associated p53 protein was phosphorylated at Ser15. These data suggest a possible association between localization of p53 protein and numerical centrosome aberrations in replicatively or prematurely senescent cells.

1. Introduction

Centrosome overduplication or centrosome amplification has been observed in many human cancers and in premalignant tissue [1–5]. Centrosome aberrations have been linked to genomic instability during tumour progression, because centrosomes dysfunction may cause abnormal spindle assembly and chromosome missegregation, leading to chromosome aneuploidy [1, 3, 5]. Some studies showed that genetic manipulations that cause centrosome amplification *in vivo* can induce tumor development [6, 7]. The mechanisms leading to centrosome aberrations have been studied extensively but still are not fully understood. However, DNA damage-induced centrosome aberrations such as inactivation, amplification, or fragmentation have been reported. For example, inhibitors of DNA replication such as aphidicolin or hydroxyurea cause centrosome inactivation or centrosome splitting, leading to spindle defects [8–10]. Deficiency of Rad51 recombinase, which is essential for DNA repair, causes supernumerary centrosomes [11]. Radiation causes centrosome amplification in tumour cells and in p53-inactivated or ATR-deficient human cells [12–14]. Several studies have suggested that centrosomal abnormalities resulting from DNA damage response cause mitotic errors and cell death, thus preventing the propagation of damaged cells that might be transformed into malignant cells. However, it is still not clear whether centrosome aberrations are part of the defence mechanism that inhibits carcinogenesis or undesirable pathological phenomena.

Our previous study showed that abnormal mitotic cells with supernumerary (>2/cell) centrosomes increase with replicative senescence in human fibroblasts, especially in a polyploid subpopulation [15]. Moreover, such numerical centrosome aberrations correlated significantly with chromosome misalignment in metaphase cells, suggesting that chromosomal instability with aging might be attributable to centrosome aberrations that are induced with cellular aging.

Recent studies have shown that several proteins involved in the DNA damage response, such as BRCA1, ATM, and p53, localize at centrosomes and regulate cell cycle or centrosome duplication [16–19]. P53 localizes at centrosomes during mitosis, and Ser15 phosphorylation of p53 by ATM, which is a DNA damage response, is required for this localization [20]. Centrosomally localized p53 regulates centrosome duplication in a manner independent of its transactivation function [17]. This study investigated a possible association between p53 localization and numerical centrosome aberrations in senescent cells by examining the localization of p53 protein at centrosomes in mitotic cells from young and near-senescent human fibroblasts. In particular, the effect of polyploidization during cellular senescence on centrosome aberrations and centrosomal localization of p53 was investigated.

2. Materials and Methods

TIG-1 normal human fibroblast cells (21 population doubling levels) were obtained from the Health Science Resources Bank (Tokyo, Japan) and grown in minimum essential medium with α modification (MEM-α; Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with heat-inactivated 10% (v/v) fetal bovine serum. Cells were incubated in a 5% (v/v) CO_2 atmosphere at 37°C and passaged every 3 or 4 days, so that cells never exceeded subconfluent density. For immunofluorescence studies, cells were seeded onto sterile glass slides, placed into culture dishes, and incubated for 2 days.

Replicative senescence was achieved by serial passaging until cells stopped proliferation. Premature senescence was induced by brief exposure of the cells to 200 μM H_2O_2 diluted in Hanks Balanced Salt Solution. The senescent phenotype was evaluated based on the doubling time by counting the cell number at each passage. In some experiments, expression of senescence-associated β-galactosidase (SA-β-gal) was examined using a Senescence Detection Kit (BioVision, CA, USA) to confirm the phenotype. To examine cell cycle profiles, DNA content of the cells was analyzed using CycleTEST Plus (Becton Dickinson Bioscience, USA) and a flow cytometer (FACSCanto II, Becton Dickinson Bioscience, USA).

To detect the localization of centrosomes and p53 protein, cells were fixed with 2% (v/v) formaldehyde, permeabilized with 0.25% (v/v) Triton X-100, and incubated for 1 h at room temperature with specific primary antibodies diluted in PBS containing 2% (w/v) BSA. Primary antibodies were mouse anti-α-tubulin (clone DM1A, 1 : 500 dilution, Sigma, T9025), rabbit anticentrin-2 (N-17, 1 : 800 dilution, Santa Cruz Biotechnology, SC-27793-R), mouse anti-p53 (clone DO-7, 1 : 100 dilution, Santa Cruz Biotechnology, SC-47698), mouse anti-p53 phosphorylated (pSer15), (clone 261352, 1 : 100 dilution, R&D Systems, MAB 1839), and rabbit anti-γ-H2AX (pSer139) (1 : 200 dilution, R&D Systems, AF2288). For fluorescent microscopy, cells treated with primary antibodies were incubated with Alexa Fluor 488-conjugated goat antimouse IgG (Molecular Probes, 1 : 500 dilution) and Alexa Fluor 555-conjugated goat antirabbit IgG (Molecular Probes, 1 : 500 dilution) for 1 h at room temperature. Cells were treated with RNase (1 mg/mL) for 30 min, and cell nuclei were counterstained with 5 μg/mL DAPI. Multiple slides were made from the same culture because the number of mitotic cells decreases as cells approach senescence. The specificity of the primary antibodies was confirmed by comparing it to the reaction with isotype control antibodies. In some experiments, another monoclonal antibody for p53, mouse anti-p53, clone DO-1 (Santa Cruz Biotechnology, SC-126), was also used to confirm the specificity of the reaction to p53 protein. Mouse monoclonal anti-p53 antibodies clone DO-7 and DO-1 are widely used and specificity of the antibodies has been established [21].

Cell cycle profiles were obtained by the use of a laser scanning cytometer (LSC-2, Olympus, Japan). DNA content was measured by DAPI fluorescence using violet (405 nm) laser excitation and a blue channel filter (460–485 nm), then subpopulations of interest were recalled, and localization of chromosomes, centrosomes, and p53 protein in mitotic cells was observed by DAPI, Alexa Fluor 555, and Alexa Fluor 488 fluorescence using appropriate filter sets, respectively. Centrosome number per mitotic cell and frequencies of centrosomal localization of p53 in mitotic cells were analyzed by preparing multiple slides from the same culture to obtain as many mitotic cells as possible.

3. Results

TIG-1 human fibroblasts showed growth retardation after 70 population doubling levels (PDLs) and entered a state of replicative senescence after 80 PDLs. Growth-arrested cells showed typical senescent phenotypes, such as enlarged size and β-galactosidase expression (Figure 1(a)). Cells exposed to 200 μM H_2O_2 showed the same phenotypes at the 4th day after exposure, indicating that premature senescence was induced by oxidative damage. Cell cycle analysis showed increased frequencies of polyploid cells (with 8C DNA) concomitant with replicative senescence and premature senescence (Figures 1(a), 1(b), and 1(c)).

Most diploid mitotic cells at early passages had two centrosomes, each of which consists of two centrioles recognized by anticentrin-2 antibody, with bipolar spindles. More than 60% of the polyploid mitotic cells (8C DNA or more) at early passages also had two centrosomes, although all other polyploid cells had more than two centrosomes (Figures 2(a) and 2(b)). In contrast, cells at later passages or the 4th day after exposure to H_2O_2 showed increased frequencies of mitotic cells with supernumerary (>2/cell) centrosomes, especially in a polyploid subpopulation (Figures 2(a) and 2(b)). These cells often showed incomplete spindles and misaligned chromosomes (Figure 2(a)). Most of the extra centrosomes with incomplete spindles also had two centrioles similar to centrosomes with bipolar spindles.

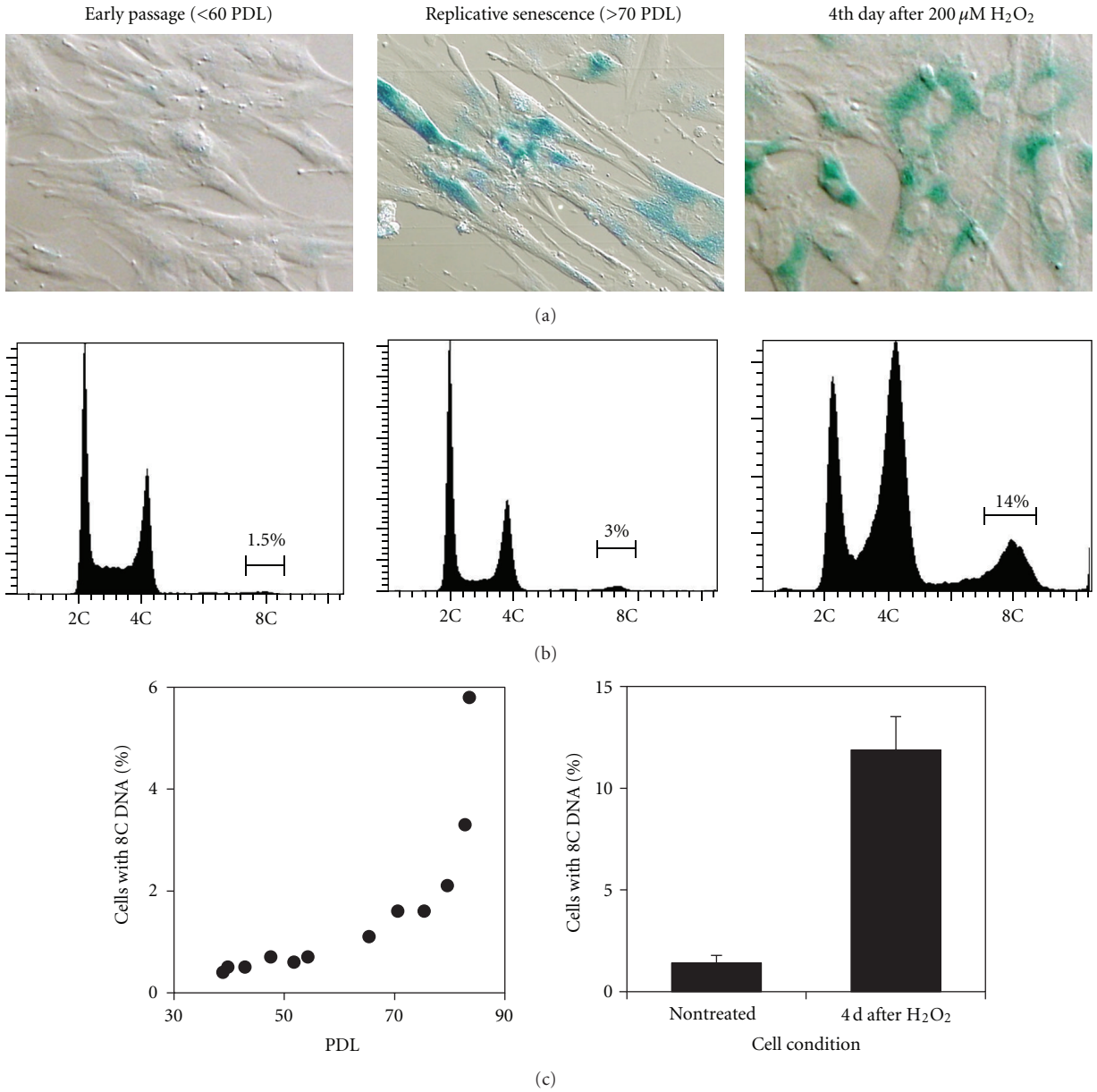


Figure 1: Polyploidization associated with cellular senescence in TIG-1 cells. (a) Differential interference contrast images overlaid with colouring reaction by senescence-associated β-galactosidase. (b) DNA histograms obtained by flow cytometer. (c) Change in frequencies of polyploid cells (with 8C DNA) by population doubling levels in serially passaged cells (left panel) and in cells exposed to 200 μM H_2O_2 (right panel).

Immunohistochemical analysis showed that p53-positive foci were frequently present in mitotic and in interphase cells. Some foci were localized at centrosomes, and others were on the chromosomes (Figure 3(a)). P53 at centrosomes was always recognized as a single focus and appears to localize in the PCM (pericentriolar material) or on one of two centrioles in a centrosome. The mean number of p53-positive foci per cell was higher in the polyploid subpopulation compared to the diploid subpopulation (Figure 3(b)). Mitotic cells at the 4th day after exposure to H_2O_2 showed an increased number of p53-positive foci per cell in both subpopulations compared to untreated early passage cells (Figure 3(b)). The frequency of centrosomes with associated p53 protein was about 10% in untreated early passage cells regardless of DNA ploidy, while mitotic cells at later passages showed increased frequencies of p53-associated centrosomes, especially in diploid subpopulations (Figure 3(c)). Interestingly, supernumerary centrosomes showed higher frequencies of p53 localization compared to normally duplicated (2 centrosomes/cell) centrosomes, especially in diploid subpopulations irrespective of cell condition (Figure 3(d)). Centrosome-associated p53 foci were consistently recognized by antibody against p53 phosphorylated at Ser15 (Figure 4), while p53 foci on chromosomes were not

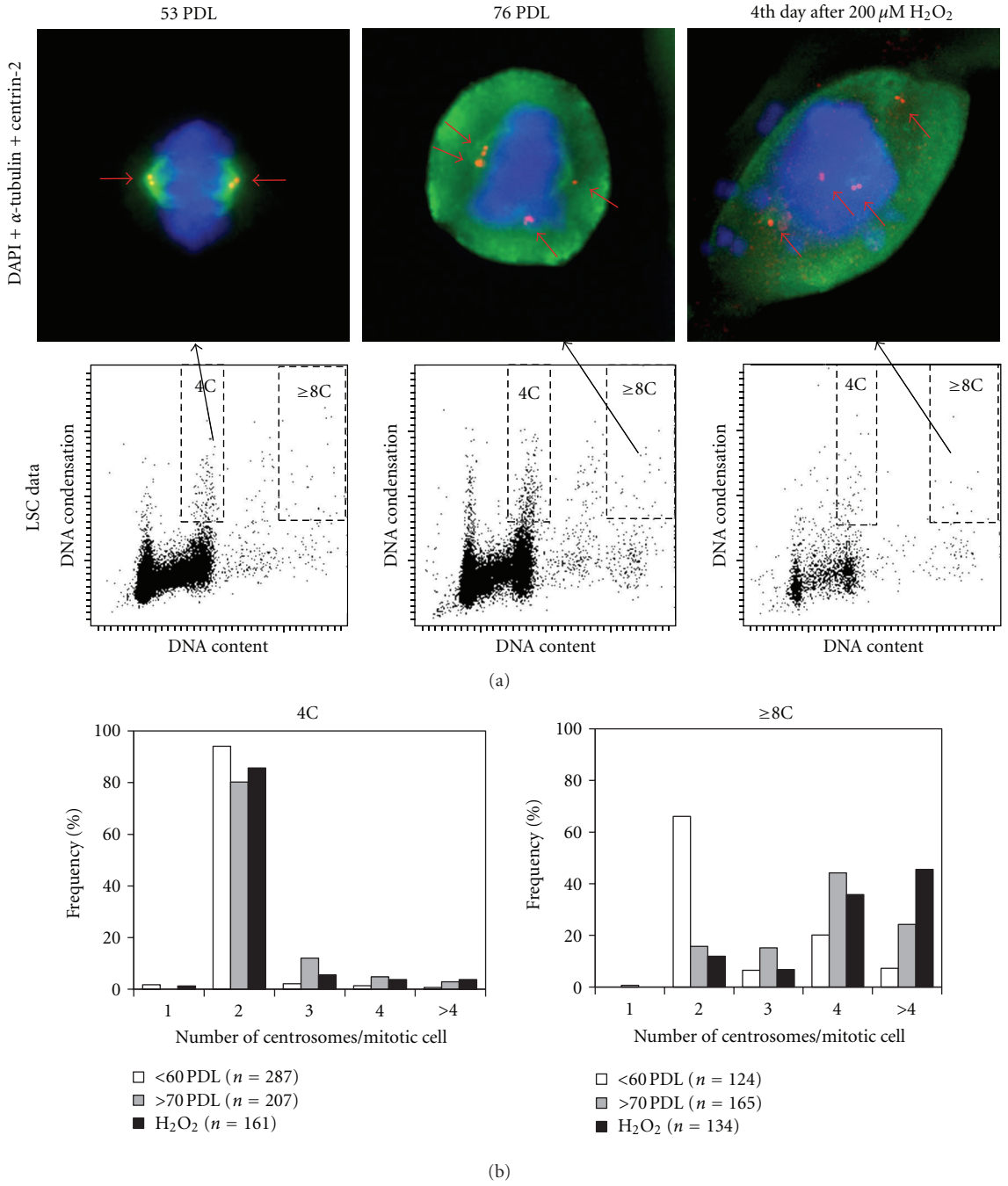


Figure 2: Numerical centrosome aberrations associated with cellular senescence. (a) Upper left panel shows a representative mitotic cell with normal morphology in a diploid subpopulation from early passage, and upper middle and upper right panels show abnormal mitotic cells with supernumerary centrosomes in a polyploid subpopulation from late passage and the 4th day after exposure to H_2O_2. Small red arrows indicate localization of centrosomes. Chromosomes, centrosomes, and mitotic spindles are labelled with DAPI (blue), anticentrin2 antibody (red), and anti-α-tubulin antibody (green), respectively. Lower panels show corresponding LSC data. (b) Histograms showing frequencies of mitotic cells with different centrosome numbers in a diploid subpopulation (4C) and a polyploid subpopulation (≥8C).

phosphorylated at Ser15. These p53 foci on chromosomes were observed more often in the polyploid subpopulation compared to the diploid subpopulation, and they were often colocalized with phosphorylated histone H2AX (γ-H2AX) foci (data not shown).

4. Discussion

This study showed that mitotic cells with supernumerary (>2/cell) centrosomes increase with cellular aging induced by exposure to H_2O_2 as well as by serial passaging, especially

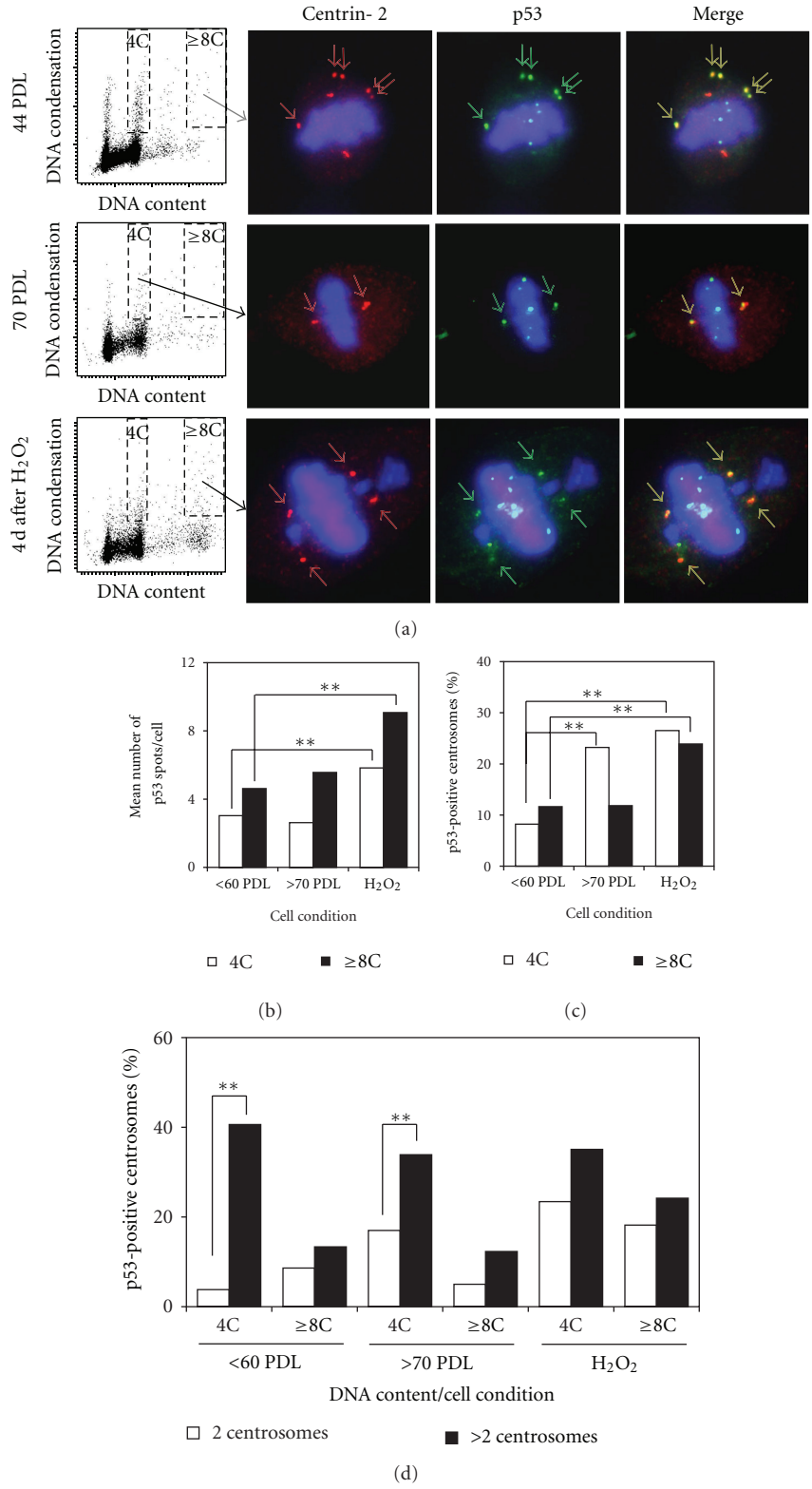


FIGURE 3: Localization of p53 protein in mitotic cells. (a) Representative mitotic cells showing localization of centrosomes and p53-positive foci from early passages (upper panels), late passages (middle panels), and the 4th day after exposure to H_2O_2 (lower panels). Arrows indicate centrosomes with p53 proteins. Chromosomes (blue) and centrosomes (red) are labelled as in Figure 2, and p53 protein is labelled with anti-p53 antibody (green). (b) Mean numbers of p53-positive foci in mitotic cells. Each column is the mean of at least 50 mitotic cells from 4 to 6 experiments. $^{**}P < 0.01$ with unpaired t-test. (c) Mean frequencies of p53-positive centrosomes. Each column is the frequency calculated using pooled data from 4 to 6 experiments. $^{**}P < 0.01$ with Chi-square test. (d) Mean frequencies of p53-positive centrosomes according to centrosome numbers per cell. Each column is the frequency calculated using pooled data from 4 to 6 experiments. $^{**}P < 0.01$ with Chi-square test.

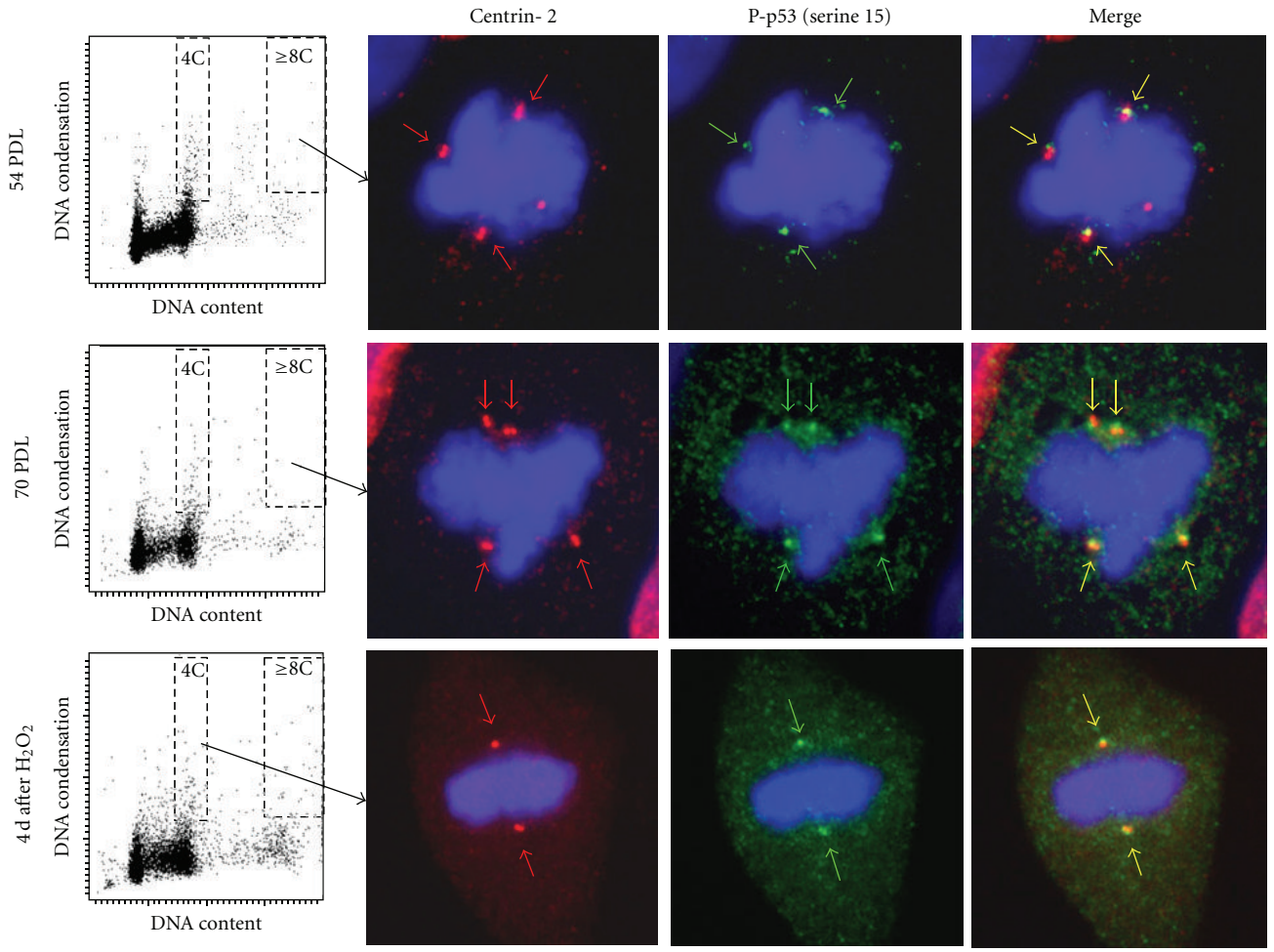


Figure 4: Localization of p53 phosphorylated at Ser15. Representative mitotic cells showing centrosomal localization of phosphorylated p53 (pSer 15) from early passages (upper panels), late passages (middle panels), and the 4th day after exposure to H_2O_2 (lower panels). Chromosomes (blue) and centrosomes (red) are labelled as in Figure 2, and phosphorylated p53 (pSer 15) is labelled with antiphosphorylated p53 (pSer 15) antibody (green).

in a polyploid subpopulation. Because cellular senescence is defined as permanent growth arrest by telomere attrition (replicative senescence) or various stresses (premature senescence) [22–24], the changes associated with cellular senescence in mitotic cells may have occurred in a near-senescent state. Recent studies have shown that cellular senescence is triggered by the DNA damage response induced by telomere attrition or oxidative DNA damage [23–26]. Therefore, cells in a near-senescent state may have extensive DNA damage that causes centrosome aberrations. Supernumerary centrosomes (>2 centrosomes/cell) could be formed due to several different mechanisms including overduplication in diploid cells, reduplication in the course of polyploidization, centrosome fragmentation, or centriole separation. However, supernumerary centrosomes observed in this study are not considered to be formed mainly by centrosome fragmentation or centriole separation, because most of the extra centrosomes consisted of two centrioles as recognized by anticentrin-2 antibody. The point of note is the relationship between numerical centrosome aberrations and DNA ploidy. Because centrosomes are duplicated in synchrony with DNA duplication, endoreduplication of DNA in polyploid cells may result in multiple centrosomes [5, 27]. However, more than 60% of polyploid mitotic cells at early passages had only two centrosomes, while the majority of polyploid mitotic cells formed in association with cellular senescence had more than two centrosomes (Figure 2(b)). In this point, we have recently shown that proliferative tetraploid cells established from normal human fibroblasts had only two centrosomes with bipolar spindles and grew at the same rate as diploid parent cells [28]. Therefore, mechanisms leading to the formation of bipolar spindles with two centrosomes in polyploid cells should exist. Mechanisms other than polyploidization may also be responsible for the numerical centrosome aberrations induced by cellular aging. Centrosome overduplication during cell cycle arrest by DNA damage response should also be considered as a possible mechanism for numerical aberrations of the centrosomes, as suggested by other investigators [11, 29, 30].

The present study shows that p53 protein localizes at centrosomes as shown by others [16, 17, 20, 31, 32], although its role is still not clear. The most significant finding of the present study is that the centrosomal localization of p53 correlated with numerical centrosome aberrations. Supernumerary centrosomes showed consistently higher frequencies of p53 localization compared to normally duplicated centrosomes, although statistical significance was observed only in diploid subpopulations. This correlation suggests that the centrosomal localization of p53 in human fibroblasts may be significantly associated with numerical centrosome aberrations. Moreover, Ser15 phosphorylation of p53 localized at centrosomes suggests that this may be associated with the DNA damage response, because p53 is known to be phosphorylated at Ser15 by ATM in DNA damage response after radiation [33, 34]. Although the significance of p53 localization at supernumerary centrosomes is not clear, it is possible that this localization may be promoted by DNA damage response. However, results of this study are somewhat inconsistent with other studies. The frequency of centrosomes with p53 protein in untreated cells was relatively lower compared to those reported in other studies. But the cells used in other studies were mouse cells in which human p53 genes were introduced [17, 32] or a lymphoblastoid cell line [16, 20, 35]. Therefore, the difference in the frequency of p53 positive centrosomes in untreated cells between this study and other studies might be cell- or species-specific. But the validity of this assumption is not certain, because the frequency of p53 localization to centrosomes in normal human fibroblasts has not been reported so far. It is also possible that sensitivity of the immunofluorescence procedure employed in this study was not sufficient for the complete detection of p53 protein on centrosomes. The majority of signals showing p53 protein on centrosomes were relatively weak.

Another point that needs to be considered is the role of p53 phosphorylation in p53 localization at centrosomes during mitosis. The studies using a lymphoblastoid cell line have shown that ATM-dependent phosphorylation of p53 at serine 15 is required for centrosomal localization of p53, and this phosphorylated p53 is rapidly dephosphorylated upon association with centrosomes [16, 20]. They showed that treatment of cells with serine-phosphatase inhibitor NaF before immunostaining is necessary to detect Ser 15 phosphorylated endogenous p53. In our experiments, however, centrosome-associated p53 foci seemed to be consistently phosphorylated at Ser15 in the absence of phosphatase inhibitors irrespective of DNA ploidy or whether centrosomes were normally duplicated or overduplicated. Although the reason for this discrepancy is not clear, it is possible that ATM-dependent Ser 15 phosphorylation of p53 in DNA damage response promoted localization of p53 at supernumerary centrosomes. Furthermore, we speculate that p53 protein localized at supernumerary centrosomes inactivates their function as the microtubule organizing centres, although this speculation lacks supporting data. Some studies published recently have shown that p53 is necessary to ensure bipolar mitosis and maintain chromosomal stability of tetraploid cells, which would otherwise cause multipolar mitosis due to supernumerary centrosomes [36, 37]. These observations regarding p53 function in maintaining chromosomal stability favor the above hypothesis.

The centrosome function in regulation of cell cycle and mitosis is increasingly being recognized, and many proteins implicated in such regulation have been shown to localize at centrosomes [19, 38–43]. They include cell cycle regulators such as cyclin-dependent kinases or cyclins, mitotic regulators such as Aurora kinases or TACC (transforming acidic coiled-coil), and mitotic checkpoint kinases such as BubR1 or Mad2. Expression and localization of those regulators may be affected directly or indirectly by p53 activation in DNA damage response, leading to control of cell cycle and mitosis. On the other hand, deregulation of those factors may activate p53 and p21 as well, causing premature cellular senescence to minimize chromosomal instability [44, 45]. Functions of centrosomes in regulating cell cycle and mitosis are not fully understood, however, and the significance of p53 localization at centrosomes should be defined more clearly in the near future.

Genetic instability of polyploid cells, which could lead to the formation of aneuploid cells, is well known [27, 46, 47], and an increase with age of polyploid cells *in vivo* and *in vitro* has been reported [48–51]. Therefore, age-associated chromosomal instability and tumorigenesis might be attributable to an increased frequency of polyploid cells with age. Results of the present study reveal that numerical centrosome aberration in polyploid cells is significantly associated with cellular aging induced by oxidative stress as well as repeated DNA replication. This association may lead to age-associated chromosomal instability and tumorigenesis. In addition, p53 protein activated during the DNA damage response is suggested to be associated with supernumerary centrosomes during mitosis, which may contribute to maintain chromosomal stability.

References

[1] A. Krämer, S. Schweizer, K. Neben et al., "Centrosome aberrations as a possible mechanism for chromosomal instability in non-Hodgkin's lymphoma," *Leukemia*, vol. 17, no. 11, pp. 2207–2213, 2003.

[2] G. A. Pihan, J. Wallace, Y. Zhou, and S. J. Doxsey, "Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas," *Cancer Research*, vol. 63, no. 6, pp. 1398–1404, 2003.

[3] K. Fukasawa, "Centrosome amplification, chromosome instability and cancer development," *Cancer Letters*, vol. 230, no. 1, pp. 6–19, 2005.

[4] M. Giehl, A. Fabarius, O. Frank et al., "Centrosome aberrations in chronic myeloid leukemia correlate with stage of disease and chromosomal instability," *Leukemia*, vol. 19, no. 7, pp. 1192–1197, 2005.

[5] E. A. Nigg, "Origins and consequences of centrosome aberrations in human cancers," *International Journal of Cancer*, vol. 119, no. 12, pp. 2717–2723, 2006.

[6] M. A. Ko, C. O. Rosario, J. W. Hudson et al., "Plk4 haploinsufficiency causes mitotic infidelity and carcinogenesis," *Nature Genetics*, vol. 37, no. 8, pp. 883–888, 2005.

[7] R. Basto, K. Brunk, T. Vinadogrova et al., "Centrosome amplification can initiate tumorigenesis in flies," *Cell*, vol. 133, no. 6, pp. 1032–1042, 2008.

[8] O. C. M. Sibon, A. Kelkar, W. Lemstra, and W. E. Theurkauf, "DNA-replication/DNA-damage-dependent centrosome inactivation in *Drosophila* embryos," *Nature Cell Biology*, vol. 2, no. 2, pp. 90–95, 2000.

[9] H. M. J. Hut, W. Lemstra, E. H. Blaauw, G. W. A. Van Cappellen, H. H. Kampinga, and O. C. M. Sibon, "Centrosomes split in the presence of impaired DNA integrity during mitosis," *Molecular Biology of the Cell*, vol. 14, no. 5, pp. 1993–2004, 2003.

[10] O. C. M. Sibon, "Centrosomes as DNA damage regulators," *Nature Genetics*, vol. 34, no. 1, pp. 6–7, 2003.

[11] H. Dodson, E. Bourke, L. J. Jeffers et al., "Centrosome amplification induced by DNA damage occurs during a prolonged G2 phase and involves ATM," *EMBO Journal*, vol. 23, no. 19, pp. 3864–3873, 2004.

[12] N. Sato, K. Mizumoto, M. Nakamura et al., "A possible role for centrosome overduplication in radiation-induced cell death," *Oncogene*, vol. 19, no. 46, pp. 5281–5290, 2000.

[13] K. Kawamura, N. Morita, C. Domiki et al., "Induction of centrosome amplification in p53 siRNA-treated human fibroblast cells by radiation exposure," *Cancer Science*, vol. 97, no. 4, pp. 252–258, 2006.

[14] H. Löffler, T. Bochtler, B. Fritz et al., "DNA damage-induced accumulation of centrosomal Chk1 contributes to its checkpoint function," *Cell Cycle*, vol. 6, no. 20, pp. 2541–2548, 2007.

[15] S. Ohshima and A. Seyama, "Cellular aging and centrosome aberrations," *Annals of the New York Academy of Sciences*, vol. 1197, pp. 108–117, 2010.

[16] E. Oricchio, C. Saladino, S. Iacovelli, S. Soddu, and E. Cundari, "ATM is activated by default in mitosis, localizes at centrosomes and monitors mitotic spindle integrity," *Cell Cycle*, vol. 5, no. 1, pp. 88–92, 2006.

[17] K. Shinmura, R. A. Bennett, P. Tarapore, and K. Fukasawa, "Direct evidence for the role of centrosomally localized p53 in the regulation of centrosome duplication," *Oncogene*, vol. 26, no. 20, pp. 2939–2944, 2007.

[18] S. Zhang, P. Hemmerich, and F. Grosse, "Centrosomal localization of DNA damage checkpoint proteins," *Journal of Cellular Biochemistry*, vol. 101, no. 2, pp. 451–465, 2007.

[19] M. Shimada and K. Komatsu, "Emerging connection between centrosome and DNA repair machinery," *Journal of Radiation Research*, vol. 50, no. 4, pp. 295–301, 2009.

[20] A. Tritarelli, E. Oricchio, M. Ciciarello et al., "p53 localization at centrosomes during mitosis and postmitotic checkpoint are ATM-dependent and require Serine 15 phosphorylation," *Molecular Biology of the Cell*, vol. 15, no. 8, pp. 3751–3757, 2004.

[21] B. A. Bonsing, W. E. Corver, M. C. B. Gorsira et al., "Specificity of seven monoclonal antibodies against p53 evaluated with western blotting, immunohistochemistry, confocal laser scanning microscopy, and flow cytometry," *Cytometry*, vol. 28, no. 1, pp. 11–24, 1997.

[22] I. Ben-Porath and R. A. Weinberg, "When cells get stressed: an integrative view of cellular senescence," *Journal of Clinical Investigation*, vol. 113, no. 1, pp. 8–13, 2004.

[23] D. B. Lombard, K. F. Chua, R. Mostoslavsky, S. Franco, M. Gostissa, and F. W. Alt, "DNA repair, genome stability, and aging," *Cell*, vol. 120, no. 4, pp. 497–512, 2005.

[24] J.-H. Chen, C. N. Hales, and S. E. Ozanne, "DNA damage, cellular senescence and organismal ageing: Causal or correlative?" *Nucleic Acids Research*, vol. 35, no. 22, pp. 7417–7428, 2007.

[25] H. Takai, A. Smogorzewska, and T. De Lange, "DNA damage foci at dysfunctional telomeres," *Current Biology*, vol. 13, no. 17, pp. 1549–1556, 2003.

[26] R. Di Micco, M. Fumagalli, A. Cicalese et al., "Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication," *Nature*, vol. 444, no. 7119, pp. 638–642, 2006.

[27] Z. Storchova and D. Pellman, "From polyploidy to aneuploidy, genome instability and cancer," *Nature Reviews Molecular Cell Biology*, vol. 5, no. 1, pp. 45–54, 2004.

[28] S. Ohshima and A. Seyama, "Formation of bipolar spindles with two centrosomes in tetraploid cells established from normal human fibroblasts," *Human Cell*, vol. 25, no. 3, pp. 78–85, 2012.

[29] R. Kuriyama, Y. Terada, K. S. Lee, and C. L. C. Wang, "Centrosome replication in hydroxyurea-arrested CHO cells expressing GFP-tagged centrin 2," *Journal of Cell Science*, vol. 120, no. 14, pp. 2444–2453, 2007.

[30] S. L. Prosser, K. R. Straatman, and A. M. Fry, "Molecular dissection of the centrosome overduplication pathway in S-phase-arrested cells," *Molecular and Cellular Biology*, vol. 29, no. 7, pp. 1760–1773, 2009.

[31] V. B. Morris, J. Brammall, J. Noble, and R. Reddel, "p53 Localizes to the centrosomes and spindles of mitotic cells in the embryonic chick epiblast, human cell lines, and a human primary culture: an immunofluorescence study," *Experimental Cell Research*, vol. 256, no. 1, pp. 122–130, 2000.

[32] P. Tarapore, Y. Tokuyama, H. F. Horn, and K. Fukasawa, "Difference in the centrosome duplication regulatory activity among p53 'hot spot' mutants: potential role of Ser 315 phosphorylation-dependent centrosome binding of p53," *Oncogene*, vol. 20, no. 47, pp. 6851–6863, 2001.

[33] T. Caspari, "Checkpoints: how to activate p53," *Current Biology*, vol. 10, no. 8, pp. R315–R317, 2000.

[34] J.-P. Kruse and W. Gu, "Modes of p53 Regulation," *Cell*, vol. 137, no. 4, pp. 609–622, 2009.

[35] M. Ciciarello, R. Mangiacasale, M. Casenghi et al., "p53 displacement from centrosomes and p53-mediated G1 arrest following transient inhibition of the mitotic spindle," *Journal of Biological Chemistry*, vol. 276, no. 22, pp. 19205–19213, 2001.

[36] C. C. Ho, P. M. Hau, M. Marxer, and R. Y. Poon, "The requirement of p53 for maintaining chromosomal stability during tetraploidization," *Oncotarget*, vol. 1, no. 7, pp. 583–595, 2010.

[37] Q. Yi, X. Zhao, Y. Huang et al., "P53 dependent centrosome clustering prevents multipolar mitosis in tetraploid cells," *PLoS One*, vol. 6, no. 11, Article ID Article numbere27304, 2011.

[38] T. Oikawa, M. Okuda, Z. Ma et al., "Transcriptional control of BubR1 by p53 and suppression of centrosome amplification by BubR1," *Molecular and Cellular Biology*, vol. 25, no. 10, pp. 4046–4061, 2005.

[39] H. Löffler, J. Lukas, J. Bartek, and A. Krämer, "Structure meets function—centrosomes, genome maintenance and the DNA damage response," *Experimental Cell Research*, vol. 312, no. 14, pp. 2633–2640, 2006.

[40] A. R. Barr and F. Gergely, "Aurora-A: the maker and breaker of spindle poles," *Journal of Cell Science*, vol. 120, no. 17, pp. 2987–2996, 2007.

[41] H. Schatten, "The mammalian centrosome and its functional significance," *Histochemistry and Cell Biology*, vol. 129, no. 6, pp. 667–686, 2008.

[42] Y. Wang, P. Ji, J. Liu, R. R. Broaddus, F. Xue, and W. Zhang, "Centrosome-associated regulators of the G2/M checkpoint as targets for cancer therapy," *Molecular Cancer*, vol. 8, article no. 8, 2009.

[43] D. Zyss and F. Gergely, "Centrosome function in cancer: guilty or innocent?" *Trends in Cell Biology*, vol. 19, no. 7, pp. 334–346, 2009.

[44] L. Lentini, V. Barra, T. Schillaci, and A. Di Leonardo, "MAD2 depletion triggers premature cellular senescence in human primary fibroblasts by activating a P53 pathway preventing aneuploid cells propagation," *Journal of Cellular Physiology*, vol. 227, no. 9, pp. 3324–3332, 2012.

[45] S. Schmidt, L. Schneider, F. Essmann et al., "The centrosomal protein TACC3 controls paclitaxel sensitivity by modulating a premature senescence program," *Oncogene*, vol. 29, no. 46, pp. 6184–6192, 2010.

[46] P. Duesberg, A. Fabarius, and R. Hehlmann, "Aneuploidy, the primary cause of the multilateral genomic instability of neoplastic and preneoplastic cells," *IUBMB Life*, vol. 56, no. 2, pp. 65–81, 2004.

[47] N. J. Ganem, Z. Storchova, and D. Pellman, "Tetraploidy, aneuploidy and cancer," *Current Opinion in Genetics and Development*, vol. 17, no. 2, pp. 157–162, 2007.

[48] H. Fujisawa, T. Nishikawa, B. H. Zhu et al., "Accelerated aging of dermal fibroblast-like cells from the senescence-accelerated mouse (SAM): acceleration of changes in DNA ploidy associated with in vitro cellular aging," *Journals of Gerontology. Series A*, vol. 53, no. 1, pp. B11–B17, 1998.

[49] M. Wagner, B. Hampel, D. Bernhard, M. Hala, W. Zwerschke, and P. Jansen-Dürr, "Replicative senescence of human endothelial cells in vitro involves G1 arrest, polyploidization and senescence-associated apoptosis," *Experimental Gerontology*, vol. 36, no. 8, pp. 1327–1347, 2001.

[50] M. R. Jones and K. Ravid, "Vascular smooth muscle polyploidization as a biomarker for aging and its impact on differential gene expression," *Journal of Biological Chemistry*, vol. 279, no. 7, pp. 5306–5313, 2004.

[51] K. H. Walen, "Bipolar genome reductional division of human near-senescent, polyploid fibroblast cells," *Cancer Genetics and Cytogenetics*, vol. 173, no. 1, pp. 43–50, 2007.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 282438, 12 pages
doi:10.1155/2012/282438

Review Article

Nuclear and Mitochondrial DNA Repair in Selected Eukaryotic Aging Model Systems

Ricardo Gredilla,[1] Christian Garm,[2] and Tinna Stevnsner[2]

[1] *Department of Physiology, Faculty of Medicine, The Complutense University, Plaza Ramón y Cajal s/n., 28040 Madrid, Spain*
[2] *Danish Center for Aging Research, Danish Center for Molecular Gerontology, and Department of Molecular Biology and Genetics, Aarhus University, C.F. Møellers Allé 3, Building 1130, 8000 Aarhus C, Denmark*

Correspondence should be addressed to Tinna Stevnsner, tvs@mb.au.dk

Received 1 July 2012; Accepted 26 August 2012

Academic Editor: Heinz D. Osiewacz

Knowledge about the different mechanisms underlying the aging process has increased exponentially in the last decades. The fact that the basic mechanisms involved in the aging process are believed to be universal allows the use of different model systems, from the simplest eukaryotic cells such as fungi to the most complex organisms such as mice or human. As our knowledge on the aging mechanisms in those model systems increases, our understanding of human aging and the potential interventions that we could approach rise significantly. Among the different mechanisms that have been implicated in the aging process, DNA repair is one of the processes which have been suggested to play an important role. Here, we review the latest investigations supporting the role of these mechanisms in the aging process, stressing how beneficial the use of different model systems is. We discuss how human genetic studies as well as several investigations on mammalian models and simpler eukaryotic organisms have contributed to a better understanding of the involvement of DNA repair mechanisms in aging.

1. Introduction

Our cells are constantly exposed to endogenous and exogenous agents that induce damage to the cellular macromolecules such as DNA, RNA, proteins, and lipids. The sources of this damage include a broad range of agents such as industrial chemicals and combustion products present in our environment, UV radiation from the sun, and endogenous metabolic byproducts. In contrast to damaged lipids and proteins, damaged DNA, which carries the inherited genetic information of the cell, cannot be replaced. Therefore, formation of DNA lesions can have profound consequences for genomic stability due to the effect that these lesions can have on polymerase fidelity and processivity. Spontaneous reactions, such as hydrolysis, are the major sources of DNA damage. They can lead to deaminated bases and abasic sites in the DNA. Another very prominent type of endogenous DNA damage, oxidative DNA damage, is caused by reactive oxygen species (ROS), which are formed continuously as a consequence of normal aerobic metabolism during mitochondrial respiration but also by inflammatory responses. ROS can also be formed by a number of external factors including UV- and ionizing radiation and chemical mutagens. DNA repair mechanisms have evolved to remove the majority of all DNA lesions, but if these mechanisms are not sufficiently efficient, it will lead to DNA damage accumulation, which is likely to result in mutations and cellular dysfunction. Due to close proximity of the mitochondrial DNA to the inner mitochondrial membrane, the mitochondrial genome is more heavily exposed to ROS than the nuclear DNA and therefore also more likely to experience DNA damage. Thus, the mitochondrial free radical theory of aging [1] postulates that organisms age due to the accumulation of DNA damage and mutations in the mitochondrial DNA, leading to mitochondrial and eventually cellular dysfunction. This paper explores some of the recent research, which has been performed in order to uncover the relationship between DNA damage, DNA repair

mechanisms, and the aging process, and emphasis is given to the use of eukaryotic model systems for this area of research.

2. DNA Damage and Aging

Among the wide variety of known DNA lesions, 8-oxo-deoxyguanosine (8oxoG) has received a lot of attention due to its mutagenicity and because of the possible correlation between its accumulation and pathological processes like cancer, degenerative diseases, and aging. However, an increasing number of studies also include other types of DNA lesions. Numerous studies report measurement of DNA damage in nuclear and mitochondrial DNA from tissues of young and old organisms, with variable outcomes. Although still controversial, several careful studies do show that 8oxoG accumulates with age. Thus, in a recent study Gan and coworkers used a sensitive LC-MS/MS method to demonstrate that 8oxoG increases with age in DNA in a number of different mouse tissues, with the largest age-dependent increase in brain [2]. Likewise, it was shown in another recent study that the oxidative DNA lesion 8,5′-cyclopurine-2′-deoxynucleoside accumulates with age in a tissue specific manner in mouse [3]. Using a high-performance liquid chromatography—electrochemical—detector Lee and coworkers showed a positive correlation between the level of 8oxoG in DNA and age in human gastric tissue [4]. Importantly, Hudson and coworkers have shown that 8oxoG increases three-fold with age in mitochondrial DNA of rat heart [5] and a number of other studies have reported similar results for mitochondria of other tissues including postmitotic tissues (reviewed in [6]).

Beside being exposed to endogenous ROS, the DNA of for example skin cells may also be heavily exposed to environmental factors such as UV irradiation due to sun light exposure. Photodamage leads to thymine dimers, 6–4 photoproducts, and ROS that damage genomic DNA and give rise to mutations in coding or regulatory DNA sequences of critical genes. The protective repeated DNA sequences at the end of our chromosomes, the telomeres, are disproportionately damaged by both UV and ROS, due to their greater proportion of target TT and G bases compared with the chromosomes overall. Such damage is postulated to disrupt the telomeric loop, expose the TTAGGG overhang, and promote aging (reviewed in [7]). UV radiation produced ROS may also activate cell surface receptors, which eventually leads to reduced dermal matrix formation. In dermal fibroblasts, UV irradiation also induces mitochondrial DNA deletions, leading to compromised synthesis of mitochondrial proteins, further increase of reactive oxygen species (ROS), and decreased ability of the cell to generate energy.

Epidemiological studies suggest that smoking is another important environmental factor in skin aging. Exposure to tobacco smoke causes DNA single-strand breaks, aromatic adducts, and oxidative damage to DNA, chromosome aberrations, and micronuclei (reviewed in [8]). ROS in tobacco smoke also increases the expression of matrix metalloproteinases, and the elevated enzyme levels are suggested to lead to the degradation of collagen and elastic fibers in the skin [9]. Finally, DNA double strand breaks pose severe problems for cells and this type of DNA damage also seems to increase with age in various tissues [10, 11].

3. Nuclear and Mitochondrial DNA Repair Pathways

Due to the serious consequences that DNA damage accumulation may have on cellular function and survival, different pathways of DNA repair have evolved in order to prevent it. DNA repair pathways have mainly been investigated in the nucleus; however, some of the known pathways have also been described in mitochondria (Figure 1).

3.1. Nucleotide Excision Repair (NER). In nuclear DNA, the NER mechanism can remove numerous types of helix distorting and bulky lesions. Additionally, NER is central for the repair of DNA cross-links. Briefly, the NER pathway includes damage recognition, opening of the DNA helix, incision of the nucleotides surrounding the lesion, gap filling replication, and ligation. NER has two subpathways with different ways of detecting lesions: the global genome (GG) NER and transcription coupled (TC) NER.

Damage recognition of the NER is accomplished through sequential actions of multiple proteins. For GG-NER destabilization of the base pairing is detected by xeroderma pigmentosum complementation group C (XPC) protein in complex with the human homologue Rad23B (hHR23B) protein, which is suggested by many studies to be the first protein factor to arrive at the lesion, and it ensures a broad spectrum of substrate specificity. Additionally, UV-damaged DNA-binding protein (UV-DDB) recognizes particular types of lesions, such as UV-induced photoproducts, thereby recruiting XPC and extending the substrate specificity [12]. For TC-NER, damage recognition is believed to be caused by the blockage of the transcribing RNA polymerase II on the damaged DNA template. TC-NER is then initiated by the Cockayne Syndrome complementation group B (CSB) protein, later followed by the CSA gene product. In both GG-NER and TC-NER, the lesion recognition step is followed by recruitment of TFIIH. The XPB and XPD helicases from the 10-subunit TFIIH complex unwind the DNA around the lesion. The initial open complex is stabilized by XPG and XPA, verifying the lesion, and by RPA, which binds the opposite intact single-stranded DNA. The structure-specific endonucleases XPG and ERCC1/XPF cleave 3′ and 5′of the lesion, respectively. The resulting 24–32 nucleotide fragment, containing the lesion, is removed and the remaining single-strand gap filled by the replication machinery and the resulting nick is sealed by ligase I or ligase III [13, 14]. NER does not seem to take place in mitochondria (reviewed in [15]).

3.2. Base Excision Repair (BER). The BER pathway is responsible for the repair of a broad spectrum of DNA base adducts. BER takes place both in nuclei and mitochondria and is therefore the main guardian against endogenously derived DNA lesions in the nucleus as well as the mitochondria [16, 17]. Thus, the BER pathway is very critical for the

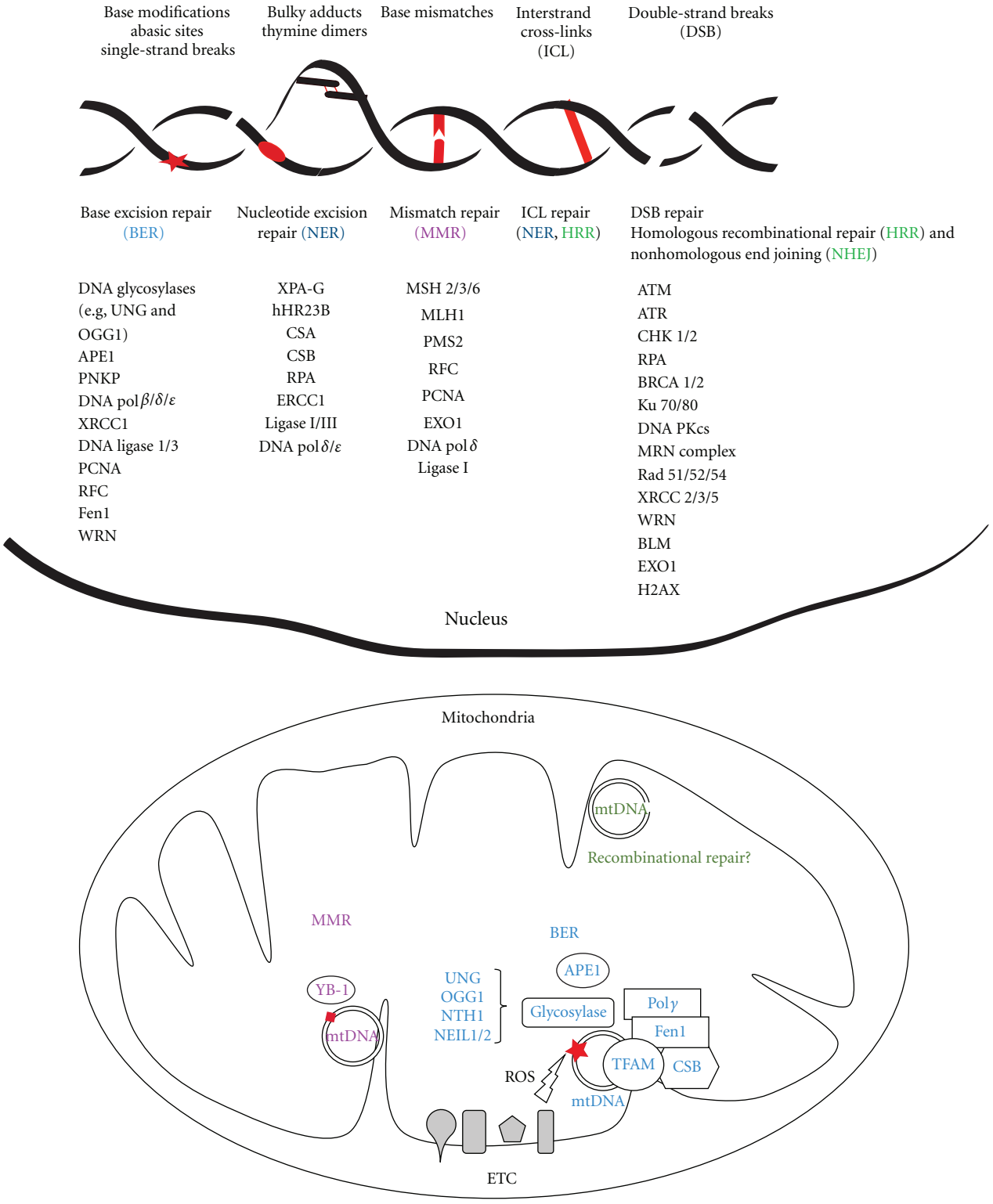


Figure 1: Nuclear and mitochondrial DNA repair mechanisms. DNA repair lesions are represented in red and DNA repair mechanisms are coded with specific colors. Only the central proteins involved in each pathway are represented. Enzymes involved in mitochondrial DNA repair are in some cases splice variants of the corresponding nuclear DNA repair enzymes. More detailed information about the different pathways is included in the text. BER: base excision repair; NER: nucleotide excision repair; MMR: mismatch repair; HRR: homologous recombinational repair; NHEJ: nonhomologous end joining; ETC: electron transport chain; ROS: reactive oxygen species.

maintenance of genomic stability [18]. As described in the introduction, one of the most prominent theories of aging, the mitochondrial free radical theory of aging, states that free radicals generated in mitochondria are involved in the intrinsic aging process, mainly due to the accumulation of oxidative damage and derived mutations in mtDNA [19, 20]. The relevance of BER mechanisms have been highlighted by studies in yeast and animal models reporting that defects in BER enzymes shorten chronological life span and are associated with aging or age-related diseases [21, 22].

Briefly, the BER pathway is initiated by removal of the modified base by a lesion-specific mono- or bifunctional DNA glycosylase, which leaves an apurinic/apyrimidinic site (AP site). AP sites are incised by AP endonuclease 1 (APE1) generating single-strand breaks (SSBs). End processing of SSBs is necessary, since they contain obstructive 3′ or 5′ termini, and this is performed by polymerase β (Polβ), APE1, or polynucleotide kinase 3′-phosphatase (PNKP) depending on the specific terminus. Completion of the BER pathway is performed by either of two subpathways: short-patch BER or long-patch BER [23]. In the nucleus, the final steps of the short-patch BER pathway include filling of the single nucleotide gap by Polβ assisted by the scaffold protein XRCC1, and subsequently ligation by ligase 3α. In the long-patch BER pathway, one or two polymerases (Polβ and Polδ/ε) fill in the 2–13 nucleotides gap assisted by additional proteins (PCNA, RFC, and Fen1) followed by ligation by ligase I. Recently, both subpathways have been described to take place in mitochondria as well [24, 25]. In mitochondria, the only polymerase in charge of the gap filling is the polymerase γ (polγ) (reviewed in [18]).

3.3. Mismatch Repair (MMR). Mismatched nucleotides cause genomic instability and are generated when errors of DNA polymerase escape their proofreading activity or, for example, polymerase slippage occurs at repetitive sequences. The MMR pathway was previously considered exclusively to take place in the nucleus, but several reports have indicated some form of mitochondrial MMR activity (reviewed in [15]). Briefly, the eukaryotic nuclear MMR pathway can be divided into four consecutive steps. (i) Recognition and binding of a mismatch by either a MSH2-MSH6 or MSH2-MSH3 heterodimeric ATPase complex. MSH2-MSH6 preferentially recognizes base-base mismatches and insertion-deletion loops of 1-2 nucleotides while MSH2-MSH3 has preference for larger insertion-deletion loops. The mismatch-bound MSH2-MSH6 (or MSH2-MSH3) complex recruits the MLH1-PMS2 complex in order to form a ternary complex. A proliferating cell nuclear antigen (PCNA) clamp recruits MMR proteins to the replication fork while the clamp loader replication factor C (RFC) loads PCNA. A strand-specific nick or gap, which may reside either 5′ or 3′ to the mismatch, is sufficient to direct repair in 5′- and 3′-directed MMR, respectively. (ii) Excision is performed by the exonuclease EXO1 in both 3′- and 5′-directed MMR. RPA binds to protect the single-stranded DNA during the excision and to facilitate the following DNA repair synthesis. (iii) Repair synthesis is accurately performed by Polδ. (iv) Ligation of the remaining nicks after DNA synthesis is performed by ligase I (reviewed in [26]). MMR proteins have been identified in yeast and coral mitochondria but MMR complexes, as we know them from the nucleus, have not yet been detected in human mitochondria. Still, human mitochondria do seem to have mismatch-repair activity, which involves proteins distinct from nuclear MMR. One of these proteins is the repair factor YB-1 [27].

3.4. Recombinational Repair. DNA double-strand breaks (DSBs) are some of the most hazardous DNA lesions, since they can lead to genome rearrangements. The DSB repair pathway is regulated by several phosphorylation events, starting immediately after DSB formation, where large numbers of the histone protein H2AX are phosphorylated (γH2AX) and accumulate in the chromatin around the break [28, 29]. Several DSB damage response proteins accumulate in foci around DSBs and send signals via signal transducers to a set of downstream effectors, which affect events like DNA repair, cell cycle checkpoints, telomere maintenance, and transcription. Two major mechanisms exist to repair DSBs: homologous recombinational repair (HRR) and nonhomologous end joining (NHEJ). The main difference between the two major DSB repair pathways is the error-prone nature of NHEJ in contrast to the error-free HRR. The balance between the DSB repair pathways differs among species, cell types, and during the phases of the cell cycle [30].

The NHEJ pathway takes place throughout the cell cycle, whereas HRR repairs DSBs during the S and G2 phase of the cell cycle [31]. Recognition of DSBs in NHEJ is performed by the Ku70/80 heterodimer, which also recruits DNA PKcs forming a DNA PK complex on both sides of the DSB [32, 33]. The DNA ends are processed followed by autophosphorylation of DNA PKcs that regulates the final ligation [34]. HRR uses homologous sequences in sister chromatids to repair DSBs, especially those formed at collapsed replication forks. Phosphorylation of BRCA1 (by ATM, ATR, and CHK2) regulates the MRN end processes. Then the 3′ssDNA ends are bound to RPA, and following several phosphorylation steps and the action of different enzymes (Rad52, BRCA2, and CDKs), the 3′ssDNA ends are bound to Rad51 leading to the formation of a nucleoprotein filament that invades a homologous sequence. The invading and complimentary strands extend and once all intermediates are repaired, the HRR pathway is terminated. DSBs are believed to contribute to mtDNA rearrangements observed during aging [35]. Although some reports have suggested mitochondrial recombinational repair in model organisms (yeast and *Drosophila melanogaster*) [36–38], our knowledge of mammalian mtDNA recombination is still limited (reviewed in [39]).

4. Human Aging and Single-Nucleotide Polymorphisms (SNPs) in Genes Related to DNA Repair Mechanisms

The involvement of genetic factors on longevity has been investigated by heritability studies using twin cohorts. The

concept of heritability is valuable for identifying to which extend individual genetic differences contribute to observed individual differences for a specific trait in a study population [40]. Several of those heritability studies have suggested that additive genetic factors account for a quarter to one-third of the variation in human lifespan [41–43]. Thus, epidemiological geneticists have been searching for genes involved in longevity, and recent population studies have investigated correlations of numerous single-nucleotide polymorphisms (SNPs) with longevity in order to identify longevity genes. One gene that has been described to strongly relate to longevity is the forkhead box O3A (FOXO3) [44, 45], which is a transcription factor involved in apoptosis and protection from oxidative stress [46]. Additionally, FOXO3 regulates the stress resistance of cells by facilitating the repair of damaged DNA [47].

Similar studies with other genes have led to the conclusion that it may be possible to identify genetic variants that affect lifespan, inspiring human geneticists to investigate different candidate genes that may be involved in human longevity. Genes related to the maintenance of DNA stability are among those candidate genes.

Initial findings were obtained by analyzing single or a few SNPs in selected candidate genes. The human exonuclease 1 (EXO1) gene was identified as a potential longevity candidate gene by investigating SNPs in approximately 400 German and French centenarians [48]. EXO1 is a 5′-3′ exonuclease participating in MMR and homologous recombination [49]. Interestingly, EXO1 also interacts with the WRN protein. As will be described in more detail later, WRN protein is crucial for the maintenance of genomic stability [50]. Additionally, an association study on SNPs in the *WRN* gene suggested associations between WRN polymorphisms, longevity, and age-associated diseases in Mexican and Finnish populations [51]. Hence, *WRN* is suggested to be a longevity candidate gene. The antioxidant enzymes superoxide dismutases (SOD1 and SOD2) are responsible for converting superoxide radicals, which are harmful to macromolecules and to oxygen and hydrogen peroxide. Variations in *SOD1* and *SOD2* were shown to influence human longevity, and thus also suggested as candidate genes for longevity [52].

Despite that some studies suggest central DNA repair and antioxidant genes as candidate longevity genes, it has been difficult to replicate these findings in independent study populations. Thus, identification of universal longevity polymorphisms is a difficult task, due to the fact that genetics of longevity seems to be extremely complicated.

It is now widely accepted that complex traits, such as longevity, are determined by numerous genes with small effects, and results from single SNP analysis provide limited biological insight. Instead of analyzing single SNPs, recent studies have investigated combined effect of a group of SNPs located in genes involved in the same biological pathway. Successive to the HapMap project [53], the tagging SNP approach is considered a stronger approach than investigating few gene variations, since the most common gene variations in the entire gene is covered. The most comprehensive study of large collections of longevity candidate variations to date has identified novel SNPs to be associated with human longevity [54]. By a case control design, including 1089 oldest-old and 736 middle-aged, Danes, Soerensen and coworkers investigated several rare allele variants in genes involved in DNA metabolism, such as BER genes (*NTHL1*, *Polβ*, and *WRN*) and DSB repair genes (*RAD52*, *H2AFX*, *XRCC5,* and *WRN*). By a longitudinal study approach, SNPs located in genes coding for DNA repair and antioxidant gene products were identified to associate with longevity [54]. Rare allele variants of *MLH1*, involved in MMR, and *XRCC5*, involved in DSB repair, as well as rare allele variants of *XDH* and *TXNRD1*, pro- and antioxidant enzymes, were advantageous, whereas the presence of rare allele variants in *H2AFX*, related to nucleosome formation and DNA damage signaling, appeared disadvantageous for longevity. Together, the studies suggest new candidate variations for human longevity located in pro-/antioxidant, DNA damage signaling and DNA repair genes, and highlight the importance of functional studies of the molecular effects of these newly presented candidate variants [54].

Increased telomere length has been associated with longevity in human leukocytes [55], and decreased telomere length in leukocytes has been associated with increased mortality [56]. At the telomere ends, T-loops are formed, which stabilize the telomeric region and prevent the ends from being recognized as DNA breaks by DNA repair proteins. The shelterin complex holds the T-loop together, protecting the telomeres [57]. Absence of T-loops will allow NHEJ at the telomeric ends, resulting in chromosomal fusion. The telomere length is regulated by telomerases and associated factors [58]. Recent studies have investigated the associations between telomere length and SNPs in telomerase and shelterin genes [55, 59–62]. The most promising results have been reported in relation to *TERC* and *TERT* genes; interestingly two of these studies have also described associations between the *TERT* and *TERC* genes and human longevity [55, 61].

5. Age Regulation of DNA Repair in Human Population Studies

Previous studies investigating SSBR in peripheral blood mononuclear cells (PBMCs) from differently aged donors suggest minor or no effect of age [63–66]; however, the endogenous level of SSBs was found to be significantly higher at old age [67–69]. An age-related decline of NHEJ functions in brain tissue has been suggested from animal studies [70, 71]. In line with these results, PBMCs from elderly humans showed reduced nuclear localization and DNA binding of the NHEJ specific Ku70/80 complex compared to PBMCs from young humans [72]. In another study, PMBCs showed an age-related decline of Ku70 levels [73], and a different study reported declining levels of Ku80 with age [74]. Using a host cell reactivation assay, Wei and coworkers found that the human NER capacity in PMBCs declines with approximately 0.6% per year [75]. Together, these studies suggest biological changes in DNA repair with age, while powerful functional studies are missing.

6. Animal Models as a Useful Approach to Human Aging

As previously described, DNA repair pathways have evolved as important systems for maintenance of DNA stability and cell survival. Proteins in the main DNA repair pathways are highly conserved [76, 77], and such high degree of conservation indicates that DNA repair pathways are fundamental mechanisms in cell survival. As observed in other DNA repair pathways, many of the genes involved in the BER pathway are highly conserved from bacteria to humans [78–80].

Since aging mechanisms have been proposed to be universal [81], animal models have become an excellent tool for investigation of the molecular mechanisms involved in the human aging process, including DNA repair pathways. One of the main advantages of animal models is that most of them have relatively short life span and that genetic modification is possible. Along with mammalian models, aging research studies and DNA metabolism include unicellular organisms and multicellular eukaryotes such as *D. melanogaster*, *Caenorhabditis elegans*, [82–85], or filamentous fungi like *Podospora anserina* [80, 86]. Investigations in these models have contributed notably to the understanding of the role of DNA repair mechanisms in the aging process in humans, particularly those taking place in mitochondria.

One of the first studies using mammalian models for investigating the relation between aging and DNA repair mechanisms was the one performed by Hart and Setlow [87]. They investigated the repair of UV-light induced DNA damage in fibroblasts of different mammalian species, reporting that DNA repair activity was inversely correlated to the maximum life span potential (MLSP) [87]. In a similar recent investigation on *C. elegans* strains showing different longevities, long-lived strains showed higher rates of UV-induced DNA damage repair than the wildtype nematodes [83]. Comparative studies using different mammalian species have also been used in order to investigate the correlation between BER mechanisms and MLSP. Brown and Stuart reported no correlation, when nuclear BER activities were analyzed in mammalian dermal fibroblasts [88]. Up to now, no investigation has been performed analyzing the correlation between mitochondrial BER and MLSP.

Compiling evidence supports that during aging alterations in BER capacity occur, age-related deficiencies in those mechanisms play an important role in cell function and survival, particularly in postmitotic tissues. As mentioned above, the mtBER pathway is highly conserved among mammalian species, and mice are widely used for investigating the role of mtBER in the aging process. Mitochondrial BER capacity has been described to be organ-specific, with the brain being one of the tissues with the lowest capacity [89]. Various studies have investigated the role of changes in mitochondrial BER in age-related functional decline, showing tissue specific age-related changes. Although some studies have observed a reduction in mtBER with aging in rat liver mitochondria [90], most of the investigations in murine mitochondria from hepatic and cardiac tissues have reported increases in APE1 activity and increases or no changes in DNA glycosylase activities during aging [91–93]. On the other hand, a general decline in DNA glycosylase activity has been observed in brain cortical mitochondria in rats [94] and mice [95, 96]. These results support the idea that mtBER plays a critical role in the maintenance of the central nervous system during aging [97]. We have reported significant differences in mtBER among various mouse brain regions during aging. Thus, we found that in cortical mitochondria, DNA glycosylase activities peaked at middle age followed by a significant drop at old age. However, only minor changes were observed in hippocampal mitochondria during the whole lifespan of the animals. Mitochondrial AP endonuclease activity increased in old animals in both brain regions [96]. The cortical region and the cerebellum have been described to accumulate less mtDNA lesions with aging and to be more resistant to oxidative stress conditions [98–100]. Interestingly, those regions showed higher BER capacity than hippocampus, which has been described to be a much more vulnerable region in the brain [98, 99]. A recent investigation also supports the relevance of mtBER in brain aging and stresses the importance of the central nervous system heterogeneity in these processes, since the age-related decline in brain mtBER seems to occur specifically at the synapses [101]. Although investigations on mtBER in humans are scarce, results obtained on mitochondrial, nuclear, or total BER from central nervous system cell cultures or even postmortem neuronal human tissue suggest that the results obtained from mouse studies can be transferred into humans. Thus, increasing Ogg1 activity in mitochondria from oligodendrocytes after targeting hOGG1 into mitochondria increases cell survival and protects against induced-oxidative stress [102]. Moreover, Weissman and coworkers reported that a significant decline in total BER takes place in the cortical area of Alzheimer's patients [103]. Postmitotic tissues are specially affected during aging, and an age-related decline has also been reported in mtBER in skeletal muscle [104], which has been suggested to contribute to age-related sarcopenia. Studies in the aging model *P. anserina* have also shown that aging is associated with a decrease in mitochondrial BER [80], likely contributing to the observed mtDNA instability in the aged fungi [105].

An important approach in the study of the role of DNA repair in the aging process is the development of several knockout (KO) mice. Among them, those models being deficient in essential enzymes in the BER pathway have resulted in embryonic lethality, such as APE1 or Polβ or ligase III [106–108], revealing the importance of these enzymes in cellular survival. Since DNA glycosylases have a certain grade of overlapping activity, KO mice for these enzymes generally result in elevated levels of DNA lesions but no apparent aging phenotype, although some KO mice show higher incidence of cancer [109–111].

Mice mutated in Ku70 or Ku80, proteins involved in repair of DSBs, display an early aging phenotype [112], and are therefore an interesting model for studying the role of NHEJ in aging. Also, Ercc1 and Xpf mutant mice, with a defect in NER and cross-link repair, display reduced lifespan and a broad range of aging-associated changes at an early age, and thus provides another valuable model for studying the role of DNA repair mechanisms in aging [113]. Mice carrying

a specific XPD point mutation found in several patients suffering from trichothiodystrophy (TTD) recapitulate the human disorder to a great extent, but in addition they display pathology consistent with accelerated aging (reviewed in [114]).

A mouse model developed in the last decade, the mitochondrial mutator mouse, appears as an interesting model in which to investigate the causative link between mtDNA mutation accumulation and aging, and the role that mtBER may play in the process [115, 116]. Moreover, the generation of this knock-in mouse expressing proof-reading deficient Polγ, but conserved replicative function, is considered by some authors as an important support of accelerated mtDNA mutation rate resulting in increased aging rate [117, 118]. In various tissues of this mouse model, mtDNA point mutations as well as mtDNA deletions accumulate at a much higher rate than in the wildtype mice [115, 116]. However, whether it is the accumulation of mtDNA deletions or point mutations that drive the premature aging phenotype and the precise mechanisms are still discussed [119, 120].

Some animal models have also been used in order to investigate the role of DNA repair mechanisms on age-related diseases, mainly age-related neurodegenerative diseases. Thus, different mouse models have been created in attempts to elucidate the underlying molecular mechanisms for disorders such as Alzheimer's (AD), Parkinson's (PD), or Huntington's disease (HD), and some of them have been used to investigate the potential role of DNA repair in those disorders [14, 121]. Investigations of age-related neurodegenerative diseases on humans are normally limited to postmortem tissue samples; consequently, animal models of those diseases are very valuable. However, the results observed in animal models do not always match those found in humans. For instance, although a general decline in BER has been described in postmortem cortical tissue in AD patients [103], no changes have been observed in BER mechanisms when the investigations have been performed in the APP and 3xTgAD mouse models [101, 122]. Interestingly, these particular models, unlike humans, do not display neurodegeneration despite the fact that both amyloid-β plaques and neurofibrillary tangles accumulate. Two well-established mouse models for HD have related DNA repair mechanisms with the progression of the disease. Whereas investigations on the HdhQ111 knock-in mice have supported the role of mismatch repair proteins in HD [123, 124], studies on R6/1 mice have revealed the contribution of BER enzymes, particularly OGG1, to CAG expansion in somatic cells that takes place in HD [121]. A recent report suggests that CSB may also be involved in promoting CAG repeat expansion in HD [125, 126].

7. Premature Aging Syndromes

Finally, another important approach to human aging is the study of genetically inherited premature aging syndromes. Such syndromes have been widely used as valuable tools in understanding the normal aging process [127]. Similarly to age-related neurodegenerative diseases, the investigation of human tissues in these syndromes is limited due to scarcely available tissue samples. Thus, research on mouse models and established human cell lines has been critical for understanding some of the mechanisms involved in premature aging syndromes and furthermore increased the understanding of the normal aging process in humans. The DNA repair defective disease Cockayne syndrome (CS), which is a segmental premature aging syndrome, is associated with severe developmental deficiencies and neurodegeneration. Although transcription coupled NER (TC-NER) is a prominent DNA repair pathway affected in this syndrome, an increasing number of investigations indicate that BER is affected as well [128]. In the last years, the investigations that have been carried out on different models of CS, particularly on the models of CSB, have dramatically improved our knowledge about this syndrome and the several roles that the CSB protein plays in genomic stability and cellular survival. Along with the alterations in TC-NER, deficiency in the repair of 8oxoG was initially reported both in whole cell extracts [129] and in mitochondria from mammalian CSB-deficient cells [130]. Moreover, CSB seems to play a role in general mitochondrial maintenance [131]. In accordance, the CSB protein, which was thought to be present exclusively in the nucleus, has recently been shown to localize to mitochondria [132]. This investigation suggests that CSB protein plays a direct role in mtBER by interacting and stabilizing BER proteins in the protein-DNA complexes associated with the inner mitochondrial membrane when mtDNA repair takes place. In addition, it has recently been reported that CSB-deficient cells show higher free radical production and accumulation of damaged mitochondria together with altered mitochondrial autophagy [133]. Thus, the authors suggest that CSB may act as an mtDNA damage sensor, inducing mitochondrial autophagy in response to stress.

Werner syndrome (WS) is another genetically inherited premature aging syndrome, which has been studied intensely with regard to the molecular role of the affected gene product, WRN. The syndrome is characterized by premature graying and thinning of hair, cataracts, diabetes mellitus, osteoporosis, and a number of other typical age-associated deficiencies. The affected WRN protein is a member of the so-called RecQ helicases and orthologs exist in a wide variety of organisms such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *C. elegans*, and *Xenopus laevis* (reviewed in [134]). Based on cellular and biochemical studies of WRN-deficient cell lines, the WRN protein has been suggested to have multiple roles in DNA repair. Hence, the protein interacts with several DNA repair proteins, which are involved in BER, NHEJ, or HHR. This is in agreement with the fact that WS cells show phenotypes such as nonhomologous chromosome exchanges and large chromosomal deletions, caused by deficiency of DSBR [135], and accumulation of, for example, Fapy lesions, which are induced by ROS [136].

In conclusion increasing evidence suggests that DNA repair mechanisms are involved in the aging process. Additional studies using various model systems will help us gain an even better understanding on the functional relationship between these associations.

Authors' Contributions

All authors contributed equally to this paper.

References

[1] D. Harman, "Role of free radicals in aging and disease," *Annals of the New York Academy of Sciences*, vol. 673, pp. 126–141, 1992.

[2] W. Gan, B. Nie, F. Shi et al., "Age-dependent increases in the oxidative damage of DNA, RNA, and their metabolites in normal and senescence-accelerated mice analyzed by LC-MS/MS: urinary 8-oxoguanosine as a novel biomarker of aging," *Free Radical Biology and Medicine*, vol. 52, no. 9, pp. 1700–1707, 2012.

[3] J. Wang, C. L. Clauson, P. D. Robbins et al., "The oxidative DNA lesions 8, 5′-cyclopurines accumulate with aging in a tissue-specific manner," *Aging Cell*, vol. 11, no. 4, pp. 714–716, 2012.

[4] B. M. Lee, S. J. Kwack, and H. S. Kim, "Age-related changes in oxidative DNA damage and benzo(a)pyrene diolepoxide-I (BPDE-I)-DNA adduct levels in human stomach," *Journal of Toxicology and Environmental Health A*, vol. 68, no. 19, pp. 1599–1610, 2005.

[5] E. K. Hudson, B. A. Hogue, N. C. Souza-Pinto et al., "Age-associated change in mitochondrial DNA damage," *Free Radical Research*, vol. 29, no. 6, pp. 573–579, 1998.

[6] H. C. Lee and Y. H. Wei, "Mitochondria and aging," *Advances in Experimental Medicine and Biology*, vol. 942, pp. 311–327, 2012.

[7] M. Yaar and B. A. Gilchrest, "Photoageing: mechanism, prevention and therapy," *British Journal of Dermatology*, vol. 157, no. 5, pp. 874–887, 2007.

[8] K. Husgafvel-Pursiainen, "Genotoxicity of environmental tobacco smoke: a review," *Mutation Research*, vol. 567, no. 2-3, pp. 427–445, 2004.

[9] A. Morita, "Tobacco smoke causes premature skin aging," *Journal of Dermatological Science*, vol. 48, no. 3, pp. 169–175, 2007.

[10] N. P. Singh, C. E. Ogburn, N. S. Wolf, G. Van Belle, and G. M. Martin, "DNA double-strand breaks in mouse kidney cells with age," *Biogerontology*, vol. 2, no. 4, pp. 261–270, 2001.

[11] U. Swain and K. Subba Rao, "Study of DNA damage via the comet assay and base excision repair activities in rat brain neurons and astrocytes during aging," *Mechanisms of Ageing and Development*, vol. 132, no. 8–9, pp. 374–381, 2011.

[12] K. Sugasawa, "Multiple DNA damage recognition factors involved in mammalian nucleotide excision repair," *Biochemistry*, vol. 76, no. 1, pp. 16–23, 2011.

[13] J. de Boer and J. H. J. Hoeijmakers, "Nucleotide excision repair and human syndromes," *Carcinogenesis*, vol. 21, no. 3, pp. 453–460, 2000.

[14] D. K. Jeppesen, V. A. Bohr, and T. Stevnsner, "DNA repair deficiency in neurodegeneration," *Progress in Neurobiology*, vol. 94, no. 2, pp. 166–200, 2011.

[15] R. Gredilla, V. A. Bohr, and T. Stevnsner, "Mitochondrial DNA repair and association with aging—an update," *Experimental Gerontology*, vol. 45, no. 7-8, pp. 478–488, 2010.

[16] H. E. Krokan, H. Nilsen, F. Skorpen, M. Otterlei, and G. Slupphaug, "Base excision repair of DNA in mammalian cells," *FEBS Letters*, vol. 476, no. 1-2, pp. 73–77, 2000.

[17] J. H. J. Hoeijmakers, "Genome maintenance mechanisms for preventing cancer," *Nature*, vol. 411, no. 6835, pp. 366–374, 2001.

[18] R. Gredilla, "DNA damage and base excision repair in mitochondria and their role in aging," *Journal of Aging Research*, vol. 2011, Article ID 257093, 2010.

[19] D. Harman, "Free radical theory of aging," *Mutation Research*, vol. 275, no. 3–6, pp. 257–266, 1992.

[20] K. C. Kregel and H. J. Zhang, "An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations," *American Journal of Physiology*, vol. 292, no. 1, pp. R18–R36, 2007.

[21] M. J. Maclean, R. Aamodt, N. Harris et al., "Base excision repair activities required for yeast to attain a full chronological life span," *Aging Cell*, vol. 2, no. 2, pp. 93–104, 2003.

[22] A. Marusyk and J. DeGregori, "Declining cellular fitness with age promotes cancer initiation by selecting for adaptive oncogenic mutations," *Biochimica et Biophysica Acta*, vol. 1785, no. 1, pp. 1–11, 2008.

[23] D. M. Wilson III and V. A. Bohr, "The mechanics of base excision repair, and its relationship to aging and disease," *DNA Repair*, vol. 6, no. 4, pp. 544–559, 2007.

[24] L. Zheng, M. Zhou, Z. Guo et al., "Human DNA2 is a mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates," *Molecular Cell*, vol. 32, no. 3, pp. 325–336, 2008.

[25] W. C. Copeland and M. J. Longley, "DNA2 resolves expanding flap in mitochondrial base excision repair," *Molecular Cell*, vol. 32, no. 4, pp. 457–458, 2008.

[26] J. Jiricny, "The multifaceted mismatch-repair system," *Nature Reviews Molecular Cell Biology*, vol. 7, no. 5, pp. 335–346, 2006.

[27] N. C. de Souza-Pinto, P. A. Mason, K. Hashiguchi et al., "Novel DNA mismatch-repair activity involving YB-1 in human mitochondria," *DNA Repair*, vol. 8, no. 6, pp. 704–719, 2009.

[28] A. Muslimovic, I. H. Ismail, Y. Gao, and O. Hammarsten, "An optimized method for measurement of gamma-H2AX in blood mononuclear and cultured cells," *Nature Protocols*, vol. 3, no. 7, pp. 1187–1193, 2008.

[29] W. M. Bonner, C. E. Redon, J. S. Dickey et al., "GammaH2AX and cancer," *Nature Reviews Cancer*, vol. 8, no. 12, pp. 957–967, 2008.

[30] M. Shrivastav, C. A. Miller, L. P. De Haro et al., "DNA-PKcs and ATM co-regulate DNA double-strand break repair," *DNA Repair*, vol. 8, no. 8, pp. 920–929, 2009.

[31] M. Shrivastav, L. P. De Haro, and J. A. Nickoloff, "Regulation of DNA double-strand break repair pathway choice," *Cell Research*, vol. 18, no. 1, pp. 134–147, 2008.

[32] P. Calsou, C. Delteil, P. Frit, J. Drouet, and B. Salles, "Coordinated assembly of Ku and p460 subunits of the DNA-dependent protein kinase on DNA ends is necessary for XRCC4-ligase IV recruitment," *Journal of Molecular Biology*, vol. 326, no. 1, pp. 93–103, 2003.

[33] S. Yoo and W. S. Dynan, "Geometry of a complex formed by double strand break repair proteins at a single DNA end: recruitment of DNA-PKcs induces inward translocation of Ku protein," *Nucleic Acids Research*, vol. 27, no. 24, pp. 4679–4686, 1999.

[34] W. D. Block, Y. Yu, D. Merkle et al., "Autophosphorylation-dependent remodeling of the DNA-dependent protein kinase catalytic subunit regulates ligation of DNA ends," *Nucleic Acids Research*, vol. 32, no. 14, pp. 4351–4357, 2004.

[35] H. Fukui and C. T. Moraes, "Mechanisms of formation and accumulation of mitochondrial DNA deletions in aging neurons," *Human Molecular Genetics*, vol. 18, no. 6, pp. 1028–1036, 2009.

[36] M. A. Graziewicz, M. J. Longley, and W. C. Copeland, "DNA polymerase γ in mitochondrial DNA replication and repair," *Chemical Reviews*, vol. 106, no. 2, pp. 383–405, 2006.

[37] F. Morel, M. Renoux, P. Lachaume, and S. Alziari, "Bleomycin-induced double-strand breaks in mitochondrial DNA of Drosophila cells are repaired," *Mutation Research*, vol. 637, no. 1-2, pp. 111–117, 2008.

[38] A. Sickmann, J. Reinders, Y. Wagner et al., "The proteome of Saccharomyces cerevisiae mitochondria," *Proceedings of the National Academy of Sciences United States of America*, vol. 100, no. 23, pp. 13207–13212, 2003.

[39] P. Liu and B. Demple, "DNA repair in mammalian mitochondria: much more than we thought?" *Environmental and Molecular Mutagenesis*, vol. 51, no. 5, pp. 417–426, 2010.

[40] J. van Dongen, P. E. Slagboom, H. H. M. Draisma et al., "The continuing value of twin studies in the omics era," *Nature Reviews Genetics*, vol. 13, no. 9, pp. 640–653, 2012.

[41] M. McGue, J. W. Vaupel, N. Holm, and B. Harvald, "Longevity is moderately heritable in a sample of Danish twins born 1870–1880," *Journals of Gerontology*, vol. 48, no. 6, pp. B237–B244, 1993.

[42] A. M. Herskind, M. McGue, N. V. Holm, T. I. A. Sørensen, B. Harvald, and J. W. Vaupel, "The heritability of human longevity: a population-based study of 2872 Danish twin pairs born 1870–1900," *Human Genetics*, vol. 97, no. 3, pp. 319–323, 1996.

[43] B. Ljungquist, S. Berg, J. Lanke, G. E. McClearn, and N. L. Pedersen, "The effect of genetic factors for longevity: a comparison of identical and fraternal twins in the Swedish Twin Registry," *Journals of Gerontology A*, vol. 53, no. 6, pp. M441–M446, 1998.

[44] B. J. Willcox, T. A. Donlon, Q. He et al., "*FOXO3A* genotype is strongly associated with human longevity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 37, pp. 13987–13992, 2008.

[45] M. Soerensen, S. Dato, K. Christensen et al., "Replication of an association of variation in the *FOXO3A* gene with human longevity using both case-control and longitudinal data," *Aging Cell*, vol. 9, no. 6, pp. 1010–1017, 2010.

[46] B. Ponugoti, G. Dong, and D. T. Graves, "Role of forkhead transcription factors in diabetes-induced oxidative stress," *Experimental Diabetes Research*, vol. 2012, Article ID 939751, 7 pages, 2012.

[47] H. Tran, A. Brunet, J. M. Grenier et al., "DNA repair pathway stimulated by the forkhead transcription factor *FOXO3A* through the Gadd45 protein," *Science*, vol. 296, no. 5567, pp. 530–534, 2002.

[48] A. Nebel, F. Flachsbart, A. Till et al., "A functional EXO1 promoter variant is associated with prolonged life expectancy in centenarians," *Mechanisms of Ageing and Development*, vol. 130, no. 10, pp. 691–699, 2009.

[49] S. E. Liberti and L. J. Rasmussen, "Is hEXO1 a cancer predisposing gene?" *Molecular Cancer Research*, vol. 2, no. 8, pp. 427–432, 2004.

[50] S. Sharma, J. A. Sommers, H. C. Driscoll, L. Uzdilla, T. M. Wilson, and R. M. Brosh, "The exonucleolytic and endonucleolytic cleavage activities of human exonuclease 1 are stimulated by an interaction with the carboxyl-terminal region of the Werner syndrome protein," *Journal of Biological Chemistry*, vol. 278, no. 26, pp. 23487–23496, 2003.

[51] E. Castro, S. D. Edland, L. Lee et al., "Polymorphisms at the Werner locus: II., 1074Leu/Phe, 1367Cys/Arg, longevity, and atherosclerosis," *American Journal of Medical Genetics A*, vol. 95, no. 4, pp. 374–380, 2000.

[52] A. I. Yashin, G. De Benedictis, J. W. Vaupel et al., "Genes and longevity: lessons from studies of centenarians," *Journal of Gerontology*, vol. 55, no. 7, pp. B319–B328, 2000.

[53] International HapMap Consortium, "The International HapMap Project," *Nature*, vol. 426, no. 6968, pp. 789–796, 2003.

[54] M. Soerensen, S. Dato, Q. Tan et al., "Human longevity and variation in GH/IGF-1/insulin signaling, DNA damage signaling and repair and pro/antioxidant pathway genes: cross sectional and longitudinal studies," *Experimental Gerontology*, vol. 47, no. 5, pp. 379–387, 2012.

[55] G. Atzmon, M. Cho, R. M. Cawthon et al., "Evolution in health and medicine Sackler colloquium: genetic variation in human telomerase is associated with telomere length in Ashkenazi centenarians," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, supplement 1, pp. 1710–1717, 2010.

[56] M. Kimura, J. V. B. Hjelmborg, J. P. Gardner et al., "Telomere length and mortality: a study of leukocytes in elderly danish twins," *American Journal of Epidemiology*, vol. 167, no. 7, pp. 799–806, 2008.

[57] T. de Lange, "Shelterin: the protein complex that shapes and safeguards human telomeres," *Genes and Development*, vol. 19, no. 18, pp. 2100–2110, 2005.

[58] K. Collins and J. R. Mitchell, "Telomerase in the human organism," *Oncogene*, vol. 21, no. 4, pp. 564–579, 2002.

[59] D. Levy, S. L. Neuhausen, S. C. Hunt et al., "Genome-wide association identifies OBFC1 as a locus involved in human leukocyte telomere biology," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 20, pp. 9293–9298, 2010.

[60] V. Codd, M. Mangino, P. Van Der Harst et al., "Common variants near TERC are associated with mean telomere length," *Nature Genetics*, vol. 42, no. 3, pp. 197–199, 2010.

[61] M. Soerensen, M. Thinggaard, M. Nygaard et al., "Genetic variation in TERT and TERC and human leukocyte telomere length and longevity: a cross-sectional and longitudinal analysis," *Aging Cell*, vol. 11, no. 2, pp. 223–227, 2012.

[62] J. Deelen, H. W. Uh, R. Monajemi et al., "Gene set analysis of GWAS data for human longevity highlights the relevance of the insulin/IGF-1 signaling and telomere maintenancepathways," *Age*. In press.

[63] W. U. Müller, T. Bauch, G. Stüben, H. Sack, and C. Streffer, "Radiation sensitivity of lymphocytes from healthy individuals and cancer patients as measured by the comet assay," *Radiation and Environmental Biophysics*, vol. 40, no. 1, pp. 83–89, 2001.

[64] M. Zana, A. Szécsényi, A. Czibula et al., "Age-dependent oxidative stress-induced DNA damage in Down's lymphocytes," *Biochemical and Biophysical Research Communications*, vol. 345, no. 2, pp. 726–733, 2006.

[65] A. R. Trzeciak, J. Barnes, N. Ejiogu et al., "Age, sex, and race influence single-strand break repair capacity in a human population," *Free Radical Biology and Medicine*, vol. 45, no. 12, pp. 1631–1641, 2008.

[66] A. R. Trzeciak, J. G. Mohanty, K. D. Jacob et al., "Oxidative damage to DNA and single strand break repair capacity: relationship to other measures of oxidative stress in a population cohort," *Mutation Research*, vol. 736, no. 1-2, pp. 93–103, 2012.

[67] Ü. Mutlu-Türkoğlu, E. Ilhan, S. Öztezcan, A. Kuru, G. Aykaç-Toker, and M. Uysal, "Age-related increases in plasma malondialdehyde and protein carbonyl levels and lymphocyte DNA

damage in elderly subjects," *Clinical Biochemistry*, vol. 36, no. 5, pp. 397–400, 2003.

[68] V. Humphreys, R. M. Martin, B. Ratcliffe et al., "Age-related increases in DNA repair and antioxidant protection: a comparison of the Boyd Orr Cohort of elderly subjects with a younger population sample," *Age and Ageing*, vol. 36, no. 5, pp. 521–526, 2007.

[69] P. Møller, "Assessment of reference values for DNA damage detected by the comet assay in human blood cell DNA," *Mutation Research*, vol. 612, no. 2, pp. 84–104, 2006.

[70] K. Ren and S. Peña De Ortiz, "Non-homologous DNA end joining in the mature rat brain," *Journal of Neurochemistry*, vol. 80, no. 6, pp. 949–959, 2002.

[71] V. N. Vyjayanti and K. S. Rao, "DNA double strand break repair in brain: reduced NHEJ activity in aging rat neurons," *Neuroscience Letters*, vol. 393, no. 1, pp. 18–22, 2006.

[72] D. Frasca, P. Barattini, D. Tirindelli et al., "Effect of age on DNA binding of the ku protein in irradiated human peripheral blood mononuclear cells (PBMC)," *Experimental Gerontology*, vol. 34, no. 5, pp. 645–658, 1999.

[73] Y. J. Ju, K. H. Lee, J. E. Park et al., "Decreased expression of DNA repair proteins Ku70 and Mre11 is associated with aging and may contribute to the cellular senescence," *Experimental and Molecular Medicine*, vol. 38, no. 6, pp. 686–693, 2006.

[74] S. Scarpaci, D. Frasca, P. Barattini, L. Guidi, and G. Doria, "DNA damage recognition and repair capacities in human naïve and memory T cells from peripheral blood of young and elderly subjects," *Mechanisms of Ageing and Development*, vol. 124, no. 4, pp. 517–524, 2003.

[75] Q. Wei, G. M. Matanoski, E. R. Farmer et al., "DNA repair and aging in basal cell carcinoma: a molecular epidemiology study," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 4, pp. 1614–1618, 1993.

[76] L. Aravind, D. R. Walker, and E. V. Koonin, "Conserved domains in DNA repair proteins and evolution of repair systems," *Nucleic Acids Research*, vol. 27, no. 5, pp. 1223–1242, 1999.

[77] L. Gros, M. K. Saparbaev, and J. Laval, "Enzymology of the repair of free radicals-induced DNA damage," *Oncogene*, vol. 21, no. 58, pp. 8905–8925, 2002.

[78] L. C. Olsen, R. Aasland, C. U. Wittwer, H. E. Krokan, and D. E. Helland, "Molecular cloning of human uracil-DNA glycosylase, a highly conserved DNA repair enzyme," *EMBO Journal*, vol. 8, no. 10, pp. 3121–3125, 1989.

[79] H. E. Krokan, R. Standal, and G. Slupphaug, "DNA glycosylases in the base excision repair of DNA," *Biochemical Journal*, vol. 325, no. 1, pp. 1–16, 1997.

[80] M. Soerensen, R. Gredilla, M. Müller-Ohldach et al., "A potential impact of DNA repair on ageing and lifespan in the ageing model organism Podospora anserina: decrease in mitochondrial DNA repair activity during ageing," *Mechanisms of Ageing and Development*, vol. 130, no. 8, pp. 487–496, 2009.

[81] B. L. Strehler, *Time, Cells and Aging*, Academic Press, New York, NY, USA, 1977.

[82] B. F. Oliveira, J. A. Nogueira-Machado, and M. M. Chaves, "The role of oxidative stress in the aging process," *The Scientific World Journal*, vol. 10, pp. 1121–1128, 2010.

[83] M. Hyun, J. Lee, K. Lee, A. May, V. A. Bohr, and B. Ahn, "Longevity and resistance to stress correlate with DNA repair capacity in Caenorhabditis elegans," *Nucleic Acids Research*, vol. 36, no. 4, pp. 1380–1389, 2008.

[84] J. N. Meyer, W. A. Boyd, G. A. Azzam, A. C. Haugen, J. H. Freedman, and B. Van Houten, "Decline of nucleotide excision repair capacity in aging Caenorhabditis elegans," *Genome Biology*, vol. 8, no. 5, article R70, 2007.

[85] H. Morinaga, S. I. Yonekura, N. Nakamura, H. Sugiyama, S. Yonei, and Q. M. Zhang-Akiyama, "Purification and characterization of Caenorhabditis elegans NTH, a homolog of human endonuclease III: essential role of N-terminal region," *DNA Repair*, vol. 8, no. 7, pp. 844–851, 2009.

[86] C. Q. Scheckhuber and H. D. Osiewacz, "Podospora anserina: a model organism to study mechanisms of healthy ageing," *Molecular Genetics and Genomics*, vol. 280, no. 5, pp. 365–374, 2008.

[87] R. W. Hart and R. B. Setlow, "Correlation between deoxyribonucleic acid excision repair and life span in a number of mammalian species," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 71, no. 6, pp. 2169–2173, 1974.

[88] M. F. Brown and J. A. Stuart, "Correlation of mitochondrial superoxide dismutase and DNA polymerase β in mammalian dermal fibroblasts with species maximal lifespan," *Mechanisms of Ageing and Development*, vol. 128, no. 11-12, pp. 696–705, 2007.

[89] B. Karahalil, B. A. Hogue, N. C. De Souza-Pinto, and V. A. Bohr, "Base excision repair capacity in mitochondria and nuclei: tissue-specific variations," *FASEB Journal*, vol. 16, no. 14, pp. 1895–1902, 2002.

[90] R. Leclère, R. Torregrossa, C. Garcia, E. Vara, J. A. F. Treguerres, and R. Gredilla, "Aging, estrogens and mitochondrial DNA repair in rat liver," in *Advances in Comparative Endocrinology, Volume 6*, M. D. Delgado, Ed., 2012, In press.

[91] S. Mitra, T. Izumi, I. Boldogh, K. K. Bhakat, R. Chattopadhyay, and B. Szczesny, "Intracellular trafficking and regulation of mammalian AP-endonuclease 1 (APE1), an essential DNA repair protein," *DNA Repair*, vol. 6, no. 4, pp. 461–469, 2007.

[92] N. C. Souza-Pinto, D. L. Croteau, E. K. Hudson, R. G. Hansford, and V. A. Bohr, "Age-associated increase in 8-oxodeoxyguanosine glycosylase/AP lyase activity in rat mitochondria," *Nucleic Acids Research*, vol. 27, no. 8, pp. 1935–1942, 1999.

[93] N. C. de Souza-Pinto, B. A. Hogue, and V. A. Bohr, "DNA repair and aging in mouse liver: 8-oxodG glycosylase activity increase in mitochondrial but not in nuclear extracts," *Free Radical Biology and Medicine*, vol. 30, no. 8, pp. 916–923, 2001.

[94] D. Chen, G. Cao, T. Hastings et al., "Age-dependent decline of DNA repair activity for oxidative lesions in rat brain mitochondria," *Journal of Neurochemistry*, vol. 81, no. 6, pp. 1273–1284, 2002.

[95] S. Z. Imam, B. Karahalil, B. A. Hogue, N. C. Souza-Pinto, and V. A. Bohr, "Mitochondrial and nuclear DNA-repair capacity of various brain regions in mouse is altered in an age-dependent manner," *Neurobiology of Aging*, vol. 27, no. 8, pp. 1129–1136, 2006.

[96] R. Gredilla, C. Garm, R. Holm, V. A. Bohr, and T. Stevnsner, "Differential age-related changes in mitochondrial DNA repair activities in mouse brain regions," *Neurobiology of Aging*, vol. 31, no. 6, pp. 993–1002, 2010.

[97] L. Weissman, N. C. de Souza-Pinto, T. Stevnsner, and V. A. Bohr, "DNA repair, mitochondria, and neurodegeneration," *Neuroscience*, vol. 145, no. 4, pp. 1318–1329, 2007.

[98] M. R. Brown, P. G. Sullivan, K. A. Dorenbos, E. A. Modafferi, J. W. Geddes, and O. Steward, "Nitrogen disruption of

synaptoneurosomes: an alternative method to isolate brain mitochondria," *Journal of Neuroscience Methods*, vol. 137, no. 2, pp. 299–303, 2004.

[99] C. R. Filburn, W. Edris, M. Tamatani, B. Hogue, I. Kudryashova, and R. G. Hansford, "Mitochondrial electron transport chain activities and DNA deletions in regions of the rat brain," *Mechanisms of Ageing and Development*, vol. 87, no. 1, pp. 35–46, 1996.

[100] S. K. Hota, K. Barhwal, S. B. Singh, and G. Ilavazhagan, "Differential temporal response of hippocampus, cortex and cerebellum to hypobaric hypoxia: a biochemical approach," *Neurochemistry International*, vol. 51, no. 6-7, pp. 384–390, 2007.

[101] R. Gredilla, L. Weissman, J. L. Yang, V. A. Bohr, and T. Stevnsner, "Mitochondrial base excision repair in mouse synaptosomes during normal aging and in a model of Alzheimer's disease," *Neurobiology of Aging*, vol. 33, no. 4, pp. 694–707, 2010.

[102] N. M. Druzhyna, S. B. Hollensworth, M. R. Kelley, G. L. Wilson, and S. P. LeDoux, "Targeting human 8-oxoguanine glycosylase to mitochondria of oligodendrocytes protects against menadione-induced oxidative stress," *GLIA*, vol. 42, no. 4, pp. 370–378, 2003.

[103] L. Weissman, D. G. Jo, M. M. Sørensen et al., "Defective DNA base excision repair in brain from individuals with Alzheimer's disease and amnestic mild cognitive impairment," *Nucleic Acids Research*, vol. 35, no. 16, pp. 5545–5555, 2007.

[104] B. Szczesny, A. W. Tann, and S. Mitra, "Age- and tissue-specific changes in mitochondrial and nuclear DNA base excision repair activity in mice: susceptibility of skeletal muscles to oxidative injury," *Mechanisms of Ageing and Development*, vol. 131, no. 5, pp. 330–337, 2010.

[105] C. Borghouts, E. Kimpel, and H. D. Osiewacz, "Mitochondrial DNA rearrangements of Podospora anserina are under the control of the nuclear gene grisea," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 20, pp. 10768–10773, 1997.

[106] D. L. Ludwig, M. A. MacInnes, Y. Takiguchi et al., "A murine AP-endonuclease gene-targeted deficiency with post-implantation embryonic progression and ionizing radiation sensitivity," *Mutation Research*, vol. 409, no. 1, pp. 17–29, 1998.

[107] N. Hance, M. I. Ekstrand, and A. Trifunovic, "Mitochondrial DNA polymerase gamma is essential for mammalian embryogenesis," *Human Molecular Genetics*, vol. 14, no. 13, pp. 1775–1783, 2005.

[108] N. Puebla-Osorio, D. B. Lacey, F. W. Alt, and C. Zhu, "Early embryonic lethality due to targeted inactivation of DNA ligase III," *Molecular and Cellular Biology*, vol. 26, no. 10, pp. 3935–3941, 2006.

[109] A. Klungland, I. Rosewell, S. Hollenbach et al., "Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13300–13305, 1999.

[110] M. T. A. Ocampo, W. Chaung, D. R. Marenstein et al., "Targeted deletion of mNth1 reveals a novel DNA repair enzyme activity," *Molecular and Cellular Biology*, vol. 22, no. 17, pp. 6111–6121, 2002.

[111] M. K. Chan, M. T. Ocampo-Hafalla, V. Vartanian et al., "Targeted deletion of the genes encoding NTH1 and NEIL1 DNA N-glycosylases reveals the existence of novel carcinogenic oxidative damage to DNA," *DNA Repair*, vol. 8, no. 7, pp. 786–794, 2009.

[112] H. Li, H. Vogel, V. B. Holcomb, Y. Gu, and P. Hasty, "Deletion of Ku70, Ku80, or both causes early aging without substantially increased cancer," *Molecular and Cellular Biology*, vol. 27, no. 23, pp. 8205–8214, 2007.

[113] S. Q. Gregg, A. R. Robinson, and L. J. Niedernhofer, "Physiological consequences of defects in ERCC1-XPF DNA repair endonuclease," *DNA Repair*, vol. 10, no. 7, pp. 781–791, 2011.

[114] K. Diderich, M. Alanazi, and J. H. J. Hoeijmakers, "Premature aging and cancer in nucleotide excision repair-disorders," *DNA Repair*, vol. 10, no. 7, pp. 772–780, 2011.

[115] A. Trifunovic, A. Wredenberg, M. Falkenberg et al., "Premature ageing in mice expressing defective mitochondrial DNA polymerase," *Nature*, vol. 429, no. 6990, pp. 417–423, 2004.

[116] G. C. Kujoth, A. Hiona, T. D. Pugh et al., "Medicine: mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging," *Science*, vol. 309, no. 5733, pp. 481–484, 2005.

[117] G. C. Kujoth, P. C. Bradshaw, S. Haroon, and T. A. Prolla, "The role of mitochondrial DNA mutations in mammalian aging," *PLoS Genetics*, vol. 3, no. 2, article e24, 2007.

[118] A. Kukat and A. Trifunovic, "Somatic mtDNA mutations and aging—facts and fancies," *Experimental Gerontology*, vol. 44, no. 1-2, pp. 101–105, 2009.

[119] D. Edgar and A. Trifunovic, "The mtDNA mutator mouse: dissecting mitochondrial involvement in aging," *Aging*, vol. 1, no. 12, pp. 1028–1032, 2009.

[120] M. Vermulst, J. Wanagat, and L. A. Loeb, "On mitochondria, mutations, and methodology," *Cell Metabolism*, vol. 10, no. 6, article 437, 2009.

[121] J. L. Yang, P. Sykora, D. M. Wilson III, M. P. Mattson, and V. A. Bohr, "The excitatory neurotransmitter glutamate stimulates DNA repair to increase neuronal resiliency," *Mechanisms of Ageing and Development*, vol. 132, no. 8–9, pp. 405–411, 2011.

[122] L. Weissman, N. C. de Souza-Pinto, M. P. Mattson, and V. A. Bohr, "DNA base excision repair activities in mouse models of Alzheimer's disease," *Neurobiology of Aging*, vol. 30, no. 12, pp. 2080–2081, 2009.

[123] V. C. Wheeler, L. A. Lebel, V. Vrbanac, A. Teed, H. T. te Riele, and M. E. MacDonald, "Mismatch repair gene Msh2 modifies the timing of early disease in HdhQ111 striatum," *Human Molecular Genetics*, vol. 12, no. 3, pp. 273–281, 2003.

[124] E. Dragileva, A. Hendricks, A. Teed et al., "Intergenerational and striatal CAG repeat instability in Huntington's disease knock-in mice involve different DNA repair genes," *Neurobiology of Disease*, vol. 33, no. 1, pp. 37–47, 2009.

[125] I. V. Kovtun, K. O. Johnson, and C. T. McMurray, "Cockayne syndrome B protein antagonizes OGG1 in modulating CAG repeat length in vivo," *Aging*, vol. 3, no. 5, pp. 509–514, 2011.

[126] A. V. Goula, B. R. Berquist, D. M. Wilson III, V. C. Wheeler, Y. Trottier, and K. Merienne, "Stoichiometry of base excision repair proteins correlates with increased somatic CAG instability in striatum over cerebellum in Huntington's disease transgenic mice," *PLoS Genetics*, vol. 5, no. 12, Article ID e1000749, 2009.

[127] V. A. Bohr, M. Sander, and K. H. Kraemer, "Rare diseases provide rare insights into DNA repair pathways, TFIIH, aging and cancer," *DNA Repair*, vol. 4, no. 2, pp. 293–302, 2005.

[128] T. Stevnsner, M. Muftuoglu, M. D. Aamann, and V. A. Bohr, "The role of Cockayne Syndrome group B (CSB) protein in base excision repair and aging," *Mechanisms of Ageing and Development*, vol. 129, no. 7-8, pp. 441–448, 2008.

[129] G. Dianov, C. Bischoff, M. Sunesen, and V. A. Bohr, "Repair of 8-oxoguanine in DNA is deficient in Cockayne syndrome group B cells," *Nucleic Acids Research*, vol. 27, no. 5, pp. 1365–1368, 1999.

[130] T. Stevnsner, S. Nyaga, N. C. De Souza-Pinto et al., "Mitochondrial repair of 8-oxoguanine is deficient in Cockayne syndrome group B," *Oncogene*, vol. 21, no. 57, pp. 8675–8682, 2002.

[131] P. O. Osenbroch, P. Auk-Emblem, R. Halsne et al., "Accumulation of mitochondrial DNA damage and bioenergetic dysfunction in CSB defective cells," *FEBS Journal*, vol. 276, no. 10, pp. 2811–2821, 2009.

[132] M. D. Aamann, M. M. Sorensen, C. Hvitby et al., "Cockayne syndrome group B protein promotes mitochondrial DNA stability by supporting the DNA repair association with the mitochondrial membrane," *FASEB Journal*, vol. 24, no. 7, pp. 2334–2346, 2010.

[133] M. Scheibye-Knudsen, M. Ramamoorthy, P. Sykora et al., "Cockayne syndrome group B protein prevents the accumulation of damaged mitochondria by promoting mitochondrial autophagy," *Journal of Experimental Medicine*, vol. 209, no. 4, pp. 855–869, 2012.

[134] M. L. Rossi, A. K. Ghosh, and V. A. Bohr, "Roles of Werner syndrome protein in protection of genome integrity," *DNA Repair*, vol. 9, no. 3, pp. 331–344, 2010.

[135] D. K. Singh, B. Ahn, and V. A. Bohr, "Roles of RECQ helicases in recombination based DNA repair, genomic stability and aging," *Biogerontology*, vol. 10, no. 3, pp. 235–252, 2009.

[136] A. Das, I. Boldogh, W. L. Jae et al., "The human Werner syndrome protein stimulates repair of oxidative DNA base damage by the DNA glycosylase NEIL1," *Journal of Biological Chemistry*, vol. 282, no. 36, pp. 26591–26602, 2007.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 321653, 19 pages
doi:10.1155/2012/321653

Review Article

Pleiotropic Cellular Functions of PARP1 in Longevity and Aging: Genome Maintenance Meets Inflammation

Aswin Mangerich and Alexander Bürkle

Molecular Toxicology Group, Department of Biology, University of Konstanz, 78457 Konstanz, Germany

Correspondence should be addressed to Aswin Mangerich, aswin.mangerich@uni-konstanz.de
and Alexander Bürkle, alexander.buerkle@uni-konstanz.de

Received 1 June 2012; Accepted 25 July 2012

Academic Editor: Paula Ludovico

Aging is a multifactorial process that depends on diverse molecular and cellular mechanisms, such as genome maintenance and inflammation. The nuclear enzyme poly(ADP-ribose) polymerase 1 (PARP1), which catalyzes the synthesis of the biopolymer poly(ADP-ribose), exhibits an essential role in both processes. On the one hand, PARP1 serves as a genomic caretaker as it participates in chromatin remodelling, DNA repair, telomere maintenance, resolution of replicative stress, and cell cycle control. On the other hand, PARP1 acts as a mediator of inflammation due to its function as a regulator of NF-κB and other transcription factors and its potential to induce cell death. Consequently, PARP1 represents an interesting player in several aging mechanisms and is discussed as a longevity assurance factor on the one hand and an aging-promoting factor on the other hand. Here, we review the molecular mechanisms underlying the various roles of PARP1 in longevity and aging with special emphasis on cellular studies and we briefly discuss the results in the context of *in vivo* studies in mice and humans.

1. Genomic Stability, Inflammation, and Aging

Aging has been defined as a progressive postmaturational decline in physiological capacity, accompanied by an increased susceptibility to disease and an increased mortality risk. Mechanisms to maintain genomic stability are thought to counteract the aging process, whereas inflammation is considered a driving force of human aging [1].

A large body of evidence supports the theory that genomic instability acts as a causative factor in the aging process, which is evident from the fact that most mouse models of premature aging as well as human progeria syndromes are related to defects in mechanisms of genomic maintenance [2]. This may be attributed to the fact that DNA serves as a blueprint of all cellular RNAs and proteins. Any acquired change in its sequence, which may arise from molecular damage, is permanent and thus may have irreversible consequences. For this reason nature invested in a sophisticated network of various mechanisms (i) to maintain genome integrity, such as DNA repair and cell cycle control, and (ii) to withdraw heavily damaged cells from the body, such as apoptosis and cellular senescence. However, even if these mechanisms may be very efficient they cannot cope with all the insults induced in the genome, leading to a gradual accumulation of DNA damage and mutations, thus contributing to organismic aging [2].

On the other hand, a direct relationship exists between physiological aging and increasing incidence of chronic inflammatory diseases. In its acute form, inflammation acts as a protective mechanism in response to pathogen invasion or tissue damage and helps to restore physiological integrity and function. However, in its chronic form, inflammation can exert detrimental effects on the cellular as well as the organismic level. Chronically inflamed tissue is characterized by infiltration of immune cells, neovascularization, fibrosis, and often tissue damage and necrosis [3]. The innate immune system, especially the mononuclear phagocyte system, is the most important mediator of chronic inflammation. Monocytes originate from the myeloid hematopoietic cell lineage in bone marrow. In the blood stream, monocytes are recruited by specific stimuli into different tissues, where they differentiate into phagocytic

macrophages. Macrophages participate in the killing of invading microorganisms and emerging tumor cells through the production of reactive oxygen or nitrogen species (ROS and RNS). In addition, macrophages secrete cytokines, which play a key role in the regulation of multiple immune functions, especially inflammatory responses [3]. During aging, the continuous pressure on the immune system caused by repeated antigen stimulation, such as infections, food antigens, allergens, and self antigens, leads to an increase in activated cells and secretion of proinflammatory cytokines, such as TNFα [4]. These circulating proinflammatory factors may keep the immune system in a state of chronic low-level activation, a phenomenon described as "inflammaging" [5, 6]. Eventually, this causes "immunosenescence," that is, an age-related decline in the capacity of adaptive immunity, consisting of more specific responses carried out by B and T cells [7]. Thus, with advanced age, the immune system undergoes a gradual remodeling in the attempt to reestablish a new balance that assures survival, however, favoring the development of chronic inflammatory conditions [5, 6, 8, 9].

DNA damage and inflammation are inevitably linked by the production of reactive chemical species, such as ROS and RNS. Cellular ROS and RNS production occurs constantly under physiological as well as pathophysiological conditions as a consequence of electron leakage of the mitochondrial electron transport chain and via enzymes such as NADPH oxidase, nitric oxide synthases, and xanthine oxidase. The "free radical theory of aging" posits that aging and its related diseases are the net consequence of free radical-induced damage and the inability to counterbalance these changes by antioxidative defenses and sufficient DNA repair [10]. Chronic inflammation results in the generation of a broad spectrum of ROS and RNS by activated macrophages and neutrophils, which damage cellular macromolecules including DNA [11, 12]. Conversely, the generation of ROS and RNS activates redox sensitive transcription factors, such as NF-κB, resulting in the generation of proinflammatory molecules. Moreover, DNA damage can induce cellular senescence, a tumor suppressive mechanism that is also associated with aging, leading to the secretion of inflammatory cytokines, a paracrine effect known as senescence-associated secretory phenotype (SASP) [5, 13]. Altogether, this can trigger a positive feedback loop that amplifies the processes of inflammation, damage, and destruction in target cells and organs, leading to an organismic decline and death over time. For example, chronic inflammation has been associated with an age-related decline in the function of hematopoietic and mesenchymal stem cells [14, 15] and has been implicated as a mediator of almost all of the aging-associated diseases, such as vascular diseases, diabetes, neurodegenerative diseases, and cancer [3, 5, 6, 9].

As discussed below, the nuclear enzyme PARP1 represents a factor that works at the interface between genomic maintenance and inflammation. Therefore, PARP1 may act in an antagonistic pleiotropic way, that is, functioning as a longevity assurance factor at younger age or in physiological conditions and as an aging-promoting factor at older age or in pathophysiological conditions. In this paper, we will discuss the numerous cellular functions of PARP1 in the context of mechanisms of longevity and aging and will put this into an organismic perspective by briefly summarizing *in vivo* studies in mice and humans.

2. PARP1 and Poly(ADP-ribosyl)ation

Poly(ADP-ribosyl)ation is a posttranslational modification of proteins that occurs in most eukaryotic organisms. The reaction is carried out by enzymes of the family of poly(ADP-ribose) polymerases (PARPs) by using NAD^+ as a substrate to synthesize the linear or branched biopolymer poly(ADP-ribose) (PAR), which consist of up to 200 ADP-ribose subunits (Figure 1) [16]. PARP activation leads to covalent modification of various proteins with PAR including PARPs themselves, as some of them catalyze their automodification. Covalent linkage is mediated either through attachment on glutamate, aspartate, or lysine residues of the acceptor proteins [16]. Apart from covalent modification, some proteins can also bind preexisting ADP-ribose chains in a noncovalent fashion, and this binding is mediated via at least three different PAR binding motifs. Those include (i) a 20 amino acid motif, (ii) distinct macrodomains, and (iii) a PAR-binding zinc finger, all of which fulfill diverse cellular functions [17–22]. Whereas the PAR-binding macrodomains and zinc fingers are present in a limited number of human proteins (<50), the 20-aa motif has been identified in several hundred human protein sequences [17, 18]. This weakly conserved motif consists of (i) a cluster rich in basic amino acids and (ii) a pattern of hydrophobic amino acids interspersed with basic residues [17, 18]. Most of the putative PAR-binding proteins identified are involved in a wide spectrum of cellular mechanisms such as genomic maintenance, chromatin remodeling, transcription, replication, RNA metabolism, inflammation, cell cycle control, and cell death [18]. In general, poly(ADP-ribosyl)ation modulates target protein function by modifying enzymatic activities or interactions with other macromolecules such as DNA, RNA, or proteins [23].

Importantly, the cellular existence of PAR is transient, since the polymer is rapidly hydrolyzed by PARPs catabolic counterpart, poly(ADP-ribose) glycohydrolase (PARG). PARG possesses both exo- and endoglycosidic activities and is encoded by a single gene giving rise to at least five different splice variants with distinct subcellular localizations [24–26]. In addition, a second enzyme was identified with weak PARG activity, that is, ADP-ribose-arginine protein hydrolase 3 (ARH3), with evidence that this enzyme is associated with PAR degradation in mitochondria [27, 28].

The PARP gene family consists of 17 homologues in the human genome [16]. PARP1 is the founding member of the gene family. It exhibits key roles in the regulation of nuclear and cellular functions and can be activated either by DNA damage, posttranslational protein modifications, or potentially by direct protein-protein interactions [29]. The strongest stimulation of PARP1 activity is mediated by its binding to DNA strand breaks which induces its catalytic activation as a monomer or dimer by several hundred-fold [30–33]. Under these conditions, PARP1 accounts for >75% of the overall cellular poly(ADP-ribosyl)ation capacity

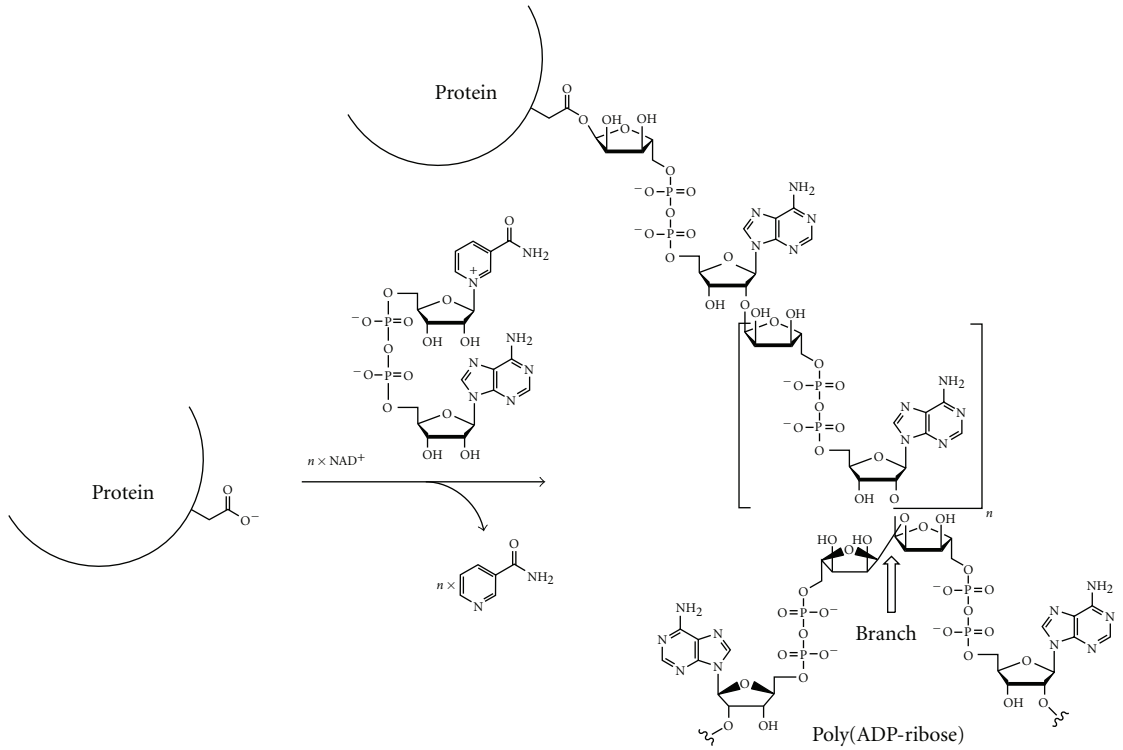


Figure 1: Poly(ADP-ribosyl)ation. PARPs cleave the glycosidic bond of NAD^+ between nicotinamide and ribose followed by the covalent modification of acceptor proteins with an ADP-ribosyl unit. PARPs also catalyze an adduct elongation, giving rise to linear polymers with chain lengths of up to 200 ADP-ribosyl units, characterized by their unique ribose $(1'' \rightarrow 2')$ ribose phosphate-phosphate backbone. At least some of the PARP family members also catalyze a branching reaction by creating ribose $(1''' \rightarrow 2'')$ ribose linkages.

[34, 35]. Apart from direct DNA damage-dependent PARP1 activation, its activity is also regulated by posttranslational modifications such as phosphorylation, acetylation, and sumoylation [36–40]. Moreover, PARP1 activity is subject to regulation by direct protein-protein interactions [41–43].

Three nonexclusive mechanisms of the cellular functions of PARP1 can be distinguished: (i) functions that rely on the enzymatic activity of PARP1 and the subsequent covalent modification or noncovalent interaction of nuclear proteins with PAR; (ii) direct interactions of proteins with PARP1 via protein-protein interaction, for example, via the BRCT domain; (iii) intervention in the cellular NAD^+ metabolism by excessive PARP1 stimulation and potential signaling functions of free PAR or its derivatives. The consequences of these actions with regard to modulation of genomic maintenance, chromatin structure, inflammation, and cell death are discussed below.

3. PARP1 in Genomic Maintenance

It is estimated that thousands of DNA damage lesions occur in a mammalian cell per day, all of which need to be repaired to ensure genomic stability and longevity. In mammals, at least six major DNA repair pathways exist, that is, O^6-methyl guanine methyltransferase (MGMT), base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and DNA double-strand break (DSB) repair including the subpathways homologous recombination (HR) and nonhomologous end-joining (NHEJ) [2].

Except for the MGMT and MMR pathways, there is evidence that PARP1 is involved in all of these repair mechanisms, and therefore, PARP1 is considered a general caretaker of genomic stability [29]. Of note, the recruitment of PARP1 to sites of DNA damage and the subsequent production of PAR can occur within seconds and is one of the fastest DNA damage responses [44, 45]. Apart from this direct involvement in several DNA repair mechanisms, PARP1 participates in genomic maintenance through its role as a regulator of chromatin structure and cell cycle regulation (Figure 2).

Several cellular studies support a role of PARP1 as a general cell survival factor upon genotoxic stimuli: transdominant inhibition of PARP1 by overexpression of its DNA binding domain potentiates cytotoxicity upon treatment of cells with alkylating agents and ionizing radiation [46]. Moreover, PARP1-deficient cells exhibit an enhanced sensitivity to alkylating agents [47, 48] and show increased frequencies of sister chromatid exchanges, both under basal conditions and upon treatment with alkylating agents [49, 50]. Consistent with this, overexpression studies demonstrated that PARP1 acts as a negative regulator of alkylation-induced sister chromatid exchange [51], and *ex vivo* supplementation of human PBMC with the NAD^+ precursor nicotinic acid enhances cellular poly(ADP-ribosyl)ation and improves cell viability upon induction of genotoxic stress [52].

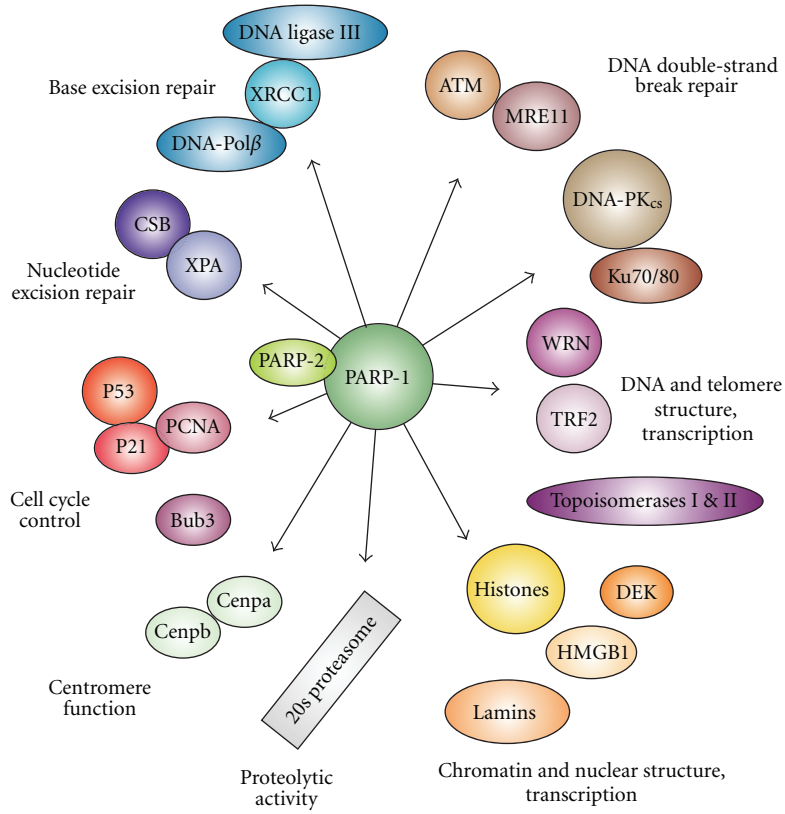


Figure 2: PARP1, some interaction partners, and their role in genomic maintenance. ATM indicates ataxia telangiectasia mutated; Bub3, budding uninhibited by benzimidazoles 2; Cenpa/b, centromeric protein a/b; CSB, Cockayne syndrome type B; DEK, DEK oncogene; DNA-Polβ, DNA polymerase β; DNA-PK$_{CS}$, DNA-activated protein kinase catalytic subunit; HMGB1, high mobility group box 1; Ku70/80, Ku antigens 70/80 kDa subunit; MRE11, meiotic recombination 11; p21, cyclin-dependent kinase inhibitor 1A; p53, tumor suppressor protein p53; PCNA, proliferating cell nuclear antigen; TRF2, telomeric repeat binding factor 2; WRN, Werner syndrome protein; XRCC1, X-ray repair complementing defective in Chinese hamster 1; XPA, xeroderma pigmentosum complementation group A.

3.1. PARP1 and Chromatin Regulation. Chromatin is a complex of DNA and proteins with a dynamic structure and is involved in replication, transcription, and other fundamental cellular processes. Structural and functional alterations of chromatin are widely associated with aging from yeast to mammals [53]. The molecular mechanisms leading to chromatin disturbances in aging are largely unknown, but may be related to accumulation of unrepaired DNA damage. On the other hand, alterations in chromatin structure increase the susceptibility to DNA damage, suggesting the presence of a positive feedback mechanism of DNA damage leading to chromatin rearrangements which, in turn, sensitizes DNA as a substrate for further damage. Moreover, there is evidence that chromatin defects lead to alterations in transcriptional programs thereby contributing to the aging process [53].

PARP1 acts as a structural and regulatory component of chromatin, both in undamaged cells and upon genotoxic stress. It may either regulate chromatin structure directly by poly(ADP-ribosyl)ation of chromatin components, or indirectly by controlling the recruitment of chromatin remodeling factors [54]. Many PAR acceptor and binding proteins contribute to chromatin and nuclear architecture such as histones, lamins, high-mobility group (HMG) proteins, heterochromatin protein 1 (HP1), and the DEK protein [54–59]. It was proposed that PARP1 induces a histone-shuttling mechanism, based on findings that poly(ADP-ribosyl)ation of polynucleosomes causes relaxation of chromatin structure and that activity of PARG degrades PAR from modified histones [60–63]. According to this model, DNA-bound histones dissociate from DNA upon poly(ADP-ribosyl)ation, causing an open chromatin structure and guiding repair factors to sites of DNA damage. Upon degradation of PAR by PARG, DNA reassociates with histones, thereby restoring the condensed chromatin structure. Moreover, upon DNA damage, PARP1 activation leads to the recruitment of the histone variant macroH2A1.1 to the site of the damage, which transiently causes chromatin rearrangements and dynamically modulates the DNA damage response [22]. Kim et al. reported that PARP1 itself can function as a component of chromatin [64], that is, histone H1 and PARP1 bind in a competitive and mutually exclusive manner to nucleosomes *in vitro*. Thereby, PARP1 promotes the local compaction of chromatin into higher order structures, which are associated with transcriptional repression. The authors suggested that PARP1 modulates the chromatin architecture and gene transcription through its intrinsic enzymatic activity in a DNA damage-independent manner; that is, PARP1 activation and automodification

trigger its release from chromatin, thereby facilitating chromatin decondensation and gene transcription by RNA polymerase II. Subsequent cellular studies demonstrated that PARP1 could replace histone H1 at RNA polymerase II-transcribed promoters, which was associated with actively transcribed genes [65].

Apart from a functional interplay between PARP1 with histones, an interesting physical and functional interaction exists between PARP1 and DEK. The DEK protein is a major nonhistone chromatin component with functions in DNA metabolism and repair on a cellular, and carcinogenesis and autoimmunity on an organismic level. DEK is often found to be upregulated in tumor tissue, and high levels of DEK favor cell immortalization by inhibiting senescence and apoptosis. Consistently, DEK deficient cells are prone to induction of senescence in the response to genotoxic stress [66]. We and others have shown that PARP1 poly(ADP-ribosyl)ates DEK. Moreover, DEK interacts with PAR in a non-covalent manner which regulates its DNA binding affinity and multimerization with possible implications in response to genotoxic stress and gene transcription. In terms of gene transcription, DEK is released from chromatin upon poly(ADP-ribosyl)ation to permit transcriptional initiation [56, 59, 67]. Whether DEK itself or its interplay with PARP1 has a direct role in aging mechanisms remains to be clarified.

PARP1 activation is also necessary for the exchange of histone H1 with high-mobility group B (HMGB) proteins, which are non-histone chromatin-associated proteins that bend DNA and recruit transcription factors to their DNA targets [68]. Interestingly, during inflammation, HMGB1 can be secreted by activated cells, where it inhibits phagocytic uptake of dying cells by macrophages. HMGB1 secreted upon specific stimuli, that is, TLR4 stimulation is highly poly(ADP-ribosyl)ated, which enhances the inhibitory effect of HMGB1 on macrophage-dependent phagocytosis. This indicates a regulatory role of PAR in such inflammatory mechanisms with potential implications in mechanisms of aging as discussed below [69].

Importantly, not only structural components of the chromatin are regulated by poly(ADP-ribosyl)ation, PAR also serves as an important factor in the regulation of chromatin remodeling factors, such as ALC1 and NURD [21, 70–72]. For example, the recruitment of the NURD chromatin remodeling complex to sites of DNA lesions depends on the synthesis of PAR. Interestingly, this complex was identified as an important modulator of aging-associated chromatin defects, and loss of several NURD components and function was evident during human premature aging [73].

The role of PARP1 in gene transcription and chromatin remodeling was impressively demonstrated in a *Drosophila* study [74]. The authors revealed that PARP1 is crucial for puff formation in giant polytene chromosomes. Puff formation arises from local relaxation of the chromatin structure and is associated with actively transcribed regions [74]. Ju et al. provided interesting mechanistic evidence linking PARP1-dependent initiation of transcription and its function in DNA binding [68]. According to this work, PARP1 acts in concert with another binding partner, that is, topoisomerase II. Topoisomerase II introduces a transient double strand break at the promoter, which leads to PARP1 binding and activation. The subsequent rapid but transient poly(ADP-ribosyl)ation triggers chromatin relaxation and initiation of transcription.

Together, these findings suggest a functional interplay of PARP1 with chromatin components and associated remodeling factors, implying an active role of PARP1 in chromatin function and transcriptional regulation during the aging process. Gene profiling data support such a hypothesis, since PARP1 deficiency alters expression of genes involved in cell cycle progression, DNA replication, oxidative stress, cancer initiation, and aging [75, 76]. The detailed spatial and temporal characteristics of these mechanisms, however, remain to be determined.

3.2. PARP1 in DNA Repair. As discussed above, a substantial body of evidence demonstrates a causative role of DNA repair and genome maintenance mechanisms in mammalian longevity.

Base excision repair (BER) is the major DNA repair pathway that acts on damage that occurs during cellular metabolism including damage from ROS, methylation, deamination, and hydroxylation. The levels of many of these lesions increase with age including the well-studied lesion 8-oxoguanine. Moreover, BER activity decreases with age in multiple tissues [77]. The core BER reaction is initiated by a DNA single-strand break (SSB) upon excision of the damaged bases by DNA glycosylases [78]. PARP1 detects such SSB via its second zinc finger (ZFII), thus triggering its enzymatic activation [79, 80]. Moreover, PARP1 physically cooperates with 8-oxoguanine-DNA glycosylase, which further stimulates PARP1 activity [81]. Importantly, the recruitment of the BER loading platform X-ray repair complementing factor 1 (XRCC1) is completely dependent on poly(ADP-ribsosyl)ation [82, 83]. Thus, PARP1 and PAR are required for the assembly and stability of XRCC1 nuclear foci after DNA damage [83]. Furthermore, XRCC1 and PARP1 interact with DNA polymerase-β and DNA ligase III, forming a multiprotein complex consisting of the major BER factors [84–86].

The finding that PARP1-deficient cells still synthesized PAR led to the identification of an additional nuclear PARP, that is, PARP2, which is also activated upon genotoxic stimuli [34, 35]. PARP1 and PARP2 homo- and heterodimerize and work at least partially in a redundant fashion, since only double-knockout mice show embryonic lethality [87, 88]. This notion is supported by the fact that PARP2 also participates in BER and interacts physically and functionally with XRCC1, DNA polymerase-β, and DNA ligase III. Recruitment studies indicate a role of PARP2 in later steps of BER repair, as proposed by the following model for spatiotemporal accumulation of BER factors: SSBs are detected by the DNA binding domain of PARP1, leading to its activation, production of PAR, and chromatin relaxation. Subsequently, additional PARP1 molecules are attracted, causing amplification of the signal. At the "point of repulsion," PARP1 then dissociates from the DNA, enabling the recruitment of the BER loading platform XRCC1, PARP2,

and further DNA repair factors. This triggers resealing of the DNA lesion and reestablishment of genomic integrity [89].

Nucleotide excision repair is responsible for the removal of bulky helix-distorting DNA adducts, which are caused by UV irradiation and endogenous metabolites [78]. The functional role of the NER as a longevity assurance mechanism is impressively represented by the fact that patients with defects in a subset of NER proteins, that is, CSA and CSB (Cockayne syndrome) and XPB, XPD, TTDA (trichothiodystrophy), as well as corresponding mouse models, show in some tissues a strong premature aging phenotype [2]. Although the role of PARP1 in NER is not very well established, at least two NER factors, the DNA-dependent ATPase Cockayne syndrome group B (CSB) protein and the DNA lesion recognition protein xeroderma pigmentosum group A (XPA), were identified as PAR binding factors [17, 90, 91]. CSB also physically interacts with PARP1 and its ATPase activity is inhibited by poly(ADP-ribosyl)ation. Consistently, there is some evidence from cell culture studies that PARP1 is involved in NER of UV photo-damage products [92, 93].

DNA double strand breaks (DSBs) arise from ionizing radiation, free radicals, chemicals, or during replication of a SSB through collapsed replication forks. They represent the most cytotoxic form of DNA damage and, if unrepaired, they can trigger apoptosis, senescence, or genomic instability. Mammalian cells repair DSBs via two mechanisms: homologous recombination (HR) utilizes the sister chromatid or chromosome for error-free repair of the DSB, whereas nonhomologous end-joining (NHEJ) simply reattaches free DNA ends without using a template. For this reason, NHEJ is prone to microdeletions or insertions which can cause frameshift mutations [78]. Whether HR or NHJE is employed depends on the species, cell type, and cell cycle phase [94]. In both pathways, PARP1 already participates at very early stages. PARP1 and the DSB sensing complexes MRN (MRE11/Rad50/NBS1) (involved in HR) and Ku70/80 (involved in NHEJ) were shown to interact with and compete for binding at free DNA ends, with PARP1 potentially guiding these proteins to the damaged site [44, 95]. PARP1 also physically and functionally interacts with two phosphatidyl inositol 3-like protein kinases ATM (involved in HR) and DNA-PK_{cs} (involved in NHEJ), which are crucial for DSB signaling [96–99]. It was suggested that PARP1 serves as a general DNA-damage-detecting molecule, which potentially mediates a switch between the NHEJ and the HR pathways [94]. Consistent with this, PARP1 functions in a NHEJ back up pathway [95, 100], and several reports demonstrated an antirecombinogenic activity of PARP1 [101–103]. Some information on the role of PARP1 in DSB repair was obtained from studies investigating the possible use of PARP inhibitors as anticancer drugs following the concept of synthetic lethality. According to this concept, PARP1 inhibition alone shows no cytotoxic effect on HR proficient cells, but causes cytotoxicity in HR-defective cells, for example, *BRCA* mutant cancer cells. This cytotoxicity is thought to be induced by accumulation of unrepaired SSBs, which are converted to DSBs by collisions with the replication machinery which cannot be repaired due to the lack of HR, ultimately triggering cell death [104]. A recent study challenged this view by presenting an alternative model based on the finding that coinactivation of NHEJ rescued the synthetic lethal effect in BRCA1-deficient cells indicating that PARP1 catalytic activity regulates NHEJ activity, thus preventing NHEJ components from binding to sites of DNA damage [105]. Yet another study reported that loss of PARP1 activity itself can inhibit HR by suppressing the expression of BRCA1 and RAD51, two key factors in HR [106]. Moreover, PARP1 is activated at stalled forks to mediate Mre11-dependent replication restart by HR [107]. Another level of complexity is added by recent work demonstrating that SIRT6, a mammalian homolog of the yeast Sir2 deacetylase which functions as a longevity regulator in yeast, is recruited to sites of DSBs. There, SIRT6 appears to stimulate DSB repair via NHEJ and HR. Of note, SIRT6 interacts with PARP1 and stimulates its activity thereby enhancing DSB repair upon oxidative stress [108]. It is important to note that the results mentioned above are not necessarily mutually exclusive, but more work is necessary to define the exact molecular mechanisms by which PARP1 participates in DSB repair and which role this may have during aging.

3.3. PARP1, Telomere Maintenance, and the WRN Protein. Telomeres are repetitive sequences at the end of the chromosomes and function as a buffer to prevent loss of coding sequences during DNA replication. They are capped by a protein complex known as shelterin, which tightly regulates the telomeric structure by interaction with several DNA repair proteins and the telomere-elongating reverse transcriptase, telomerase. Deterioration of telomeres represents an important factor during human aging [109].

The role of PARP1 in the regulation of telomere length is well established. *In vivo*, a substantial loss of telomeric DNA by 30% was observed in the first generation of *Parp*$1^{-/-}$ mice [110]. Gomez et al. reported that PARP1 is dispensable for the capping of normal telomeres, but is specifically recruited to eroded telomeres, where it might help to protect chromosomes against end-to-end fusions and genomic instability [111]. Our group demonstrated in various cell culture systems that pharmacological inhibition of poly(ADP-ribosyl)ation or PARP1 knockdown via RNA interference leads to a rapid decrease in telomere length and stabilization at a lower level. Importantly, neither the length of the single-stranded telomeric overhang nor telomerase activity was affected by PARP1 inhibition. Interestingly, release from PARP inhibition led to a fast regain in telomere length in telomerase-positive cells indicating that PARP1 activity is an important determinant in telomere length regulation [112]. On a molecular level, the function of PARP1 in telomere length regulation presumably depends on its interaction with the telomeric repeat binding factor 2 (TRF2). TRF2 is a key component of the shelterin complex and is responsible for telomeric stability, length regulation, and suppression of unscheduled activity of the double-strand break repair machinery by maintaining the t-loop [113]. PARP1 interacts with and modifies TRF2, and the poly(ADP-ribosyl)ation of TRF2 affects its binding to telomeric DNA [111, 114].

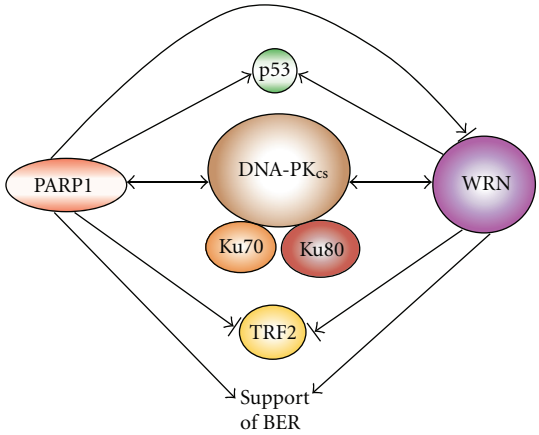


Figure 3: Interaction map between PARP1 and Werner syndrome protein (WRN). The two proteins share many overlapping interaction pathways. There is a reciprocal interaction with DNA-PK (double-headed arrow) and p53, stimulation of base excision repair (BER, one-headed arrow), and inhibition of TRF2-DNA binding (blocked arrow). PARP1 also inhibits WRN functions if in an unmodified state (reproduced from [113]).

Another PARP1 interaction partner that is involved in telomere regulation is the RecQ helicase WRN [115]. Patients with the rare autosomal recessive disorder Werner syndrome, in which the *WRN* gene is mutated, display genomic instability and telomere shortening on the cellular and premature aging on the organismic level with symptoms resembling normal human aging in many aspects including cataracts, graying of hair and alopecia, atherosclerosis, osteoporosis, and higher cancer incidence. The premature aging phenotype of these patients appears to be at least partially dependent on telomere length, since human symptoms were only recapitulated in mice with short telomeres, that is, WRN/telomerase double-knockout mice [115, 116]. (*N.B.* Mice usually exhibit considerably longer telomeres (~40 kb) than humans (5–15 kb)). On a cellular level, fibroblasts derived from WS patients display genomic instability and a reduced replicative lifespan. This phenotype is in accordance with experimental data demonstrating that WRN is involved in multiple aspects of DNA metabolism, such as DNA replication, genomic maintenance, and telomere regulation [115]. WRN functions as a 3′-5′ helicase and additionally as a 3′-5′ exonuclease. Proper enzymatic activity of WRN seems to be crucial for maintaining genomic integrity, since pharmacological inhibition of WRN's helicase activity causes DSBs and apoptosis [117]. WRN and PARP1 directly interact with each other physically and PARP1 modulates WRN's exonuclease and helicase activities [118, 119]. Upon automodification of PARP1, the inhibition of WRN's exonuclease and helicase activities is released suggesting that PARP1 regulates the timing of WRN activity towards its substrates [113]. The regulation of PARP1 and WRN appears to be reciprocal, because poly(ADP-ribosyl)ation is impaired in WRN deficient cells indicating that WRN is required to regulate PARP1-dependent poly(ADP-ribosyl)ation [120]. Moreover, other factors than PARP1 and WRN are involved in these mechanisms, because WRN and PARP1 share many interaction partners, including DNA-PK, P53, and TRF2 (Figure 3). For example, PARP1, WRN, and DNA-PK (including Ku70/80 and DNA-PK$_{cs}$) can form a complex, in which PAR-modified Ku70/80 inhibits WRN [121]. Furthermore, both PARP1 and WRN have positive impact on telomere length, presumably by regulating the binding of TRF2 to the t-loop. Genetic cooperation between PARP1 and WRN was demonstrated *in vivo*, because mice with deficiencies in both proteins display higher rates of chromatid breaks, chromosomal rearrangements, and cancer than each of the single-mutant mice [122]. Moreover, double mutants appear to have reduced median and maximum lifespan, despite the fact that these mice were on a telomerase-positive genetic background and telomere lengths of single-mutant MEFs did not differ significantly from the double-mutant MEFs. This finding suggests that telomere-independent functions of WRN and PARP1 exist in the mouse to maintain organismic longevity. (*N.B.* In contrast, wild-type MEFs showed 30–40% longer telomeres). In conclusion, since PARP1 and WRN share many interaction partners and both proteins participate in other DNA repair pathways such as BER and NHEJ, they probably synergistically collaborate to maintain overall genomic stability and ensure longevity.

3.4. PARP1 during DNA Replication, Mitosis, and Cell Cycle Control. The WRN helicase also participates in the response to replicative stress, a cellular stressor that was linked to mammalian aging due to its ability to drive cells, including stem cells, into senescence and apoptosis [123, 124]. Replication forks contain several proteins such as helicases and polymerases, forming the so-called replisome. Usually, progression of the replication fork continues until it encounters a replication fork barrier such as DNA-protein complexes or SSBs. In this case, the replicative helicase progresses much more slowly, so that the fork is "stalled." If this goes along with the disassembly of the replisome the fork "collapses" and a DSBs is formed [125]. WRN and PARP1 are involved in the reactivation of stalled replication forks. Specifically, PARP1 binds to and is activated at stalled replication forks and mediates the recruitment of Mre11, a key component of the MRN complex. Mre11 may collaborate with WRN helicase to resect DNA ends for RAD51 loading and subsequent HR repair to promote replication fork restart after release from replication blocks [44, 107, 125, 126]. In accordance with these data, a recent study demonstrated that PARP activity is required for effective replication fork restart upon treatment of cells with sublethal dosis of the replication stress-inducing topoisomerase 1 inhibitor camptothecin [127].

After DNA replication is completed, proper mitotic regulation is crucial to ensure genomic integrity [128]. During mitosis, the spindle pole formation requires the centrosome, whereas the centromere is the chromosomal region that organizes the kinetochore, thus enabling the attachment of the mitotic spindle microtubules. First, evidence for a role of poly(ADP-ribosyl)ation in spindle regulation was obtained from a study with *Xenopus laevis* egg extracts showing that PAR is a component of the mitotic spindle and is required for

its assembly and function, although this was attributed to the enzymatic activity of another PARP family member, that is, tankyrase-1 [129, 130]. With regards to PARP1, it was shown that haploinsufficiency for PARP1 is related to centrosome duplication and chromosomal instability [131]. Consistent with this, PARP1 localizes to the centrosome [132, 133]. Moreover, PARP1 and PARP2 are present at centromeres and interact with the constitutive centromere proteins Cenpa, Cenpb and the spindle check point protein Bub3 [134, 135]. The physical and functional relationship of PARP1 to the centrosome and the centromere links DNA damage surveillance to the mitotic spindle checkpoint. The notion that mitotic spindle checkpoint proteins play an important role to ensure mammalian longevity is supported by studies demonstrating that mice with low levels of the mitotic checkpoint protein BubR1 and mice haploinsufficient for Bub3 and Rae1—another mitotic checkpoint gene—age prematurely (*N.B.* A complete knockout of these genes results in embryonic lethality in the mouse) [136, 137].

Because severe DNA damage or mitotic misregulation can cause genomic instability leading to tumor formation, a complex cellular security network has evolved to counteract carcinogenesis. This signaling network can stop the cell cycle at different stages, thereby either inducing DNA repair, or eradicating or neutralizing heavily damaged cells by apoptosis or senescence, respectively. To this end, apoptosis and senescence are powerful tumor-suppressive mechanisms, but on the other hand, both pathways can lead to depletion of the regenerative cell pool, thus promoting tissue degeneration and organ failure, which are hallmarks of aging [138]. One of the most important regulators of cell cycle progression and induction of senescence/apoptosis is the transcription factor P53. Consequently, mouse studies demonstrated that P53 deficiency leads to premature death due to tumor development, whereas constantly active P53 protects against cancer at the cost of a premature aging phenotype [138]. Consistent with the role of PARP1 and P53 as caretakers and guardians of the genome, respectively, PARP1 and P53 synergistically cooperate *in vivo* in telomere and chromosomal maintenance as well as in tumor suppression [139–143]. Many functional interactions between PARP1 and P53 during DNA damage response and apoptosis exist, such as delayed P53 transactivation potential in PARP1-deficient cells [144–147]. In addition to its function as a positive regulator of gene expression, P53 also acts as a gene-specific transcriptional transrepressor. Interestingly, P53-mediated transrepression of the *MTA1* gene (MTA1, metastasis associated protein 1), a component of a nucleosome remodeling complex which is associated with very aggressive tumor phenotypes, depends on functional poly(ADP-ribosyl)ation of P53 [148]. On the other hand, poly(ADP-ribosyl)ation of P53 is also able to inhibit its binding to its transcriptional consensus sequence, indicating that multifaceted regulatory mechanisms exist between PARP1 and P53 [149, 150]. Kanai et al. suggested a mechanism of PARP1-dependent regulation of P53 activity. According to this study, poly(ADP-ribosyl)ation induces structural changes in P53 that mask its nuclear export sequence, resulting in an accumulation of P53 in the nucleus, where it exerts its transactivational functions. Accordingly, a P53 mutant in which acceptor sites were mutated was localized to the cytoplasm to a greater extent than wildtype P53 [151].

In conclusion, there is ample evidence that PARP1 modulates P53 stability, intracellular localization, and transcriptional activity with likely implications in the induction of apoptosis and senescence on a cellular and therefore aging and longevity on an organismic level. However, studying the combined role of PARP1 and P53 in the aging process is complicated by the situation that mouse models with deficiencies in both tumor-suppressor genes show cancer-dependent premature death unrelated to other signs of premature aging. The development of sophisticated conditional mouse models with spatiotemporal-controlled expression of PARP1 and P53 may represent an approach to overcome these hurdles.

3.5. PARP1 as a Longevity Assurance Factor. As discussed in the preceding sections, PARP1 acts as a general caretaker of genomic stability and is associated with various factors, whose involvement in mechanisms of aging and longevity are well established. This indicates a potential role of PARP1 as a longevity assurance factor which is supported by *in vivo* studies as briefly discussed in the following.

There is a large body of evidence showing a positive correlation of poly(ADP-ribosyl)ation capacity and mammalian longevity. Previously, we demonstrated that poly(ADP-ribosyl)ation capacity in peripheral blood mononuclear cells (PBMCs) of 13 mammalian species strongly correlates with their maximum lifespan, for example, maximum poly(ADP-ribosyl)ation levels were five times higher in humans than in rodents [152]. Interestingly, these differences in poly(ADP-ribosyl)ation are not associated with different enzyme levels, but are rather influenced by an higher poly(ADP-ribosyl)ation capacity of the human PARP1 enzyme in comparison to its mouse ortholgue [153]. Moreover, poly(ADP-ribosyl)ation capacity in PBMCs declines with age in humans and rodents [152, 154]. Interestingly, humans exhibiting an exceptional long lifespan, that is, centenarians, display a significantly higher poly(ADP-ribosyl)ation capacity than the average population [155], which is comparable to those of young subjects [156].

Apart from these correlative studies, studies with *Parp*1$^{-/-}$ mice indicate a role of PARP1 as a longevity assurance factor. Thus, *Parp*1$^{-/-}$ mice and cells derived thereof are hypersensitive to DNA-damaging agents and *Parp*1$^{-/-}$ cells display increased spontaneous genomic instability as measured by the frequency of sister chromatid exchanges, chromosome aberrations, and micronuclei formation, which confirmed that PARP1 functions as a general caretaker of the genome [157]. In support of the view that PARP1 counteracts the aging process is the finding that *Parp*1$^{-/-}$ mice age is moderately faster compared to wild-type control animals [158]. Moreover, this and various other studies supported the notion that PARP1 acts as a tumor-suppressor gene, since PARP1 deficiency enhances carcinogenesis during aging and upon induction by DNA damaging agents [143, 158–161]. Consistently, data from human studies showed that a hypomorphic PARP1 polymorphism (V762A) serves

as a risk factor in the development of some types of human cancers [162–166].

In conclusion, PARP1 participates in various genome maintenance mechanisms, such as chromatin remodeling, DNA repair, reactivation of stalled replication forks, telomere maintenance, and cell cycle control. Consistent with its role as a general caretaker of the genome, there is strong evidence from *in vivo* studies indicating that PARP1 indeed functions as a longevity assurance factor. On the other hand, the interaction of PARP1 with key regulators of immune function, such as NF-κB, and its potential to induce cell death may contribute to aging-promoting mechanisms as discussed in the following sections.

4. PARP1 in Inflammation and Cell Death

First, evidence that PARP1 contributes to inflammation and the development of related pathologies was revealed by genetic studies in *Parp*1$^{-/-}$ mice, because these animals are protected from several inflammation and cell death-associated pathologies such as ischemic infarction, collagen-induced arthritis, and LPS-induced septic shock [157, 167]. Moreover, *Parp*1$^{-/-}$ animals are resistant to MPTP-induced Parkinson's disease and streptozotocin-induced diabetes mellitus [168–171]. The molecular and cellular mechanisms underlying these phenotypic results and their possible implications in mechanisms of aging and longevity are discussed below.

4.1. PARP1, NF-κB, and Inflammation. Various studies demonstrated that PARP1 participates in the regulation of transcriptional processes, either via general chromatin remodeling or through specific interaction and regulation of a wide range of transcription factors [171, 172].

Maybe the best studied interaction is that of PARP1 with NF-κB. The transcription factor NF-κB is considered a master regulator in controlling gene expression upon proinflammatory stimuli. NF-κB is composed of dimeric combinations of Rel family members with the major subunits p65 and p50. In nonstimulated cells, NF-κB is located in the cytoplasm via the binding to the inhibitory IκB proteins. Upon proinflammatory stimuli, IκB proteins are phosphorylated by IκB kinases (IKKs), which cause their degradation by the ubiquitin/proteasome system. Subsequently, NF-κB is translocated to the nucleus, where it can activate the transcription of a number of genes, especially inflammatory genes [173]. Apart from the regulation of NF-κB by its subcellular localization, its action is tightly regulated within the nucleus by posttranslational modifications and interaction with transcriptional cofactors. Importantly, NF-κB-dependent gene expression is associated with aging in the mouse as well as in humans [174]. Recently, it was shown that hyperactive NF-κB signaling contributes to premature aging in the mouse [175], and blocking of NF-κB in aged mice was sufficient to reverse some features of skin aging [174, 176]. In accordance with these studies, pharmacological inhibition of NF-κB prolongs lifespan of *Drosophila melanogaster* by ~15% [177]. Cellular studies showed that NF-κB-dependent gene transcription can be induced by genotoxic stress and gene transcription studies in conditionally immortalized human fibroblast suggested that NF-κB signaling plays a causal role in the development of senescence [178]. In addition, NF-κB signaling was implicated in maintaining cellular senescence, because NF-κB-deficient fibroblasts escape senescence earlier and immortalize at a faster rate [179]. On the other hand, NF-κB-dependent gene transcription can be induced by genotoxic stress and is required for the transcription of many SASP factors [5]. In summary, there is substantial evidence that NF-κB plays a crucial role in aging and age-related diseases [180].

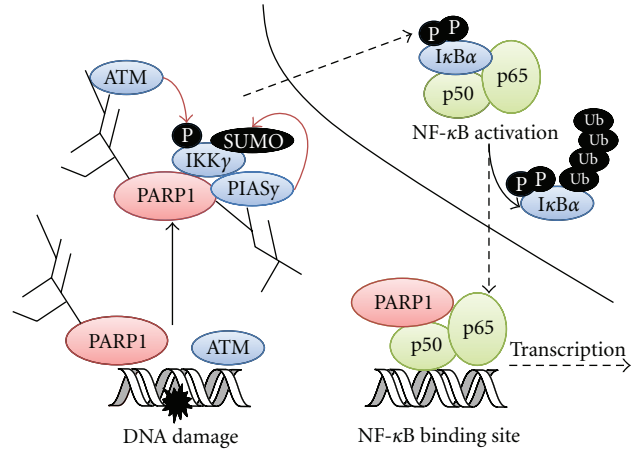


Figure 4: Simplified model of PARP1-dependent mechanisms of NF-κB activation. For details see text. Scheme based on [167, 185].

The expression and activation patterns of PARP1 and NF-κB are remarkably similar in various tissues. A direct role of PARP1 in NF-κB-mediated transcription was emphasized by the finding that expression of NF-κB-dependent proinflammatory mediators, such as TNFα, IL6, or iNOS, is impaired in *Parp*1$^{-/-}$ mice [167, 181]. PARP1 physically interacts with both major subunits of NF-κB, that is, p65 and p50, and is required for NF-κB-dependent gene transcription (Figure 4) [182]. Moreover, PARP1 is acetylated by the histone acetylase p300/CBP upon inflammatory stimuli, leading to a stronger association with NF-κB [39]. Subsequent expression of proinflammatory mediators such as iNOS leads to the production of highly reactive chemical species that, in turn, cause extensive DNA damage in the target cell, potentially supporting a positive feedback mechanism. Importantly, in this study neither the DNA binding nor the enzymatic activity of PARP1 was necessary for direct transcriptional activation of NF-κB [183]. On the other hand, inhibition of PARP's enzymatic activity is sufficient to decrease the expression of iNOS, IL6, and TNFα in cultured cells and to reduce the expression of inflammatory mediators in mice [184]. This is consistent with a recent study demonstrating that the PARP1-dependent activation of NF-κB occurs at two levels (Figure 4). Thus, in addition to the nuclear coactivator function of PARP1 on NF-κB activity, this study identified PARP1 as a trigger for the translocation of NF-κB from the cytoplasm into the nucleus upon genotoxic stress (Figure 4) [185]. According to this

model, PARP1 is recruited to DNA strand breaks and is automodified with PAR. Upon dissociation into the nucleoplasm, PARP1 then rapidly forms a signalosome composed of the SUMO1 ligase PIASy, IKKγ (NEMO), and ATM. The signalosome is stabilized by a network of direct protein-protein interactions as well as by PAR binding of PIASy and ATM through PAR binding motifs. PAR degradation by PARG causes subsequent destabilization of the signalosome, resulting in IKKγ SUMOylation, translocation to the cytoplasm, phosphorylation of IκB proteins, and NF-kB activation. This mechanism directly links the DNA-damage-signaling functions of PARP1 to its role in inflammation-related mechanisms. Interestingly, PARP1-NF-κB signaling seems also to contribute to the activation and maintenance of the secretory phenotype of senescent cells [186]. In consequence, the associated secretion of proinflammatory factors possibly changes the tissue microenvironment and forms a site of low-level chronic inflammation with tumor and aging-promoting properties.

4.2. PARP1 and Its Role in Cell Death. Historically, two major mechanisms of mammalian cell death are distinguished, that is, apoptosis and necrosis. Apoptosis is considered as the default pathway, where cell death occurs in a controlled manner resulting in the elimination of cells by macrophages without secondary damage of the surrounding cells. In contrast, necrosis is considered an uncontrolled process which leads to disruption of cells promoting tissue inflammation [187]. Several transition states between the two pathways exist such as apoptosis inducing factor-(AIF-) dependent cell death [188]. Cell death is an important factor contributing to organismic aging, because apoptosis can lead to depletion of the regenerative cell pool and necrosis can cause chronic inflammatory conditions. PARP1 is involved in necrosis as well as in apoptosis, depending on the cell type and the intensity of DNA damage. Excessive DNA damage, as it can be triggered by pathophysiological stimuli and during NF-κB-dependent inflammatory responses, leads to an overactivation of PARP1, which induces the depletion of cellular NAD^+ pools and subsequently of ATP pools [189]. This could affect energy-dependent cellular functions resulting in necrosis, which in turn reinforce tissue inflammation leading to a vicious cycle of PARP1 activation, necrosis, and inflammation. The role of PARP1 in apoptosis is manifold depending on the cell cycle state. Two major types of apoptosis exist: caspase-dependent and caspase-independent apoptosis. On the one hand, in proliferating cells, PARP1 contributes to classical caspase-dependent apoptosis through its regulatory activity on P53. Here, after an initial synthesis of PAR, PARP1 is cleaved by caspases 3 and 7 in a 24 kD and an 89 kD fragment [190]. This occurs potentially to inactivate PARP1 and to preserve cellular ATP pools for the apoptosis program [191–193]. On the other hand, it was shown that PARP1 contributes to caspase-independent apoptosis by releasing AIF from the mitochondria [194, 195]. PAR itself acts as a signaling molecule between nucleus and mitochondria, where it binds to AIF in a non-covalent manner and then triggers its release. AIF then translocates to the nucleus, where it causes chromatin condensation, large-scale DNA fragmentation, and finally cell death [196–198].

In conclusion, three interconnected cellular mechanisms have been proposed to be responsible for the involvement of PARP1 in cell death and inflammation-related, age-related pathologies. First, PARP1 overactivation by severe DNA damage upon an initial pathological insult can lead to NAD^+ and subsequent ATP depletion causing necrotic cell death due to energy depletion [189]. Second, such an initial pathological insult or secondary necrotic disruption of cells can trigger an inflammatory response leading to further damage of the surrounding tissue. This process can be stimulated by the action of PARP1 as an essential transcriptional coactivator of the proinflammatory transcription factor NF-κB. Products of NF-κB regulated genes, for example, iNOS, participate in the production of ROS and RNS, which could support the aforementioned vicious cycle of DNA damage, subsequent PARP1 activation, and cell death potentiating inflammation and tissue damage. Third, the PAR-dependent release of apoptosis-inducing factor (AIF) from the mitochondria resulting in caspase-independent apoptosis may contribute to some extent to PARP1-dependent pathologies in particular neurodegenerative disorders [194, 197, 198]. Over time, these mechanisms can contribute to aging and the development of age-related pathological conditions.

4.3. PARP1 as an Aging-Promoting Factor. Evidence supporting a role of PARP1 as a driving force of inflammation on an organismic level is given by the fact that *Parp*$1^{-/-}$ mice are protected from several inflammation- and cell-death-associated diseases and that *Parp*$1^{-/-}$ mice and cells display lower expression levels of a whole spectrum of proinflammatory cytokines, adhesion molecules, and enzymes [167]. Consequently, given the role of inflammation during mammalian aging, PARP1 was postulated to act as an aging-promoting factor [199]. In line with this concept, PARP inhibition or ablation of gene transcription has beneficial effects on several age-related diseases, including aging-associated cardiac and vascular dysfunctions [184, 200, 201]. Apart from its functions as a regulator of NF-κB-dependent gene transcription, PARP1 competes with the family of type III histone deacetylases of sirtuins (i.e., in humans and mice SIRT1-7) for the same substrate, that is, NAD^+. In addition, PARP1 and SIRT1 interact physically and show an antagonistic interplay on a functional level [71, 202]. Sirtuins regulate the energy homeostasis by controlling the acetylation status and activity of various enzymes and transcriptional regulators and have been identified to act as longevity factors in various species [203]. Moreover, consumption of NAD^+ links PARP1 and sirtuins to energy metabolism, which plays a fundamental role in aging mechanisms. *In vivo* studies showed that SIRT1-overexpressing mice are leaner, metabolically more active, show improved glucose tolerance, exhibit less inflammation, and are resistant to intestinal cancer development [204–208]. *Parp*$1^{-/-}$ mice exhibit increased NAD^+ content and enhanced SIRT1 activity in brown adipose and muscle tissue. Consequently, *Parp*$1^{-/-}$ mice phenocopy many aspects of SIRT1 activation, such as a

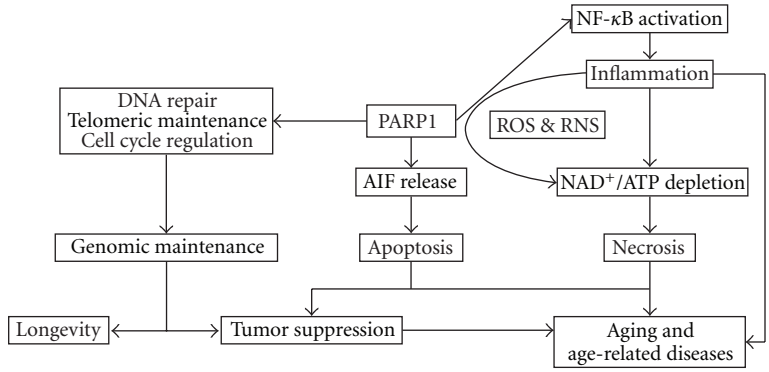


Figure 5: PARP1-related mechanisms in longevity and aging. For details see text.

higher mitochondrial content, increased energy expenditure, reduced body weight, and protection against metabolic disease [209].

Conversely, this phenotype is mirrored by the phenotype of mice with ectopic expression of hPARP1 [210]. These mice develop sporadic obesity and show impaired glucose tolerance. Furthermore, hPARP1-expressing mice exhibit impaired survival rates, which are accompanied by premature development of several inflammation and age-associated pathologies, such as nephropathy, dermatitis, pneumonitis, myocardiopathy, and hepatitis. In support of this hypothesis, *hPARP1* mice develop normocytic, normochromic anemia and show an increase in the fraction of circulating monocytes, which is suggestive of anemia of chronic inflammatory disease [211, 212]. Moreover, *hPARP1* mice show typical signs of premature aging, such as early development of kyphosis and impaired hair regrowth. In addition to a potentially altered interplay between PARP1 and sirtuins in these mice, the pathological phenotype of *hPARP1* mice might be related to an altered PARP1-NF-κB interaction leading to a continuous low-level increase in pro-inflammatory stimuli. Consistently, expression of NF-κB-dependent target genes, such as TNFα, IL1, and IL6, is dysregulated in *hPARP1* animals. This may contribute to the premature development of typical age-related chronic diseases in these mice [210].

5. Summary

Aging is a complex process which cannot be explained by a single pathway or even a set of closely related pathways. More likely, many diverse cellular functions will contribute to aging and they will do so in a highly interdependent manner [53]. As summarized here, this complexity is already represented at the level a single enzyme, that is, PARP1. PARP1 is a factor that connects DNA damage response and inflammatory mechanisms, both of which are closely associated with mammalian aging. Thus, under physiological conditions and mild genotoxic stress, PARP1 is thought to play an important role in genomic maintenance (Figure 5). On the other hand, under pathophysiological conditions, reactive chemical species are generated by activated immune cells potentially inducing DNA damage in an autocrine and paracrine fashion. If exceeding a threshold, DNA damage and the subsequent signaling can force cells into senescence. Senescent cells, in turn, possess the potential to secrete proinflammatory cytokines thereby reinforcing tissue inflammation. PARP1 is involved in these processes due to its close interplay with NF-κB at different stages during NF-κB activation. In addition, severe DNA damage can trigger overactivation of PARP1 resulting in cell death by apoptosis or necrosis (Figure 5). Debris of necrotic cells is phagocytosed by macrophages, thereby triggering a proinflammatory response, again inducing the generation of reactive chemical species. Interestingly, there is some evidence that chronic intestinal inflammation can induce systemic genotoxicity, for example, in leukocytes and hepatocytes, suggesting that local sites of inflammation can affect genomic stability and homeostasis even at an organismic level [213]. Over time, inflammation, senescence, and cell death contribute to the depletion of the regenerative cell pool and tissue dysfunction accumulating in the aging process.

There is ample evidence supporting a role of PARP1 as a longevity assurance factor on the one hand, but also as an aging-promoting factor on the other hand.The dual role of PARP1 in longevity and aging might be reflected in the moderate premature aging phenotype observed in cohorts of *Parp*$1^{-/-}$ mice [158]. Thus, it is reasonable to assume that overall aging in these mice is kept nearly in balance, due to comprised genomic integrity on the one hand, but reduced inflammatory status on the other hand. The generation of *Parp*$1^{-/-}$ mice with tissue specific reconstitution of PARP1 expression may be a suitable model to test such a hypothesis. Tissue-specific reexpression of PARP1 in cells of the innate immune system on an otherwise *Parp*$1^{-/-}$ background may lead to a more drastic accelerated aging phenotype, since PARP1-overexpressing cells of the innate immune system are expected to exhibit an enhanced inflammatory status, while cells of the remaining *Parp*$1^{-/-}$ tissues are genomically unstable. Another possibility explaining the moderate premature aging phenotype of *Parp*$1^{-/-}$ mice may be that alternative mechanisms are able to compensate for the PARP1 deficiency. Such potential backup mechanisms rely most likely on PARP2 which shares some redundancy to PARP1, as it is evident by the finding that *Parp1/Parp2* double-deficient mice are not viable. The generation of

conditional and inducible double-knockout mice may help to test this hypothesis.

Many theories of aging exist. Most of these are not mutually exclusive and although none of these is probably able to explain all characteristics of human aging, in all probability there is some truth in many of them. The "antagonistic pleitropy" theory of aging postulates the existence of pleiotropic genes and mechanisms having opposite effects on fitness at different stages of age. Thus, a gene or mechanism may be beneficial for survival in early life, when natural selection is strong, but harmful at later ages, when selection is weak or absent [214]. Mechanisms of DNA damage response as well as inflammation may support such a theory. DNA damage response, with its final end points, DNA repair, senescence, and apoptosis, is clearly beneficial at young age, as these mechanisms prevent cancer development. However, at older age, this may become detrimental, as depletion of the regenerative cell pool by senescence or apoptosis may contribute to tissue degeneration and aging. The same holds true for inflammation. At young age, inflammatory responses most likely fulfill beneficial functions, for example, acting as a first line defense against infections. (*N.B.* This is supported by the finding that some mouse models with deficiencies in NF-κB signaling are hypersensitive to infectious diseases [215]). However, at older age, continuous pressure on the immune system caused by repeated antigen stimulation leads to remodeling of the immune system with pro-inflammatory properties reinforcing the aging process and the development of age-related disease. Because PARP1 fulfills key roles in mechanisms of DNA damage response and inflammation, it is conceivable that functions of this gene act in some aspects in an antagonistic pleiotropic way, with beneficial functions in the youth and detrimental functions at old age.

In conclusion, PARP1 and the synthesis of poly(ADP-ribose) are emerging as central factors in general cellular stress response with functions in a plethora of molecular mechanisms, such as chromatin remodeling, transcription, DNA damage signaling, DNA repair, cell cycle regulation, cell death, and inflammation. As reviewed here, there is ample evidence that PARP1 fulfills numerous direct as well as indirect roles in mechanisms of aging and longevity which renders it an interesting factor to study in order to better define mechanisms of the aging process.

Acknowledgements

Our experimental work was supported by the DFG-funded Collaborative Research Center (CRC) 969, the Konstanz Research School Chemical Biology (KoRS-CB) and the International Research Training Group (IRTG) 1331. Funding for open access publishing was provided by University of Konstanz.

References

[1] B. R. Troen, "The biology of aging," *Mount Sinai Journal of Medicine*, vol. 70, no. 1, pp. 3–22, 2003.

[2] G. A. Garinis, G. T. J. van der Horst, J. Vijg, and J. H. J. Hoeijmakers, "DNA damage and ageing: new-age ideas for an age-old problem," *Nature Cell Biology*, vol. 10, no. 11, pp. 1241–1247, 2008.

[3] R. Medzhitov, "Inflammation 2010: new adventures of an old flame," *Cell*, vol. 140, no. 6, pp. 771–776, 2010.

[4] D. de Gonzalo-Calvo, K. Neitzert, M. Fernández et al., "Differential inflammatory responses in aging and disease: TNF-α and IL-6 as possible biomarkers," *Free Radical Biology and Medicine*, vol. 49, no. 5, pp. 733–737, 2010.

[5] A. Freund, A. V. Orjalo, P. Y. Desprez, and J. Campisi, "Inflammatory networks during cellular senescence: causes and consequences," *Trends in Molecular Medicine*, vol. 16, no. 5, pp. 238–246, 2010.

[6] M. De Martinis, C. Franceschi, D. Monti, and L. Ginaldi, "Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity," *FEBS Letters*, vol. 579, no. 10, pp. 2035–2039, 2005.

[7] J. Wang, H. Geiger, and K. L. Rudolph, "Immunoaging induced by hematopoietic stem cell aging," *Current Opinion in Immunology*, vol. 23, no. 4, pp. 532–536, 2011.

[8] A. Salminen, J. Huuskonen, J. Ojala, A. Kauppinen, K. Kaarniranta, and T. Suuronen, "Activation of innate immunity system during aging: NF-κB signaling is the molecular culprit of inflamm-aging," *Ageing Research Reviews*, vol. 7, no. 2, pp. 83–105, 2008.

[9] D. Sarkar and P. B. Fisher, "Molecular mechanisms of aging-associated inflammation," *Cancer Letters*, vol. 236, no. 1, pp. 13–23, 2006.

[10] D. Harman, "Free radical theory of aging: an update-increasing the functional life span," *Annals of the New York Academy of Sciences*, vol. 1067, no. 1, pp. 10–21, 2006.

[11] P. Lonkar and P. C. Dedon, "Reactive species and DNA damage in chronic inflammation: reconciling chemical mechanisms and biological fates," *International Journal of Cancer*, vol. 128, no. 9, pp. 1999–2009, 2011.

[12] A. Mangerich, C. G. Knuston, N. M. Parry et al., "Infection-induced colitis in mice causes dynamic and tissue-specific changes in stress response and DNA damage leading to colon cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 27, pp. E1820–E1829, 2012.

[13] F. Rodier, J. P. Coppé, C. K. Patil et al., "Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion," *Nature Cell Biology*, vol. 11, no. 8, pp. 973–979, 2009.

[14] S. M. Chambers, C. A. Shaw, C. Gatza, C. J. Fisk, L. A. Donehower, and M. A. Goodell, "Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation.," *PLoS biology*, vol. 5, no. 8, p. e201, 2007.

[15] G. Lepperdinger, "Inflammation and mesenchymal stem cell aging," *Current Opinion in Immunology*, vol. 23, no. 4, pp. 518–524, 2011.

[16] M. O. Hottiger, P. O. Hassa, B. Lüscher, H. Schüler, and F. Koch-Nolte, "Toward a unified nomenclature for mammalian ADP-ribosyltransferases," *Trends in Biochemical Sciences*, vol. 35, no. 4, pp. 208–219, 2010.

[17] J. M. Pleschke, H. E. Kleczkowska, M. Strohm, and F. R. Althaus, "Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins," *Journal of Biological Chemistry*, vol. 275, no. 52, pp. 40974–40980, 2000.

[18] J. P. Gagné, M. Isabelle, K. S. Lo et al., "Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes," *Nucleic Acids Research*, vol. 36, no. 22, pp. 6959–6976, 2008.

[19] I. Ahel, D. Ahel, T. Matsusaka et al., "Poly(ADP-ribose)-binding zinc finger motifs in DNA repair/checkpoint proteins," *Nature*, vol. 451, no. 7174, pp. 81–85, 2008.

[20] M. Altmeyer, S. Messner, P. O. Hassa, M. Fey, and M. O. Hottiger, "Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites," *Nucleic Acids Research*, vol. 37, no. 11, pp. 3723–3738, 2009.

[21] A. J. Gottschalk, G. Timinszky, S. E. Kong et al., "Poly(ADP-ribosyl)ation directs recruitment and activation of an ATP-dependent chromatin remodeler," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 33, pp. 13770–13774, 2009.

[22] G. Timinszky, S. Till, P. O. Hassa et al., "A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation," *Nature Structural and Molecular Biology*, vol. 16, no. 9, pp. 923–929, 2009.

[23] A. Hakmé, H. K. Wong, F. Dantzer, and V. Schreiber, "The expanding field of poly(ADP-ribosyl)ation reactions. 'Protein Modifications: beyond the Usual Suspects' Review Series," *EMBO Reports*, vol. 9, no. 11, pp. 1094–1100, 2008.

[24] M. L. Meyer-Ficca, R. G. Meyer, D. L. Coyle, E. L. Jacobson, and M. K. Jacobson, "Human poly(ADP-ribose) glycohydrolase is expressed in alternative splice variants yielding isoforms that localize to different cell compartments," *Experimental Cell Research*, vol. 297, no. 2, pp. 521–532, 2004.

[25] M. Niere, S. Kernstock, F. Koch-Nolte, and M. Ziegler, "Functional localization of two poly(ADP-ribose)-degrading enzymes to the mitochondrial matrix," *Molecular and Cellular Biology*, vol. 28, no. 2, pp. 814–824, 2008.

[26] W. K. Min, U. Cortes, Z. Herceg, W. M. Tong, and Z. Q. Wang, "Deletion of the nuclear isoform of poly(ADP-ribose) glycohydrolase (PARG) reveals its function in DNA repair, genomic stability and tumorigenesis," *Carcinogenesis*, vol. 31, no. 12, pp. 2058–2065, 2010.

[27] S. Oka, J. Kato, and J. Moss, "Identification and characterization of a mammalian 39-kDa poly(ADP-ribose) glycohydrolase," *Journal of Biological Chemistry*, vol. 281, no. 2, pp. 705–713, 2006.

[28] M. Niere, M. Mashimo, L. Agledal et al., "ADP-ribosylhydrolase 3 (ARH3), not poly-ADP-ribose glycohydrolase (PARG) isoforms, are responsible for degradation of mitochondrial matrix-associated poly-ADP-ribose," *Journal of Biological Chemistry*, vol. 287, no. 20, pp. 16088–16102, 2012.

[29] M. Rouleau, A. Patel, M. J. Hendzel, S. H. Kaufmann, and G. G. Poirier, "PARP inhibition: PARP1 and beyond," *Nature Reviews Cancer*, vol. 10, no. 4, pp. 293–301, 2010.

[30] E. Pion, G. M. Ullmann, J. C. Amé, D. Gérard, G. De Murcia, and E. Bombarda, "DNA-induced dimerization of poly(ADP-ribose) polymerase-1 triggers its activation," *Biochemistry*, vol. 44, no. 44, pp. 14670–14681, 2005.

[31] H. Mendoza-Alvarez and R. Alvarez-Gonzalez, "Poly(ADP-ribose) polymerase is a catalytic dimer and the automodification reaction is intermolecular," *Journal of Biological Chemistry*, vol. 268, no. 30, pp. 22575–22580, 1993.

[32] M. F. Langelier, J. L. Planck, S. Roy, and J. M. Pascal, "Structural basis for DNA damage-dependent poly(ADP-ribosyl)ation by human PARP-1," *Science*, vol. 336, no. 6082, pp. 728–732, 2012.

[33] A. A. Ali, R. Arribas-Bosacoma, M. Kozlowski et al., "The zinc-finger domains of PARP1 cooperate to recognize DNA strand breaks," *Nature Structural & Molecular Biology*, vol. 19, no. 7, pp. 685–692, 2012.

[34] W. M. Shieh, J. C. Amé, M. V. Wilson et al., "Poly(ADP-ribose) polymerase null mouse cells synthesize ADP-ribose polymers," *Journal of Biological Chemistry*, vol. 273, no. 46, pp. 30069–30072, 1998.

[35] J. C. Amé, V. Rolli, V. Schreiber et al., "PARP-2, a novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase," *Journal of Biological Chemistry*, vol. 274, no. 25, pp. 17860–17868, 1999.

[36] S. Messner, D. Schuermann, M. Altmeyer et al., "Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function," *FASEB Journal*, vol. 23, no. 11, pp. 3978–3989, 2009.

[37] J. W. Walker, H. B. Jijon, and K. L. Madsen, "AMP-activated protein kinase is a positive regulator of poly(ADP-ribose) polymerase," *Biochemical and Biophysical Research Communications*, vol. 342, no. 1, pp. 336–341, 2006.

[38] T. M. Kauppinen, W. Y. Chan, S. W. Suh, A. K. Wiggins, E. J. Huang, and R. A. Swanson, "Direct phosphorylation and regulation of poly(ADP-ribose) polymerase-1 by extracellular signal-regulated 1/2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 18, pp. 7136–7141, 2006.

[39] P. O. Hassa, S. S. Haenni, C. Buerki et al., "Acetylation of poly(ADP-ribose) polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-κB-dependent transcription," *Journal of Biological Chemistry*, vol. 280, no. 49, pp. 40450–40464, 2005.

[40] M. Cohen-Armon, L. Visochek, D. Rozensal et al., "DNA-independent PARP-1 activation by phosphorylated ERK2 increases Elk1 activity: a link to histone acetylation," *Molecular Cell*, vol. 25, no. 2, pp. 297–308, 2007.

[41] R. Midorikawa, Y. Takei, and N. Hirokawa, "KIF4 motor regulates activity-dependent neuronal survival by suppressing PARP-1 enzymatic activity," *Cell*, vol. 125, no. 2, pp. 371–383, 2006.

[42] F. Berger, C. Lau, and M. Ziegler, "Regulation of poly(ADP-ribose) polymerase 1 activity by the phosphorylation state of the nuclear NAD biosynthetic enzyme NMN adenylyl transferase 1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 10, pp. 3765–3770, 2007.

[43] T. Guastafierro, B. Cecchinelli, M. Zampieri et al., "CCCTC-binding factor activates PARP-1 affecting DNA methylation machinery," *Journal of Biological Chemistry*, vol. 283, no. 32, pp. 21873–21880, 2008.

[44] J. F. Haince, D. McDonald, A. Rodrigue et al., "PARP1-dependent kinetics of recruitment of MRE11 and NBS1 proteins to multiple DNA damage sites," *Journal of Biological Chemistry*, vol. 283, no. 2, pp. 1197–1208, 2008.

[45] O. Mortusewicz, J. C. Amé, V. Schreiber, and H. Leonhardt, "Feedback-regulated poly(ADP-ribosyl)ation by PARP-1 is required for rapid response to DNA damage in living cells," *Nucleic Acids Research*, vol. 35, no. 22, pp. 7665–7675, 2007.

[46] J. H. Küpper, M. Muller, M. K. Jacobson et al., "trans-Dominant inhibition of poly(ADP-ribosyl)ation sensitizes cells against γ-irradiation and N-methyl-N'-nitro-N-nitrosoguanidine but does not limit DNA replication of a polyomavirus replicon," *Molecular and Cellular Biology*, vol. 15, no. 6, pp. 3154–3163, 1995.

[47] M. Masutani, T. Nozaki, E. Nishiyama et al., "Function of poly(ADP-ribose) polymerase in response to DNA damage: gene-disruption study in mice," *Molecular and Cellular Biochemistry*, vol. 193, no. 1-2, pp. 149–152, 1999.

[48] C. Trucco, F. J. Oliver, G. De Murcia, and J. Ménissier-De Murcia, "DNA repair defect in poly(ADP-ribose) polymerase-deficient cell lines," *Nucleic Acids Research*, vol. 26, no. 11, pp. 2644–2649, 1998.

[49] J. M. De Murcia, C. Niedergang, C. Trucco et al., "Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 14, pp. 7303–7307, 1997.

[50] Z. Q. Wang, L. Stingl, C. Morrison et al., "PARP is important for genomic stability but dispensable in apoptosis," *Genes and Development*, vol. 11, no. 18, pp. 2347–2358, 1997.

[51] R. Meyer, M. Muller, S. Beneke, J.-H. Küpper, and A. Bürkle, "Negative regulation of alkylation-induced sister-chromatid exchange by poly(ADP-ribose) polymerase-1 activity," *International Journal of Cancer*, vol. 88, no. 3, pp. 351–355, 2000.

[52] K. Weidele, A. Kunzmann, M. Schmitz, S. Beneke, and A. Bürkle, "Ex vivo supplementation with nicotinic acid enhances cellular poly(ADP-ribosyl)ation and improves cell viability in human peripheral blood mononuclear cells," *Biochemical Pharmacology*, vol. 80, no. 7, pp. 1103–1112, 2010.

[53] G. Pegoraro and T. Misteli, "The central role of chromatin maintenance in aging," *Aging*, vol. 1, no. 12, pp. 1017–1022, 2009.

[54] M. Rouleau, R. A. Aubin, and G. G. Poirier, "Pol (ADP-ribosyl)ated chromatin domains: access granted," *Journal of Cell Science*, vol. 117, no. 6, pp. 815–825, 2004.

[55] D. Quénet, V. Gasser, L. Fouillen et al., "The histone subcode: poly(ADP-ribose) polymerase-1 (Parp-1) and Parp-2 control cell differentiation by regulating the transcriptional intermediary factor TIF1β and the heterochromatin protein HP1α," *FASEB Journal*, vol. 22, no. 11, pp. 3853–3865, 2008.

[56] M. J. Gamble and R. P. Fisher, "SET and PARP1 remove DEK from chromatin to permit access by the transcription machinery," *Nature Structural and Molecular Biology*, vol. 14, no. 6, pp. 548–555, 2007.

[57] D. Ditsworth, W. X. Zong, and C. B. Thompson, "Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus," *Journal of Biological Chemistry*, vol. 282, no. 24, pp. 17845–17854, 2007.

[58] S. Messner, M. Altmeyer, H. Zhao et al., "PARP1 ADP-ribosylates lysine residues of the core histone tails," *Nucleic Acids Research*, vol. 38, no. 19, Article ID gkq463, pp. 6350–6362, 2010.

[59] F. Kappes, J. Fahrer, M. S. Khodadoust et al., "DEK is a poly(ADP-ribose) acceptor in apoptosis and mediates resistance to genotoxic stress," *Molecular and Cellular Biology*, vol. 28, no. 10, pp. 3245–3257, 2008.

[60] G. G. Poirier, G. De Murcia, and J. Jongstra-Bilen, "Poly (ADP-ribosyl)ation of polynucleosomes causes relaxation of chromatin structure," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 79, no. 11, pp. 3423–3427, 1982.

[61] G. de Murcia, A. Huletsky, and D. Lamarres, "Modulation of chromatin superstructure induced by poly(ADP-ribose) synthesis and degradation," *Journal of Biological Chemistry*, vol. 261, no. 15, pp. 7011–7017, 1986.

[62] F. R. Althaus, "Poly ADP-ribosylation: a histone shuttle mechanism in DNA excision repair," *Journal of Cell Science*, vol. 102, no. 4, pp. 663–670, 1992.

[63] C. A. Realini and F. R. Althaus, "Histone shuttling by poly(ADP-ribosylation)," *Journal of Biological Chemistry*, vol. 267, no. 26, pp. 18858–18865, 1992.

[64] M. Y. Kim, S. Mauro, N. Gévry, J. T. Lis, and W. L. Kraus, "NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1," *Cell*, vol. 119, no. 6, pp. 803–814, 2004.

[65] R. Krishnakumar, M. J. Gamble, K. M. Frizzell, J. G. Berrocal, M. Kininis, and W. L. Kraus, "Reciprocal binding of PARP-1 and histone H1 at promoters specifies transcriptional outcomes," *Science*, vol. 319, no. 5864, pp. 819–821, 2008.

[66] G. M. Kavanaugh, T. M. Wise-Draper, R. J. Morreale et al., "The human DEK oncogene regulates DNA damage response signaling and repair," *Nucleic Acids Research*, vol. 39, no. 17, pp. 7465–7476, 2011.

[67] J. Fahrer, O. Popp, M. Malanga et al., "High-affinity interaction of poly(ADP-ribose) and the human DEK oncoprotein depends upon chain length," *Biochemistry*, vol. 49, no. 33, pp. 7119–7130, 2010.

[68] B. G. Ju, V. V. Lunyak, V. Perissi et al., "A topoisomerase IIβ-mediated dsDNA break required for regulated transcription," *Science*, vol. 312, no. 5781, pp. 1798–1802, 2006.

[69] M. Zerfaoui, K. Davis, S. Banerjee, A. Friggeri, C. Bell, and E. Abraham, "Poly(ADP-ribosyl)ation of high mobility group box 1 (HMGB1) protein enhances inhibition of efferocytosis," *Molecular Medicine*, vol. 18, no. 1, pp. 359–369, 2012.

[70] D. Ahel, Z. Hořejší, N. Wiechens et al., "Poly(ADP-ribose)-dependent regulation of DNA repair by the chromatin remodeling enzyme ALC1," *Science*, vol. 325, no. 5945, pp. 1240–1243, 2009.

[71] S. B. Rajamohan, V. B. Pillai, M. Gupta et al., "SIRT1 promotes cell survival under stress by deacetylation-dependent deactivation of poly(ADP-ribose) polymerase 1," *Molecular and Cellular Biology*, vol. 29, no. 15, pp. 4116–4129, 2009.

[72] D. M. Chou, B. Adamson, N. E. Dephoure et al., "A chromatin localization screen reveals poly (ADP ribose)-regulated recruitment of the repressive polycomb and NuRD complexes to sites of DNA damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 43, pp. 18475–18480, 2010.

[73] G. Pegoraro, N. Kubben, U. Wickert, H. Göhler, K. Hoffmann, and T. Misteli, "Ageing-related chromatin defects through loss of the NURD complex," *Nature Cell Biology*, vol. 11, no. 10, pp. 1261–1267, 2009.

[74] A. Tulin and A. Spradling, "Chromatin loosening by poly (ADP)-ribose polymerase (PARP) at Drosophila puff loci," *Science*, vol. 299, no. 5606, pp. 560–562, 2003.

[75] C. M. Simbulan-Rosenthal, D. H. Ly, D. S. Rosentha et al., "Misregulation of gene expression in primary fibroblasts lacking poly(ADP-ribose)polymerase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 21, pp. 11274–11279, 2000.

[76] F. Deschênes, C. Garand, and M. Lebel, "*In vivo* misregulation of genes involved in apoptosis, development and oxidative stress in mice lacking both functional Werner syndrome protein and poly(ADP-ribose) polymerase-1," *Human Molecular Genetics*, vol. 14, no. 21, pp. 3293–3308, 2005.

[77] G. Xu, M. Herzig, V. Rotrekl, and C. A. Walter, "Base excision repair, aging and health span," *Mechanisms of Ageing and Development*, vol. 129, no. 7-8, pp. 366–382, 2008.

[78] J. H. J. Hoeijmakers, "Genome maintenance mechanisms for preventing cancer," *Nature*, vol. 411, no. 6835, pp. 366–374, 2001.

[79] G. Gradwohl, J. Menissier De Murcia, M. Molinete et al., "The second zinc-finger domain of poly(ADP-ribose) polymerase determines specificity for single-stranded breaks in DNA," *Proceedings of the National Academy of Sciences of*

the United States of America, vol. 87, no. 8, pp. 2990–2994, 1990.

[80] M. Molinete, W. Vermeulen, A. Bürkle et al., "Overproduction of the poly(ADP-ribose) polymerase DNA-binding domain blocks allkylation-induced DNA repair synthesis in mammalian cells," *EMBO Journal*, vol. 12, no. 5, pp. 2109–2117, 1993.

[81] H. N. Noren, K. Kompaniez, J. Barnes, A. Lohani, and M. K. Evans, "Poly(ADP-ribose) polymerase 1 (PARP-1) binds to 8-oxoguanine-DNA glycosylase (OGG1)," *Journal of Biological Chemistry*, vol. 286, no. 52, pp. 44679–44690, 2011.

[82] M. Masson, C. Niedergang, V. Schreiber, S. Muller, J. Menissier-De Murcia, and G. De Murcia, "XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage," *Molecular and Cellular Biology*, vol. 18, no. 6, pp. 3563–3571, 1998.

[83] S. F. El-Khamisy, M. Masutani, H. Suzuki, and K. W. Caldecott, "A requirement for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative DNA damage," *Nucleic Acids Research*, vol. 31, no. 19, pp. 5526–5533, 2003.

[84] K. W. Caldecott, S. Aoufouchi, P. Johnson, and S. Shall, "XRCC1 polypeptide interacts with DNA polymerase β and possibly poly (ADP-ribose) polymerase, and DNA ligase III is a novel molecular 'nick-sensor' *in vitro*," *Nucleic Acids Research*, vol. 24, no. 22, pp. 4387–4394, 1996.

[85] J. B. Leppard, Z. Dong, Z. B. Mackey, and A. E. Tomkinson, "Physical and functional interaction between DNA ligase IIIα and poly(ADP-ribose) polymerase 1 in DNA single-strand break repair," *Molecular and Cellular Biology*, vol. 23, no. 16, pp. 5919–5927, 2003.

[86] N. F. Confer, S. R. Kumari, and R. Alvarez-Gonzalez, "Biochemical association of poly(ADP-ribose) polymerase-1 and its apoptotic peptide fragments with DNA polymerase beta.," *Chemistry & Biodiversity*, vol. 1, no. 10, pp. 1476–1486, 2004.

[87] V. Schreiber, J. C. Amé, P. Dollé et al., "Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1," *Journal of Biological Chemistry*, vol. 277, no. 25, pp. 23028–23036, 2002.

[88] J. Ménissier de Murcia, M. Ricoul, L. Tartier et al., "Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse," *EMBO Journal*, vol. 22, no. 9, pp. 2255–2263, 2003.

[89] O. Mortusewicz, J. C. Amé, V. Schreiber, and H. Leonhardt, "Feedback-regulated poly(ADP-ribosyl)ation by PARP-1 is required for rapid response to DNA damage in living cells," *Nucleic Acids Research*, vol. 35, no. 22, pp. 7665–7675, 2007.

[90] J. Fahrer, R. Kranaster, M. Altmeyer, A. Marx, and A. Bürkle, "Quantitative analysis of the binding affinity of poly(ADP-ribose) to specific binding proteins as a function of chain length," *Nucleic Acids Research*, vol. 35, no. 21, p. e143, 2007.

[91] T. Thorslund, C. Von Kobbe, J. A. Harrigan et al., "Cooperation of the Cockayne syndrome group B protein and poly(ADP-ribose) polymerase 1 in the response to oxidative stress," *Molecular and Cellular Biology*, vol. 25, no. 17, pp. 7625–7636, 2005.

[92] C. Flohr, A. Bürke, J. P. Radicella, and B. Epe, "Poly(ADP-ribosyl)ation accelerates DNA repair in a pathway dependent on Cockayne syndrome B protein," *Nucleic Acids Research*, vol. 31, no. 18, pp. 5332–5337, 2003.

[93] M. M. Ghodgaonkar, N. Zacal, S. Kassam, A. J. Rainbow, and G. M. Shah, "Depletion of poly(ADP-ribose) polymerase-1 reduces host cell reactivation of a UV-damaged adenovirus-encoded reporter gene in human dermal fibroblasts," *DNA Repair*, vol. 7, no. 4, pp. 617–632, 2008.

[94] M. Shrivastav, L. P. De Haro, and J. A. Nickoloff, "Regulation of DNA double-strand break repair pathway choice," *Cell Research*, vol. 18, no. 1, pp. 134–147, 2008.

[95] M. Wang, W. Wu, W. Wu et al., "PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways," *Nucleic Acids Research*, vol. 34, no. 21, pp. 6170–6182, 2006.

[96] R. Aguilar-Quesada, J. A. Muñoz-Gámez, D. Martín-Oliva et al., "Interaction between ATM and PARP-1 in response to DNA damage and sensitization of ATM deficient cells through PARP inhibition," *BMC Molecular Biology*, vol. 8, p. 29, 2007.

[97] J. F. Haince, S. Kozlov, V. L. Dawson et al., "Ataxia telangiectasia mutated (ATM) signaling network is modulated by a novel poly(ADP-ribose)-dependent pathway in the early response to DNA-damaging agents," *Journal of Biological Chemistry*, vol. 282, no. 22, pp. 16441–16453, 2007.

[98] T. Ruscetti, B. E. Lehnert, J. Halbrook et al., "Stimulation of the DNA-dependent protein kinase by poly(ADP-ribose) polymerase," *Journal of Biological Chemistry*, vol. 273, no. 23, pp. 14461–14467, 1998.

[99] L. Spagnolo, J. Barbeau, and N. J. Curtin, "Visualization of a DNA-PK/PARP1 complex," *Nucleic Acids Research*, vol. 40, no. 9, pp. 4168–4177, 2012.

[100] M. Audebert, B. Salles, and P. Calsou, "Involvement of poly (ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining," *Journal of Biological Chemistry*, vol. 279, no. 53, pp. 55117–55126, 2004.

[101] J. Domínguez-Bendala, M. Masutani, and J. McWhir, "Down-regulation of PARP-1, but not of Ku80 or DNA-PKcs, results in higher gene targeting efficiency," *Cell Biology International*, vol. 30, no. 4, pp. 389–393, 2006.

[102] A. S. Waldman and B. C. Waldman, "Stimulation of intrachromosomal homologous recombination in mammalian cells by an inhibitor of poly(ADP-ribosylation)," *Nucleic Acids Research*, vol. 19, no. 21, pp. 5943–5947, 1991.

[103] A. Semionov, D. Cournoyer, and T. Y. K. Chow, "1,5-Isoquinolinediol increases the frequency of gene targeting by homologous recombination in mouse fibroblasts," *Biochemistry and Cell Biology*, vol. 81, no. 1, pp. 17–24, 2003.

[104] H. E. Bryant, N. Schultz, H. D. Thomas et al., "Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase," *Nature*, vol. 434, no. 7035, pp. 913–917, 2005.

[105] A. G. Patel, J. N. Sarkaria, and S. H. Kaufmann, "Nonhomologous end joining drives poly(ADP-ribose) polymerase (PARP) inhibitor lethality in homologous recombination-deficient cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 8, pp. 3406–3411, 2011.

[106] D. C. Hegan, Y. Lu, G. C. Stacheleka, M. E. Crosbya, R. S. Bindraa, and P. M. Glazer, "Inhibition of poly(ADP-ribose) polymerase down-regulates BRCA1 and RAD51 in a pathway mediated by E2F4 and p130," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 5, pp. 2201–2206, 2010.

[107] H. E. Bryant, E. Petermann, N. Schultz et al., "PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination," *EMBO Journal*, vol. 28, no. 17, pp. 2601–2615, 2009.

[108] Z. Mao, C. Hine, X. Tian et al., "SIRT6 promotes DNA repair under stress by activating PARP1," *Science*, vol. 332, no. 6036, pp. 1443–1446, 2011.

[109] M. A. Blasco, "Telomeres and human disease: ageing, cancer and beyond," *Nature Reviews Genetics*, vol. 6, no. 8, pp. 611–622, 2005.

[110] F. D. Di Fagagna, M. P. Hande, W. M. Tong, P. M. Lansdorp, Z. Q. Wang, and S. P. Jackson, "Functions of poly (ADP-ribose) polymerase in controlling telomere length and chromosomal stability," *Nature Genetics*, vol. 23, no. 1, pp. 76–80, 1999.

[111] M. Gomez, J. Wu, V. Schreiber et al., "PARP1 is a TRF2-associated poly(ADP-ribose)polymerase and protects eroded telomeres," *Molecular Biology of the Cell*, vol. 17, no. 4, pp. 1686–1696, 2006.

[112] S. Beneke, O. Cohausz, M. Malanga, P. Boukamp, F. Althaus, and A. Bürkle, "Rapid regulation of telomere length is mediated by poly(ADP-ribose) polymerase-1," *Nucleic Acids Research*, vol. 36, no. 19, pp. 6309–6317, 2008.

[113] S. Beneke and A. Bürkle, "Survey and summary: poly(ADP-ribosyl)ation in mammalian ageing," *Nucleic Acids Research*, vol. 35, no. 22, pp. 7456–7465, 2007.

[114] M. S. O'Connor, A. Safari, D. Liu, J. Qin, and Z. Songyang, "The human Rap1 protein complex and modulation of telomere length," *Journal of Biological Chemistry*, vol. 279, no. 27, pp. 28585–28591, 2004.

[115] M. L. Rossi, A. K. Ghosh, and V. A. Bohr, "Roles of Werner syndrome protein in protection of genome integrity," *DNA Repair*, vol. 9, no. 3, pp. 331–344, 2010.

[116] S. Chang, A. S. Multani, N. G. Cabrera et al., "Essential role of limiting telomeres in the pathogenesis of Werner syndrome," *Nature Genetics*, vol. 36, no. 8, pp. 877–882, 2004.

[117] M. Aggarwal, J. A. Sommers, R. H. Shoemaker, and R. M. Brosh, "Inhibition of helicase activity by a small molecule impairs Werner syndrome helicase (WRN) function in the cellular response to DNA damage or replication stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 4, pp. 1525–1530, 2011.

[118] C. von Kobbe, J. A. Harrigan, V. Schreiber et al., "Poly(ADP-ribose) polymerase 1 regulates both the exonuclease and helicase activities of the Werner syndrome protein," *Nucleic Acids Research*, vol. 32, no. 13, pp. 4003–4014, 2004.

[119] C. Adelfalk, M. Kontou, M. Hirsch-Kauffmann, and M. Schweiger, "Physical and functional interaction of the Werner syndrome protein with poly-ADP ribosyl transferase," *FEBS Letters*, vol. 554, no. 1-2, pp. 55–58, 2003.

[120] C. Von Kobbe, J. A. Harrigan, A. May et al., "Central role for the Werner syndrome protein/poly(ADP-Ribose) polymerase 1 complex in the poly(ADP-Ribosyl)ation pathway after DNA damage," *Molecular and Cellular Biology*, vol. 23, no. 23, pp. 8601–8613, 2003.

[121] B. Li, S. Navarro, N. Kasahara, and L. Comai, "Identification and biochemical characterization of a Werner's syndrome protein complex with Ku70/80 and poly(ADP-ribose) polymerase-1," *Journal of Biological Chemistry*, vol. 279, no. 14, pp. 13659–13667, 2004.

[122] M. Lebel, J. Lavoie, I. Gaudreault, M. Bronsard, and R. Drouint, "Genetic cooperation between the Werner syndrome protein and poly(ADP-ribose) polymerase-1 in preventing chromatid breaks, complex chromosomal rearrangements, cancer in mice," *American Journal of Pathology*, vol. 162, no. 5, pp. 1559–1569, 2003.

[123] Y. Ruzankina, A. Asare, and E. J. Brown, "Replicative stress, stem cells and aging," *Mechanisms of Ageing and Development*, vol. 129, no. 7-8, pp. 460–466, 2008.

[124] W. C. Burhans and M. Weinberger, "DNA replication stress, genome instability and aging," *Nucleic Acids Research*, vol. 35, no. 22, pp. 7545–7556, 2007.

[125] R. M. Jones and E. Petermann, "Replication fork dynamics and the DNA damage response," *Biochemical Journal*, vol. 443, no. 1, pp. 13–26, 2012.

[126] Y. G. Yang, U. Cortes, S. Patnaik, M. Jasin, and Z. Q. Wang, "Ablation of PARP-1 does not interfere with the repair of DNA double-strand breaks, but compromises the reactivation of stalled replication forks," *Oncogene*, vol. 23, no. 21, pp. 3872–3882, 2004.

[127] A. Ray Chaudhuri, Y. Hashimoto, R. Herrador et al., "Topoisomerase i poisoning results in PARP-mediated replication fork reversal," *Nature Structural and Molecular Biology*, vol. 19, no. 4, pp. 417–423, 2012.

[128] J. M. Scholey, I. Brust-Mascher, and A. Mogilner, "Cell division," *Nature*, vol. 422, no. 6933, pp. 746–752, 2003.

[129] P. Chang, M. K. Jacobson, and T. J. Mitchison, "Poly(ADP-ribose) is required for spindle assembly and structure," *Nature*, vol. 432, no. 7017, pp. 645–649, 2004.

[130] P. Chang, M. Coughlin, and T. J. Mitchison, "Tankyrase-1 polymerization of poly(ADP-ribose) is required for spindle structure and function," *Nature Cell Biology*, vol. 7, no. 11, pp. 1133–1139, 2005.

[131] M. Kanai, W. M. Tong, Z. Q. Wang, and M. Miwa, "Haploinsufficiency of poly(ADP-ribose) polymerase-1-mediated poly(ADP-ribosyl)ation for centrosome duplication," *Biochemical and Biophysical Research Communications*, vol. 359, no. 3, pp. 426–430, 2007.

[132] M. Kanai, M. Uchida, S. Hanai, N. Uematsu, K. Uchida, and M. Miwa, "Poly(ADP-ribose) polymerase localizes to the centrosomes and chromosomes," *Biochemical and Biophysical Research Communications*, vol. 278, no. 2, pp. 385–389, 2000.

[133] M. Kanai, W. M. Tong, E. Sugihara, Z. Q. Wang, K. Fukasawa, and M. Miwa, "Involvement of poly(ADP-ribose) polymerase 1 and poly(ADP-ribosyl)ation in regulation of centrosome function," *Molecular and Cellular Biology*, vol. 23, no. 7, pp. 2451–2462, 2003.

[134] A. Saxena, L. H. Wong, P. Kalitsis, E. Earle, L. G. Shaffer, and K. H. A. Choo, "Poly(ADP-ribose) polymerase 2 localizes to mammalian active centromeres and interacts with PARP-1, Cenpa, Cenpb and Bub3, but not Cenpc," *Human Molecular Genetics*, vol. 11, no. 19, pp. 2319–2329, 2002.

[135] A. Saxena, R. Saffery, L. H. Wong, P. Kalitsis, and K. H. A. Choo, "Centromere proteins Cenpa, Cenpb, and Bub3 interact with poly(ADP-ribose) polymerase-1 protein and are poly(ADP-ribosyl)ated," *Journal of Biological Chemistry*, vol. 277, no. 30, pp. 26921–26926, 2002.

[136] D. J. Baker, K. B. Jeganathan, J. D. Cameron et al., "BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice," *Nature Genetics*, vol. 36, no. 7, pp. 744–749, 2004.

[137] D. J. Baker, K. B. Jeganathan, L. Malureanu, C. Perez-Terzic, A. Terzic, and J. M. A. Van Deursen, "Early aging-associated phenotypes in Bub3/Rae1 haploinsufficient mice," *Journal of Cell Biology*, vol. 172, no. 4, pp. 529–540, 2006.

[138] H. C. Reinhardt and B. Schumacher, "The p53 network: cellular and systemic DNA damage responses in aging and cancer," *Trends in Genetics*, vol. 28, no. 3, pp. 128–136, 2012.

[139] J. Wesierska-Gadek, C. Ranftler, and G. Schmid, "Physiological ageing: role of p53 and PARP-1 tumor suppressors in the

regulation of terminal senescence," *Journal of Physiology and Pharmacology*, vol. 56, supplement 2, pp. 77–88, 2005.

[140] W. M. Tong, H. Ohgaki, H. Huang, C. Granier, P. Kleihues, and Z. Q. Wang, "Null mutation of DNA strand break-binding molecule poly(ADP-ribose) polymerase causes medulloblastomas in p53-/- mice," *American Journal of Pathology*, vol. 162, no. 1, pp. 343–352, 2003.

[141] W. M. Tong, Y. G. Yang, W. H. Cao et al., "Poly(ADP-ribose) polymerase-1 plays a role in suppressing mammary tumourigenesis in mice," *Oncogene*, vol. 26, no. 26, pp. 3857–3867, 2007.

[142] R. Beneke and T. Möröy, "Inhibition of poly(ADP-ribose) polymerase activity accelerates T-cell lymphomagenesis in p53 deficient mice," *Oncogene*, vol. 20, no. 56, pp. 8136–8141, 2001.

[143] W. M. Tong, M. P. Hande, P. M. Lansdorp, and Z. Q. Wang, "DNA strand break-sensing molecule poly(ADP-ribose) polymerase cooperates with p53 in telomere function, chromosome stability, and tumor suppression," *Molecular and Cellular Biology*, vol. 21, no. 12, pp. 4046–4054, 2001.

[144] S. R. Kumari, H. Mendoza-Alvarez, and R. Alvarez-Gonzalez, "Functional interactions of p53 with poly(ADP-ribose) polymerase (PARP) during apoptosis following DNA damage: covalent poly(ADP-ribosyl)ation of p53 by exogenous PARP and noncovalent binding of p53 to the M(r) 85,000 proteolytic fragment," *Cancer Research*, vol. 58, no. 22, pp. 5075–5078, 1998.

[145] M. T. Valenzuela, R. Guerrero, M. I. Núñez et al., "PARP-1 modifies the effectiveness of p53-mediated DNA damage response," *Oncogene*, vol. 21, no. 7, pp. 1108–1116, 2002.

[146] X. Wang, K. Ohnishi, A. Takahashi, and T. Ohnishi, "Poly (ADP-ribosyl)ation is required for p53-dependent signal transduction induced by radiation," *Oncogene*, vol. 17, no. 22, pp. 2819–2825, 1998.

[147] S. Wieler, J. P. Gagné, H. Vaziri, G. G. Poirier, and S. Benchimol, "Poly(ADP-ribose) polymerase-1 is a positive regulator of the p53-mediated G1 arrest response following ionizing radiation," *Journal of Biological Chemistry*, vol. 278, no. 21, pp. 18914–18921, 2003.

[148] M. H. Lee, H. Na, E. Kim, H. Lee, and M. Lee, "Poly(ADP-ribosyl)ation of p53 induces gene-specific transcriptional repression of MTA1," *Oncogene*, January 2012.

[149] H. Mendoza-Alvarez and R. Alvarez-Gonzalez, "Regulation of p53 Sequence-specific DNA-binding by Covalent Poly (ADP-ribosyl)ation," *Journal of Biological Chemistry*, vol. 276, no. 39, pp. 36425–36430, 2001.

[150] C. M. Simbulan-Rosenthal, D. S. Rosenthal, R. Luo et al., "Poly(ADP-ribosyl)ation of p53 *in vitro* and *in vivo* modulates binding to its DNA consensus sequence," *Neoplasia*, vol. 3, no. 3, pp. 179–188, 2001.

[151] M. Kanai, K. Hanashiro, S. H. Kim et al., "Inhibition of Crm1-p53 interaction and nuclear export of p53 by poly (ADP-ribosyl)ation," *Nature Cell Biology*, vol. 9, no. 10, pp. 1175–1183, 2007.

[152] K. Grube and A. Bürkle, "Poly(ADP-ribose) polymerase activity in mononuclear leukocytes of 13 mammalian species correlates with species-specific life span," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 24, pp. 11759–11763, 1992.

[153] S. Beneke, A. L. Scherr, V. Ponath, O. Popp, and A. Bürkle, "Enzyme characteristics of recombinant poly(ADP-ribose) polymerases-1 of rat and human origin mirror the correlation between cellular poly(ADP-ribosyl)ation capacity and species-specific life span," *Mechanisms of Ageing and Development*, vol. 131, no. 5, pp. 366–369, 2010.

[154] A. Kunzmann, G. Dedoussis, J. Jajte, M. Malavolta, E. Mocchegiani, and A. Bürkle, "Effect of zinc on cellular poly (ADP-ribosyl)ation capacity," *Experimental Gerontology*, vol. 43, no. 5, pp. 409–414, 2008.

[155] M. L. Muiras, M. Müller, F. Schächter, and A. Bürkle, "Increased poly(ADP-ribose) polymerase activity in lymphoblastoid cell lines from centenarians," *Journal of Molecular Medicine*, vol. 76, no. 5, pp. 346–354, 1998.

[156] M. Chevanne, C. Calia, M. Zampieri et al., "Oxidative DNA damage repair and parp 1 and parp 2 expression in Epstein-Barr virus-immortalized B lymphocyte cells from young subjects, old subjects, and centenarians," *Rejuvenation Research*, vol. 10, no. 2, pp. 191–203, 2007.

[157] S. Shall and G. De Murcia, "Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model?" *Mutation Research*, vol. 460, no. 1, pp. 1–15, 2000.

[158] T. S. Piskunova, M. Yurova, A. Ovsyannikov et al., "Deficiency in poly(ADP-ribose) polymerase-1 (PARP-1) accelerates aging and spontaneous carcinogenesis in mice," *Current Gerontology and Geriatrics Research*, vol. 2008, p. 754190, 2008.

[159] W. M. Tong, U. Cortes, M. Prakash Hande et al., "Synergistic role of Ku80 and poly(ADP-ribose) polymerase in suppressing chromosomal aberrations and liver cancer formation," *Cancer Research*, vol. 62, no. 23, pp. 6990–6996, 2002.

[160] T. Nozaki, H. Fujihara, M. Watanabe et al., "Parp-1 deficiency implicated in colon and liver tumorigenesis induced by azoxymethane," *Cancer Science*, vol. 94, no. 6, pp. 497–500, 2003.

[161] M. Tsutsumi, M. Masutani, T. Nozaki et al., "Increased susceptibility of poly(ADP-ribose) polymerase-1 knockout mice to nitrosamine carcinogenicity," *Carcinogenesis*, vol. 22, no. 1, pp. 1–3, 2001.

[162] F. Cottet, H. Blanché, P. Verasdonck et al., "New polymorphisms in the human poly(ADP-ribose) polymerase-1 coding sequence: lack of association with longevity or with increased cellular poly(ADP-ribosyl)ation capacity," *Journal of Molecular Medicine*, vol. 78, no. 8, pp. 431–440, 2000.

[163] K. L. Lockett, M. C. Hall, J. Xu et al., "The ADPRT V762A genetic variant contributes to prostate cancer susceptibility and deficient enzyme function," *Cancer Research*, vol. 64, no. 17, pp. 6344–6348, 2004.

[164] Q. Zhang, Y. Li, X. Li et al., "PARP-1 Val762Ala polymorphism, CagA+ H. pylori infection and risk for gastric cancer in Han Chinese population," *Molecular Biology Reports*, vol. 36, no. 6, pp. 1461–1467, 2009.

[165] X. Zhang, X. Miao, G. Liang et al., "Polymorphisms in DNA base excision repair genes ADPRT and XRCC1 and risk of lung cancer," *Cancer Research*, vol. 65, no. 3, pp. 722–726, 2005.

[166] X. G. Wang, Z. Q. Wang, W. M. Tong, and Y. Shen, "PARP1 Val762Ala polymorphism reduces enzymatic activity," *Biochemical and Biophysical Research Communications*, vol. 354, no. 1, pp. 122–126, 2007.

[167] P. O. Hassa and M. O. Hottiger, "The functional role of poly(ADP-ribose)polymerase 1 as novel coactivator of NF-κB in inflammatory disorders," *Cellular and Molecular Life Sciences*, vol. 59, no. 9, pp. 1534–1553, 2002.

[168] M. Masutani, H. Suzuki, N. Kamada et al., "Poly(ADP-ribose) polymerase gene disruption conferred mice resistant to streptozotocin-induced diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 5, pp. 2301–2304, 1999.

[169] V. Burkart, Z. Q. Wang, J. Radons et al., "Mice lacking the poly(ADP-ribose) polymerase gene are resistant to pancreatic beta-cell destruction and diabetes development induced by streptozocin," *Nature Medicine*, vol. 5, no. 3, pp. 314–319, 1999.

[170] A. S. Mandir, S. Przedborski, V. Jackson-Lewis et al., "Poly (ADP-ribose) polymerase activation mediates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 10, pp. 5774–5779, 1999.

[171] A. A. Pieper, D. J. Brat, D. K. Krug et al., "Poly(ADP-ribose) polymerase-deficient mice are protected from streptozotocin-induced diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 6, pp. 3059–3064, 1999.

[172] W. L. Kraus, "Transcriptional control by PARP-1: chromatin modulation, enhancer-binding, coregulation, and insulation," *Current Opinion in Cell Biology*, vol. 20, no. 3, pp. 294–302, 2008.

[173] M. S. Hayden and S. Ghosh, "Shared principles in NF-κB signaling," *Cell*, vol. 132, no. 3, pp. 344–362, 2008.

[174] A. S. Adler, T. L. A. Kawahara, E. Segal, and H. Y. Chang, "Reversal of aging by NFκB blockade," *Cell Cycle*, vol. 7, no. 5, pp. 556–559, 2008.

[175] T. L. A. Kawahara, E. Michishita, A. S. Adler et al., "SIRT6 links histone H3 lysine 9 deacetylation to NF-κB-dependent gene expression and organismal life span," *Cell*, vol. 136, no. 1, pp. 62–74, 2009.

[176] A. S. Adler, S. Sinha, T. L. A. Kawahara, J. Y. Zhang, E. Segal, and H. Y. Chang, "Motif module map reveals enforcement of aging by continual NF-κB activity," *Genes and Development*, vol. 21, no. 24, pp. 3244–3257, 2007.

[177] A. Moskalev and M. Shaposhnikov, "Pharmacological inhibition of NF-κB prolongs lifespan of Drosophila melanogaster," *Aging*, vol. 3, no. 4, pp. 391–394, 2011.

[178] E. Rovillain, L. Mansfield, C. Caetano et al., "Activation of nuclear factor-κ B signalling promotes cellular senescence," *Oncogene*, vol. 30, no. 20, pp. 2356–2366, 2011.

[179] J. Wang, N. K. Jacob, K. J. Ladner et al., "RelA/p65 functions to maintain cellular senescence by regulating genomic stability and DNA repair," *EMBO Reports*, vol. 10, no. 11, pp. 1272–1278, 2009.

[180] J. S. Tilstra, C. L. Clauson, L. J. Niedernhofer, and P. D. Robbins, "NF-κB in aging and disease," *Aging and Disease*, vol. 2, no. 6, pp. 449–465, 2011.

[181] F. J. Oliver, J. Ménissier-de Murcia, C. Nacci et al., "Resistance to endotoxic shock as a consequence of defective NF-κB activation in poly (ADP-ribose) polymerase-1 deficient mice," *EMBO Journal*, vol. 18, no. 16, pp. 4446–4454, 1999.

[182] P. O. Hassa and M. O. Hottiger, "A role of poly (ADP-Ribose) polymerase in NF-κB transcriptional activation," *Biological Chemistry*, vol. 380, no. 7-8, pp. 953–959, 1999.

[183] P. O. Hassa, M. Covic, S. Hasan, R. Imhof, and M. O. Hottiger, "The enzymatic and DNA binding activity of PARP-1 are not required for NF-κB coactivator function," *Journal of Biological Chemistry*, vol. 276, no. 49, pp. 45588–45597, 2001.

[184] M. Altmeyer and M. O. Hottiger, "Poly(ADP-ribose) polymerase 1 at the crossroad of metabolic stress and inflammation in aging," *Aging*, vol. 1, no. 5, pp. 458–469, 2009.

[185] M. Stilmann, M. Hinz, S. C. Arslan, A. Zimmer, V. Schreiber, and C. Scheidereit, "A nuclear poly(ADP-Ribose)-dependent signalosome confers DNA damage-induced IκB kinase activation," *Molecular Cell*, vol. 36, no. 3, pp. 365–378, 2009.

[186] M. Ohanna, S. Giuliano, C. Bonet et al., "Senescent cells develop a parp-1 and nuclear factor-κB-associated secretome (PNAS)," *Genes and Development*, vol. 25, no. 12, pp. 1245–1261, 2011.

[187] R. S. Hotchkiss, A. Strasser, J. E. McDunn, and P. E. Swanson, "Cell death," *New England Journal of Medicine*, vol. 361, no. 16, pp. 1570–1583, 2009.

[188] L. E. Broker, F. A. E. Kruyt, and G. Giaccone, "Cell death independent of caspases: a review," *Clinical Cancer Research*, vol. 11, no. 9, pp. 3155–3162, 2005.

[189] N. A. Berger, J. L. Sims, D. M. Catino, and S. J. Berger, "Poly(ADP-ribose) polymerase mediates the suicide response to massive DNA damage: studies in normal and DNA-repair defective cells," *Princess Takamatsu symposia*, vol. 13, pp. 219–226, 1983.

[190] C. Negri, M. Donzelli, R. Bernardi, L. Rossi, A. Bürkle, and A. I. Scovassi, "Multiparametric staining to identify apoptotic human cells," *Experimental Cell Research*, vol. 234, no. 1, pp. 174–177, 1997.

[191] M. Tewari, L. T. Quan, K. O'Rourke et al., "Yama/CPP32β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase," *Cell*, vol. 81, no. 5, pp. 801–809, 1995.

[192] Y. A. Lazebnik, S. H. Kaufmann, S. Desnoyers, G. G. Poirier, and W. C. Earnshaw, "Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE," *Nature*, vol. 371, no. 6495, pp. 346–347, 1994.

[193] M. Germain, E. B. Affar, D. D'Amours, V. M. Dixit, G. S. Salvesen, and G. G. Poirier, "Cleavage of automodified poly(ADP-ribose) polymerase during apoptosis. Evidence for involvement of caspase-7," *Journal of Biological Chemistry*, vol. 274, no. 40, pp. 28379–28384, 1999.

[194] S. W. Yu, H. Wang, M. F. Poitras et al., "Mediation of poty(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor," *Science*, vol. 297, no. 5579, pp. 259–263, 2002.

[195] O. Cohausz, C. Blenn, M. Malanga, and F. R. Althaus, "The roles of poly(ADP-ribose)-metabolizing enzymes in alkylation-induced cell death," *Cellular and Molecular Life Sciences*, vol. 65, no. 4, pp. 644–655, 2008.

[196] Y. Wang, N. S. Kim, J. F. Haince et al., "Poly(ADP-ribose) (PAR) binding to apoptosis-inducing factor is critical for PAR polymerase-1-dependent cell death (parthanatos)," *Science Signaling*, vol. 4, no. 167, p. ra20, 2011.

[197] S.-W. Yu, S. A. Andrabi, H. Wang et al., "Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 48, pp. 18314–18319, 2006.

[198] S. A. Andrabi, S. K. No, S.-W. Yu et al., "Poly(ADP-ribose) (PAR) polymer is a signal," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 48, pp. 18308–18313, 2006.

[199] R. Aguilar-Quesada, J. A. Muñoz-Gámez, D. Martín-Oliva et al., "Modulation of transcription by PARP-1: consequences in carcinogenesis and inflammation," *Current Medicinal Chemistry*, vol. 14, no. 11, pp. 1179–1187, 2007.

[200] T. Radovits, L. Seres, D. Gero et al., "Single dose treatment with PARP-inhibitor INO-1001 improves aging-associated cardiac and vascular dysfunction," *Experimental Gerontology*, vol. 42, no. 7, pp. 676–685, 2007.

[201] T. Von Lukowicz, P. O. Hassa, C. Lohmann et al., "PARP1 is required for adhesion molecule expression in atherogenesis," *Cardiovascular Research*, vol. 78, no. 1, pp. 158–166, 2008.

[202] R. El Ramy, N. Magroun, N. Messadecq et al., "Functional interplay between parp-1 and SirT1 in genome integrity and chromatin-based processes," *Cellular and Molecular Life Sciences*, vol. 66, no. 19, pp. 3219–3234, 2009.
[203] R. H. Houtkooper, E. Pirinen, and J. Auwerx, "Sirtuins as regulators of metabolism and healthspan," *Nature Reviews Molecular Cell Biology*, vol. 13, no. 4, pp. 225–238, 2012.
[204] R. Firestein, G. Blander, S. Michan et al., "The SIRT1 deacetylase suppresses intestinal tumorigenesis and colon cancer growth," *PLoS ONE*, vol. 3, no. 4, Article ID e2020, 2008.
[205] A. S. Banks, N. Kon, C. Knight et al., "SirT1 gain of function increases energy efficiency and prevents diabetes in mice," *Cell Metabolism*, vol. 8, no. 4, pp. 333–341, 2008.
[206] L. Bordone, D. Cohen, A. Robinson et al., "SIRT1 transgenic mice show phenotypes resembling calorie restriction," *Aging Cell*, vol. 6, no. 6, pp. 759–767, 2007.
[207] P. T. Pfluger, D. Herranz, S. Velasco-Miguel, M. Serrano, and M. H. Tschöp, "Sirt1 protects against high-fat diet-induced metabolic damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 28, pp. 9793–9798, 2008.
[208] K. A. Moynihan, A. A. Grimm, M. M. Plueger et al., "Increased dosage of mammalian Sir2 in pancreatic β cells enhances glucose-stimulated insulin secretion in mice," *Cell Metabolism*, vol. 2, no. 2, pp. 105–117, 2005.
[209] P. Bai, C. Cantó, H. Oudart et al., "PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation," *Cell Metabolism*, vol. 13, no. 4, pp. 461–468, 2011.
[210] A. Mangerich, N. Herbach, B. Hanf et al., "Inflammatory and age-related pathologies in mice with ectopic expression of human PARP-1," *Mechanisms of Ageing and Development*, vol. 131, no. 6, pp. 389–404, 2010.
[211] R. Zarychanski and D. S. Houston, "Clinical paradigms: anemia of chronic disease: a harmful disorder or an adaptive, beneficial response?" *CMAJ*, vol. 179, no. 4, pp. 333–337, 2008.
[212] G. Weiss and L. T. Goodnough, "Anemia of chronic disease," *New England Journal of Medicine*, vol. 352, no. 10, pp. 1011–1059, 2005.
[213] A. M. Westbrook, B. Wei, J. Braun, and R. H. Schiestl, "Intestinal inflammation induces genotoxicity to extraintestinal tissues and cell types in mice," *International Journal of Cancer*, vol. 7, no. 6, 2011.
[214] T. B. L. Kirkwood, "Understanding the odd science of aging," *Cell*, vol. 120, no. 4, pp. 437–447, 2005.
[215] S. Gerondakis, R. Grumont, R. Gugasyan et al., "Unravelling the complexities of the NF-κB signalling pathway using mouse knockout and transgenic models," *Oncogene*, vol. 25, no. 51, pp. 6781–6799, 2006.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 310534, 8 pages
doi:10.1155/2012/310534

Research Article

Persistent Amplification of DNA Damage Signal Involved in Replicative Senescence of Normal Human Diploid Fibroblasts

Masatoshi Suzuki,[1] Keiji Suzuki,[1] Seiji Kodama,[2] Shunichi Yamashita,[1] and Masami Watanabe[3]

[1] *Department of Radiation Medical Sciences, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8523, Japan*
[2] *Laboratory of Radiation Biology, Department of Biological Science, Graduate School of Science, Osaka Prefecture University, Sakai, Osaka 599-8570, Japan*
[3] *Radiation Biology Center, Kyoto University, Kyoto 606-8501, Japan*

Correspondence should be addressed to Masami Watanabe, msm@orange.plala.or.jp

Received 1 June 2012; Revised 26 July 2012; Accepted 13 August 2012

Academic Editor: William C. Burhans

Foci of phosphorylated histone H2AX and ATM are the surrogate markers of DNA double strand breaks. We previously reported that the residual foci increased their size after irradiation, which amplifies DNA damage signals. Here, we addressed whether amplification of DNA damage signal is involved in replicative senescence of normal human diploid fibroblasts. Large phosphorylated H2AX foci (>1.5 μm diameter) were specifically detected in presenescent cells. The frequency of cells with large foci was well correlated with that of cells positive for senescence-associated β-galactosidase staining. Hypoxic cell culture condition extended replicative life span of normal human fibroblast, and we found that the formation of large foci delayed in those cells. Our immuno-FISH analysis revealed that large foci partially localized at telomeres in senescent cells. Importantly, large foci of phosphorylated H2AX were always colocalized with phosphorylated ATM foci. Furthermore, Ser15-phosphorylated p53 showed colocalization with the large foci. Since the treatment of senescent cells with phosphoinositide 3-kinase inhibitor, wortmannin, suppressed p53 phosphorylation, it is suggested that amplification of DNA damage signaling sustains persistent activation of ATM-p53 pathway, which is essential for replicative senescence.

1. Introduction

It is well known that normal human somatic cells have a finite replicative life span, which resulted from permanent cell cycle arrest caused by persistent activation of DNA damage checkpoint [1]. Therefore, it is presumed that unreparable and sustained DNA damage could be the trigger of replicative senescence. It has been widely accepted that shortened telomeres cause persistent activation of DNA damage checkpoints [2]. Telomeres generally form looped structure, otherwise, the telomeric DNA-ends might be sensed as DNA double-strand break (DSB) [3]. Experimentally, the relationship between telomere dysfunction and replicative senescence has been investigated by using dominant-negative TRF2 proteins. Collapse of telomere loop exposes telomeric DNA-ends, which resulted in senescence induction in normal human fibroblasts [4, 5]. Thus, it is clear that telomere dysfunction is the primary cause of replicative senescence.

As telomere dysfunction activates DNA damage checkpoint factors, DNA damage signaling could be critical for replicative senescence [6]. For example, phosphorylated H2AX foci, which are often referred to as γH2AX foci, have been treated as a surrogate marker for DNA damage signal activation, and the formation of phosphorylated H2AX foci are commonly observed in replicative senescence [7, 8]. In addition, immuno-FISH analysis, which is the combination of immunofluorescent detection of foci and telomere FISH revealed foci formation detected with telomere FISH signals in senescent cells, suggesting telomere in senescent cells causes DSB. Moreover, two genome-wide studies revealed

enrichment of H2AX phosphorylation as well as another DNA damage checkpoint factor, 53BP1, at the end of chromosome in senescent normal human fibroblasts [7, 9]. Thus, DNA damage signals triggered by telomere dysfunction appear to be critical for replicative senescence.

It is quite evident that various external stresses causing DNA damage prematurely induce senescence-like features in normal human fibroblasts. For example, ionizing radiation has been reported to induce senescence-like growth arrest (SLGA) [10]. It has been shown that persistent unreparable DSBs result in SLGA, which seems to be equivalent to DSBs located at telomere ends in replicative senescent cells [11]. In fact, we previously found persistent foci in different size in cells inducing SLGA [12]. The initial foci, which were detected immediately after irradiation, were tiny, and most initial foci rapidly disappeared thereafter. In contrast, sustained foci especially for over several days following irradiation are quite large in size, and the large foci are observed in cells underwent SLGA. Because large foci continuously amplify DNA damage signal, prolonged activation of DNA damage checkpoint should play a critical role in irreversible growth arrest. Therefore, we here addressed whether amplification of DNA damage signal is involved in replicative senescence of normal human diploid fibroblasts.

2. Materials and Methods

2.1. Cell Culture and Reagent. Normal human diploid fibroblast, HE49, was exponentially grown in Eagle's minimum essential medium (Nissui, Tokyo, Japan) supplemented 10% fetal bovine serum (Thermo Trace Ltd., VIC, Australia). Normoxic cell culture was performed at 37°C in a humidified incubator with 5% CO_2 and 95% air, and hypoxic cell culture was performed in a humidified incubator (Ikeda Scientific Co., Ltd., Tokyo, Japan) with 5% CO_2, 2% O_2, and 93% N_2 by supplying nitrogen gas from a nitrogen gas generator (Kojima, Kyoto, Japan). Population doubling level (PDL) was calculated by the following equations

$$n = \frac{\log(N/N_0)}{\log 2}, \qquad \text{PDL} = \sum n, \tag{1}$$

"N" or "N_0" indicate the counted cell number following cell culture or the seeding cell number at the plating. "n" represents population doubling level of each passage.

2.2. Immunofluorescence Staining. Cells grown on coverslips at indicated PDL were washed once with cold PBS^-, and then fixed with 4% paraformaldehyde/PBS^- solution for 10 min at room temperature followed by permeabilization with 0.5% Triton X-100/PBS^- solution for 5 min on ice. Alternatively, preextraction treatment which excluded chromatin-free nuclear protein was performed by the sequential treatments as follows, permeabilization with 0.5% Triton X-100 in cytoskeleton, CSK, (10 mM HEPES-KOH, pH 7.4, 300 mM Sucrose, 100 mM NaCl, 3 mM $MgCl_2$-$6H_2O$) buffer for 2 min on ice, fixation with 4% paraformaldehyde/CSK buffer for 20 min at room temperature, and then 0.5% NP-40/CSK buffer-treatment for 5 min at room temperature. The primary antibody was treated for 2 h at 37°C with following listed antibodies; mouse monoclonal antiphosphorylated H2AX at Ser139 antibody and rabbit polyclonal antiphosphorylated H2AX at Ser139 antibody (Upstate Biotechnology, NY, USA), rabbit polyclonal antiphosphorylated ATM at Ser1981 antibody (Rockland, PA, USA), mouse monoclonal anti-p53 (Lab Vision, CA, USA), and rabbit polyclonal antiphosphorylated p53 at Ser15 (Cell Signaling Technology, MA, USA). Following the primary antibody treatment, Alexa Fluor 488-conjugated goat antimouse or rabbit antibody or Alexa Fluor 594-conjugated antimouse or rabbit antibody (Molecular Probes, OR, USA) was treated as secondary antibody for 1 h at 37°C. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 10 ng/mL; Molecular Probes, OR, USA) or propidium iodide, PI. Images were acquired with an Olympus fluorescence microscope and then analyzed with IP Lab software (Scanalytics, VA, USA).

2.3. Immuno-FISH. Immuno-FISH assay was performed as described previously [13]. Briefly, cells were fixed, permeabilized, and stained with antiphosphorylated histone H2AX antibody as described above. After immunofluorescence staining step, labeled protein was cross-linked with 4% paraformaldehyde/PBS^- for 20 min at room temperature. The samples were then dehydrated in 70%, 90%, and 100% ethanol for 3 min each and air-dried, and DNA was denatured for 30 min on a hotplate at 80°C. After hybridization with a telomere-PNA probe for 5 h, the cells were washed three times with 70% formamide/10 mM Tris, pH 6.8, for 15 min, followed by a 5-min wash with 0.05 M Tris/0.15 M NaCl, pH 7.5/0.05% Tween 20 and a 5-min wash with PBS^-. Microscopic analysis was performed as described in the section of immunofluorescence assay.

2.4. Senescence Associated β-Galactosidase Staining. SA-β-gal staining was carried out as described previously [14]. Briefly, cells were plated into 35 mm dish and on the following day, it was fixed with 2% paraformaldehyde containing 0.2% glutaraldehyde for 5 min at room temperature. After fixation, cells were washed extensively with PBS^- and were then incubated with stain solution (40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$) containing 1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, X-gal, (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.5. Cell Cycle Analysis. Cells grown on the coverslip were fixed with ice-cold 70% ethanol for 5 min at room temperature. Following extensive wash process with PBS^-, samples were treated with PI (50 μg/mL) stain solution containing 200 μg/mL RNase for 30 min at 37°C. Cell cycle analysis was performed using a laser scanning cytometer (LSC-101, Olympus, Tokyo).

2.6. Immunoblotting. Whole cell extracts were prepared in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing 1x protease inhibitor. The membrane that transferred proteins separated by SDS-PAGE was probed with primary antibody for 2 h at room temperature followed by biotinylated antimouse or -rabbit IgG antibodies (Amersham Pharmacia Biotech, UK) as secondary antibody, the bands were visualized using alkaline phosphatase detection system (Amersham Pharmacia Biotech, UK) by addition of nitroblue tetrazolium/5-bromo-4-chloro-3′-indolyl phosphate (Roche Diagnostic, USA).

3. Result

3.1. Foci Growth of Phosphorylated H2AX in Replicative Senescence. DNA damage signal amplification in replicative senescence of normal human diploid fibroblasts were examined by immunofluorescence staining of phosphorylated histone H2AX at Ser139 at different PDLs (Figure 1(a)). Histone H2AX underwent phosphorylation and formed dotted foci in nearly 10% of cells at PDL12, when cells exponentially proliferated and most cells were negative for SA-β-gal staining (Figures 1(a), 1(b) and 2(a)). The frequency of the cells gradually elevated with increasing PDL, and it reached to nearly 80% at PDL 61, when approximately 60% of cells was positive for SA-β-gal. According to our previous criteria [15], the foci with more than 1.5 μm in diameter were judged as large foci in replicative senescence. No large foci formation was observed at PDL 12. Then, the frequency of large foci positive cell was slightly elevated over the culture days up to PDL 55, and they were formed in nearly 60% of cells at PDL 61. Approximately 65% of positive cells for H2AX phosphorylation showed large foci. The frequency of SA-β-gal positive cells was well correlated with those of the cells with large foci over culture days. These data indicate that large foci formation of DNA damage checkpoint factor correlates well with the induction of replicative senescence.

Localization of large foci was investigated by Immuno-FISH analysis that combined immunofluorescent detection of H2AX phosphorylation with telomere FISH (Figure 1(c)). Whereas large foci did not colocalize with telomere signals at PDL 21, large foci associated with telomere signals were observed in 25% at PDL 61 (Figure 1(d)). It should be mentioned that large foci were completely colocalized with foci of phosphorylated ATM, that is, active form of ATM, at any PDLs (Figure 1(e)). These data indicate that ATM-dependent DNA damage signal is amplified at the site of large foci in senescent cells, indicating that not only dysfunctional telomeres but also interstitial DNA breaks could be associated with senescence induction.

3.2. Extension of Replicative Life Span Delayed Large Foci Formation of Phosphorylated H2AX. The link between senescence induction and large foci formation was further examined in cells cultured under 2% of hypoxic condition which extended replicative life span [16]. The cells used for this study were originally cultured under normoxic condition up to PDL 21 before they were moved to hypoxic culture condition. Then, they were divided into two different culture conditions, hypoxia and normoxia. Therefore, we set day 0 in culture at PDL 21. Both cell groups were individually maintained and subcultured at the same day. PDL of both cells was equally elevated at the initial culture period, however, cell growth was completely stopped under normoxic condition approximately at 65 days, while the cells in hypoxic condition continued proliferation for more than 8 cell division, and finally arrested approximately at 80 days (Figure 2(a)). Cell cycle analysis of S phase demonstrated that growth arrest was much delayed under hypoxic condition (Figures 2(b) and 2(c)). For example, the fractions of S phase, at day 13, were similarly detected under normoxia and hypoxia, respectively. It was markedly diminished to 5% under normoxia, while the fraction still detected in 16% under hypoxia at day 59 and eventually diminished to 4% at day 93. In hypoxic condition, large foci formation was similarly observed as shown in Figure 1, however, they were detected much later compared with the cells cultured in normoxic condition (Figure 2(d)). Therefore, these data demonstrated that large foci were generated by endogenous oxidative stress, and the formation of large foci was strongly correlated with senescence induction.

3.3. Activation of ATM-p53 Pathway at the Large Foci of Phosphorylated H2AX. We next examined whether ATM-p53 pathway is involved in persistent activation of cell cycle arrest in senescent cells. In replicative senescence of HE49, accumulation of p53 accompanied with phosphorylation at Ser15 and transactivation of p21 was observed over the culture time. Especially, p53-p21 pathway was persistently upregulated when p16 was also induced (Figure 3(a)). p53 was then visualized by immunofluorescence staining following formalin fixation at indicated PDLs (Figure 3(b)). Approximately 20% of cells at PDL 21 weakly expressed p53 in nuclear (indicated as "+" in Figure 3(c)), and others were under detection level of p53. Increase of p53-expressing cells was observed at PDL 61 as detected in western blotting (Figure 3(a)), and p53 highly accumulated in 30% at PDL 61 (indicated as "+++" in Figure 3(c)). Interestingly, accumulated p53 formed colocalized foci with phosphorylated ATM foci (Figure 3(b)). p53 was also visualized in the cells receiving preextraction treatment followed by formalin fixation (Figure 3(d)). Preextraction removed chromatin-free nuclear protein and accumulating p53 in nuclear disappeared, while aggregated p53 was still detected at the sites formed large foci of phosphorylated ATM. Furthermore, Ser15-phosphorylation form of p53 was also detected at the large foci of phosphorylated ATM following preextraction (Figure 3(e)). Furthermore, the effect of ATM kinase inhibition on p53 phosphorylation at Ser15 in senescent cells revealed suppression of phosphorylation level especially at lower doses (Figure 3(f)), suggesting ATM is involved in p53 activation in replicative senescence. These data indicate ATM-p53 pathway persistently activated at the site of large foci in senescent cells.

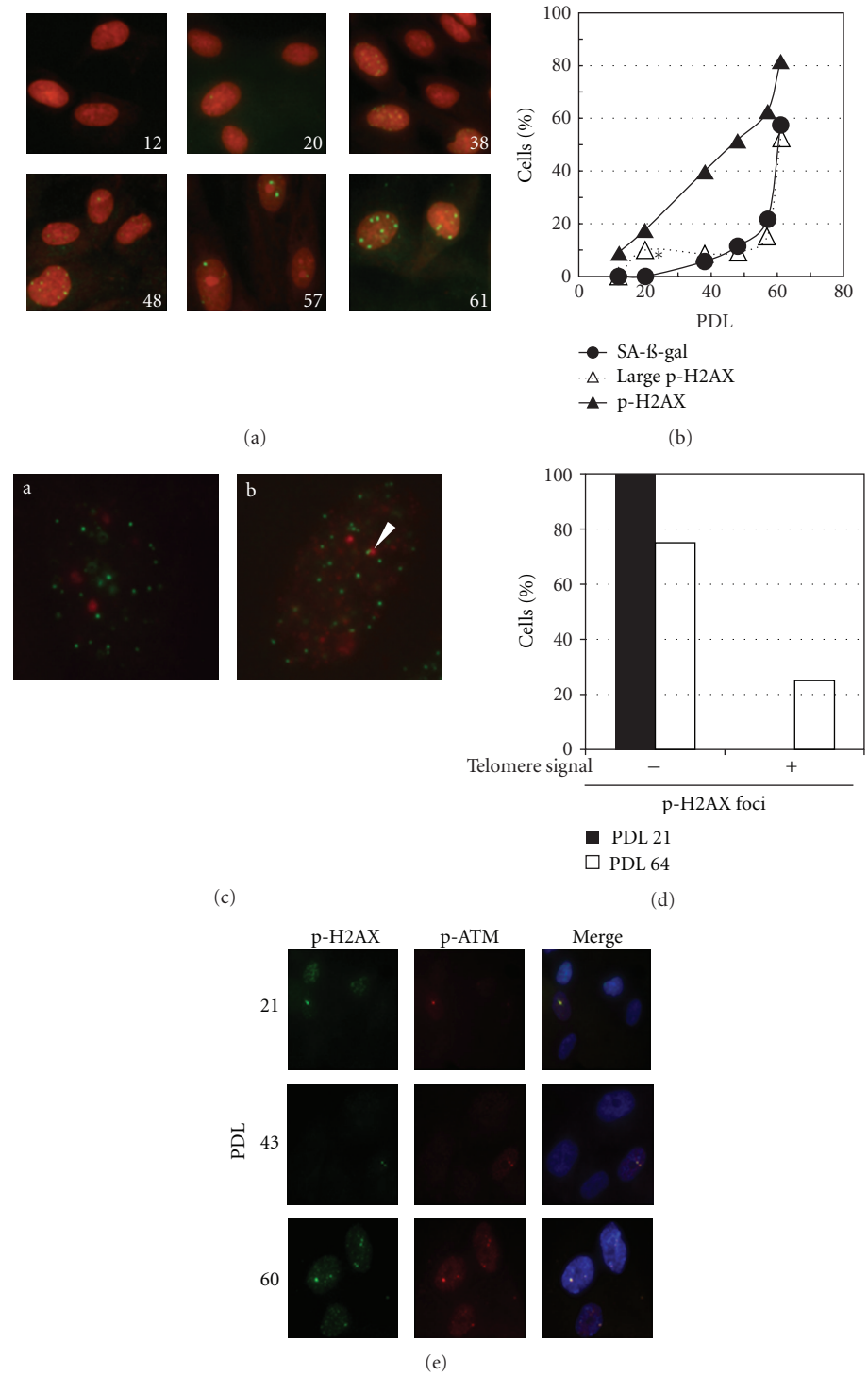


Figure 1: Foci growth of phosphorylated H2AX in replicative senescence. (a) Visualization of phosphorylated H2AX foci (green) at the different PDLs indicated by the numbers. Nuclear counterstain was performed with PI (red). (b) Induction frequency of senescent cell (closed circle), H2AX phosphorylation (closed triangle), and large foci formation (open triangle). At least 300 cells and 100 cells were analyzed for senescent induction and foci diameter, respectively. Statistical analysis of the frequency between SA-β-gal (+) and large foci (+) was performed by Welch's test. ($^*P < 0.01$) (c) Each Immuno-FISH image represented a cell with phosphorylated H2AX foci (red) in the absence (a) or the presence (b) of colocalization with telomere FISH signal (green). Arrowhead in (b) represents the phosphorylated H2AX focus accompanied with telomere FISH signal. (d) The frequency of cells in which large foci were detected with (+) or without (−) telomere signal. The data was analyzed with the cells detected large foci in both PDLs of 21 (closed bar) and of 64 (open bar (b)). At least 100 cells were investigated in both PDLs. (e) Colocalization of phosphorylated H2AX (green) and phosphorylated ATM (red) in replicative senescence. Nuclear counterstain was performed with DAPI (blue).

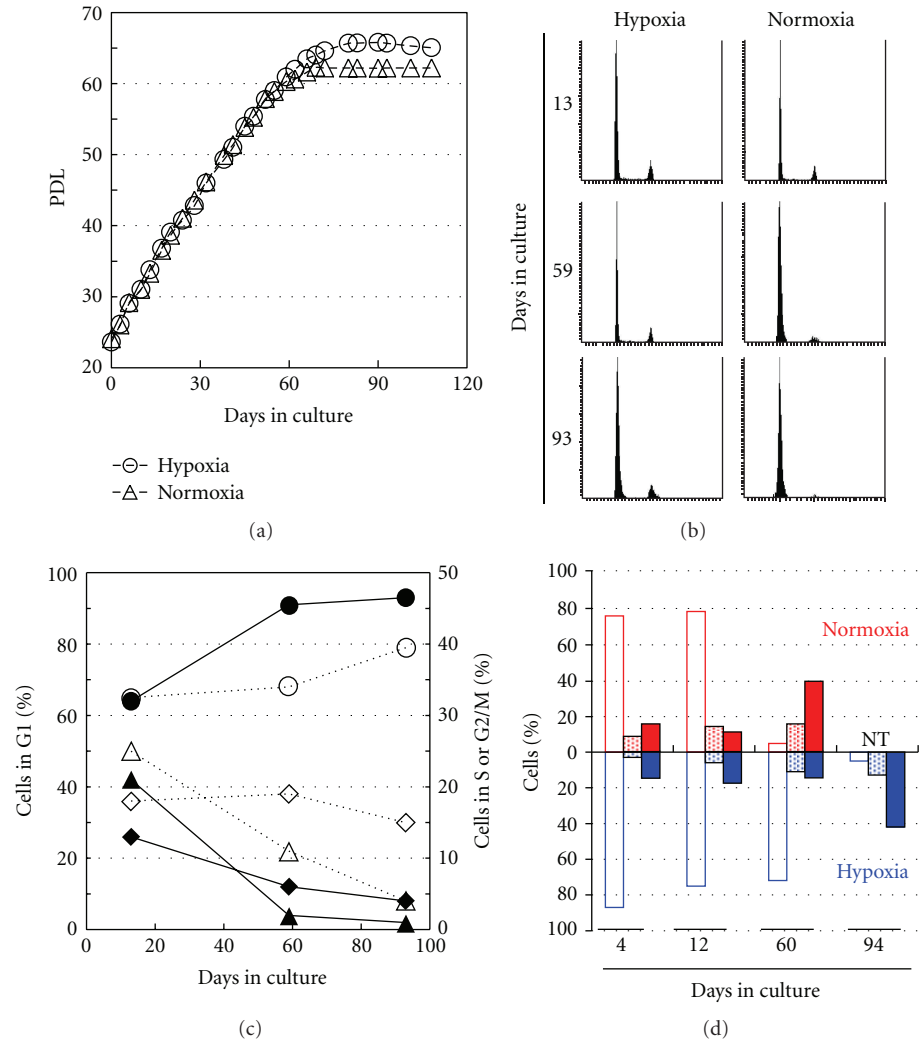


Figure 2: Extension of replicative life span correlated with delayed large foci formation of phosphorylated H2AX. (a) HE49 was cultured under normoxic or hypoxic condition and then PDL was calculated. (b and c) Cell cycle distribution under normoxic (line in c) and hypoxic (dotted line in c) conditions was analyzed by LSC at the indicated time points. Cell cycle data shown in (b) was graphed in (c). The symbols of circle, triangle, and diamond represent the frequency in G1, S, and G2 phase, respectively. The data was obtained from more than 6,000 cells. (d) Phosphorylated histone H2AX status was classified into H2AX phosphorylation negative (open bar), H2AX phosphorylation positive (dotted bar), and large foci formation (closed bar) and the frequency of each status were investigated in replicative senescence under normoxic (red) and hypoxic (blue) condition. NT indicated "not tested." At least 100 cells were analyzed in each condition.

4. Discussion

The present study demonstrates that persistent amplification of DNA damage signal is involved in replicative senescence.

It has been generally thought that prolonged activation of DNA damage response at dysfunctional telomere results in irreversible cell cycle arrest in replicative senescence [17]. Indeed, foci formation at telomeres is detected in senescent cells [7, 8]. Our current study extends such observation and adds the evidence that DNA damage signals at dysfunctional telomeres are mostly amplified.

Our previous findings demonstrate that amplification of DNA damage signal relates to persistent activation of ATM-p53 pathway sufficient for executing irreversible G1 arrest in response to ionizing radiation [11, 12]. We also demonstrated that increase in foci size was essential for amplification of DNA damage signals. In fact, residual foci, which sustain for over several days following irradiation, are larger foci, which are indispensable for proper activation of p53 [15]. The present study clearly demonstrated that formation of large foci also takes place in replicative senescent cells (Figure 1(a)). Our results are the following: (1) increase of cells with large foci is well correlated with the senescence induction (Figure 1(b)), and (2) hypoxic cell culture, which extends replicative life span, delays the formation of large foci (Figure 2), indicate mutual relationship between amplification of DNA damage signals and induction of replicative senescence.

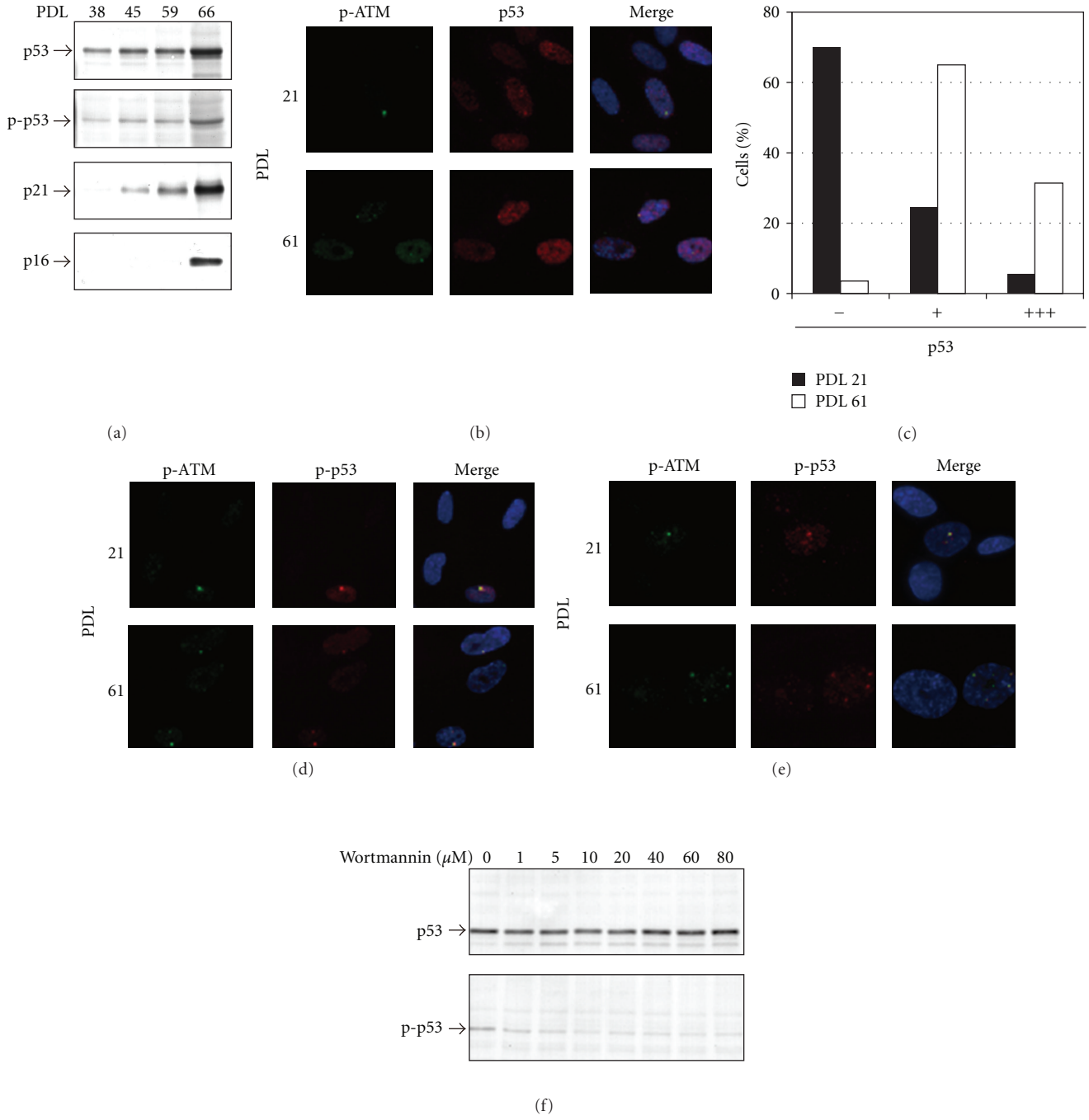


Figure 3: Activation of ATM-p53 pathway at the large foci of phosphorylated H2AX. (a) Investigation of factors relating to G1 checkpoint machinery in replicative senescence was performed by western blotting. "p-p53" indicated Ser15-phosphorylated p53. (b) p53 accumulation in cells formed phosphorylated ATM foci (p-ATM) in replicative senescence. (c) The cells detected different p53 level was scored at both PDL of 21 (closed bar) and of 64 (open bar) ($n \geq 1,000$). "−", "+", and "+++" indicated p53 negative, positive, and strong positive, respectively. Representative cells of p53 positive or strong positive were shown in upper or lower image in (b). (d and e) The cells were preextracted to remove chromatin-free nuclear protein, and then detected indicated proteins. (f) p53 phosphorylation at Ser15 was examined in senescent cells treated with a series of concentration of wortmannin.

It has been thought that telomere dysfunction results in activation of DNA damage response. Dysfunctional telomere is able to be detected by foci formation of DNA damage checkpoint factors which accompanied with telomere FISH signal, so called telomere-induced foci (TIF) [8, 13], and we also detected TIF in 25% of senescent cells (Figure 1(c)). It is generally thought that TIF represents uncapped telomere exposing telomeric DNA-ends. Therefore, it is assumed that unreparable DSBs causes prolonged activation of DNA damage response [8]. On the other hand, our previous data

represent localization of phosphorylated H2AX foci not only at DSB site but also on dicentric chromosome [18, 19]. It has also been demonstrated that telomere-telomere fusion-mediated dicentric chromosomes were formed in senescent normal human fibroblasts of MRC-5 and WI-38 [20, 21], suggesting another possibility that TIF might be reflected the region on dicentric chromosome derived from telomere fusion. Our immuno-FISH analysis indeed demonstrated that large foci without telomere-FISH signal in 75% of senescent cells (Figure 1(c)). Nakamura et al. precisely analyzed foci formation with metaphase chromosome spreads of presenescent WI-38 and BJ normal human fibroblasts [22]. They found localization of foci at the end of chromosome which lacked telomere-FISH signal in more than 50% of foci detected in presenescent metaphase spreads. Therefore, large foci formation without telomere-FISH signal in our telomere-FISH analysis might involve such foci. Alternatively, following telomere-telomere fusion, Fusion-Bridge-Breakage (FBB) cycle might initiate DSBs at interstitial chromatin region [23]. Once dysfunctional telomeres are fused and generate dicentric chromosome, two centromeres are pulled in opposite directions during anaphase. Such a chromosome regionally gets a tension, eventually, DNA break is initiated at interstitial chromatin region of dicentric chromosome. On the basis of the model, dysfunctional telomeres might be in the one mechanism of large foci formation in replicative senescence, but interstitial chromatin region could also be the candidate to serve DNA ends.

Formationof large foci activates ATM-p53 pathway, which triggers p21 transactivation. It has been represented that p53-p21 pathway as well as p16 is associated with irreversible growth arrest in senescent cells, especially p16 expression is elevated at late senescent stage [24]. We also confirmed induction of both pathways in replicative senescence (Figure 3(a)). We found that lower concentration of wortmannin treatment in senescent cells significantly suppressed Ser15-phosphorylation of p53 (Figure 3(f)). Previous reports demonstrated that IC_{50} of wortmannin treatment for ATM was approximately 5 μM [25], and ATM, but not DNA-PK, is known as a major factor for Ser15-phosphorylation of p53 *in vivo* in response to DSB [26]. Thus, it can be concluded that ATM-dependent p53 activation is amplified at large foci.

In conclusion, our data presented here provide strong correlation between large foci formation of DNA damage checkpoint factors and replicative senescence induction. Large foci could be formed at dysfunctional telomeres as well as at interstitial chromatin regions, which ensures persistent activation of DNA damage response. Thus, persistent amplification of DNA damage signals via ATM-p53 pathway plays a critical role in replicative senescence.

References

[1] L. Hayflick and P. S. Moorhead, "The serial cultivation of human diploid cell strains," *Experimental Cell Research*, vol. 25, no. 3, pp. 585–621, 1961.

[2] E. Sikora, T. Arendt, M. Bennett, and M. Narita, "Impact of cellular senescence signature on ageing research," *Ageing Research Reviews*, vol. 10, no. 1, pp. 146–152, 2011.

[3] S. Misri, S. Pandita, R. Kumar, and T. K. Pandita, "Telomeres, histone code, and DNA damage response," *Cytogenetic and Genome Research*, vol. 122, no. 3-4, pp. 297–307, 2009.

[4] A. Smogorzewska, J. Karlseder, H. Holtgreve-Grez, A. Jauch, and T. De Lange, "DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2," *Current Biology*, vol. 12, no. 19, pp. 1635–1644, 2002.

[5] J. Karlseder, D. Broccoli, D. Yumin, S. Hardy, and T. De Lange, "p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2," *Science*, vol. 283, no. 5406, pp. 1321–1325, 1999.

[6] V. Gorbunova and A. Seluanov, "Making ends meet in old age: DSB repair and aging," *Mechanisms of Ageing and Development*, vol. 126, no. 6-7, pp. 621–628, 2005.

[7] F. Di Fagagna, P. M. Reaper, L. Clay-Farrace et al., "A DNA damage checkpoint response in telomere-initiated senescence," *Nature*, vol. 426, no. 6963, pp. 194–198, 2003.

[8] O. A. Sedelnikova, I. Horikawa, D. B. Zimonjic, N. C. Popescu, W. M. Bonner, and J. C. Barrett, "Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks," *Nature Cell Biology*, vol. 6, no. 2, pp. 168–170, 2004.

[9] A. Meier, H. Fiegler, P. Mũoz et al., "Spreading of mammalian DNA-damage response factors studied by ChIP-chip at damaged telomeres," *EMBO Journal*, vol. 26, no. 11, pp. 2707–2718, 2007.

[10] M. Suzuki and D. A. Boothman, "Stress-induced premature senescence (SIPS)-influence of sips on radiotherapy," *Journal of Radiation Research*, vol. 49, no. 2, pp. 105–112, 2008.

[11] K. Suzuki, I. Mori, Y. Nakayama, M. Miyakoda, S. Kodama, and M. Watanabe, "Radiation-induced senescence-like growth arrest requires TP53 function but not telomere shortening," *Radiation Research*, vol. 155, no. 1, pp. 248–253, 2001.

[12] M. Suzuki, K. Suzuki, S. Kodama, and M. Watanabe, "Interstitial chromatin alteration causes persistent p53 activation involved in the radiation-induced senescence-like growth arrest," *Biochemical and Biophysical Research Communications*, vol. 340, no. 1, pp. 145–150, 2006.

[13] U. Herbig, W. A. Jobling, B. P. C. Chen, D. J. Chen, and J. M. Sedivy, "Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21CIP1, but not p16INK4a," *Molecular Cell*, vol. 14, no. 4, pp. 501–513, 2004.

[14] G. P. Dimri, X. Lee, G. Basile et al., "A biomarker that identifies senescent human cells in culture and in aging skin in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 20, pp. 9363–9367, 1995.

[15] M. Yamauchi, Y. Oka, M. Yamamoto et al., "Growth of persistent foci of DNA damage checkpoint factors is essential for amplification of G1 checkpoint signaling," *DNA Repair*, vol. 7, no. 3, pp. 405–417, 2008.

[16] G. Kashino, S. Kodama, Y. Nakayama et al., "Relief of oxidative stress by ascorbic acid delays cellular senescence of normal human and Werner syndrome fibroblast cells," *Free Radical Biology and Medicine*, vol. 35, no. 4, pp. 438–443, 2003.

[17] N. Ohtani, D. J. Mann, and E. Hara, "Cellular senescence: its role in tumor suppression and aging," *Cancer Science*, vol. 100, no. 5, pp. 792–797, 2009.

[18] M. Suzuki, K. Suzuki, S. Kodama, and M. Watanabe, "Phosphorylated histone H2AX foci persist on rejoined mitotic chromosomes in normal human diploid cells exposed to ionizing radiation," *Radiation Research*, vol. 165, no. 3, pp. 269–276, 2006.

[19] E. J. Hall, "Molecular biology in radiation therapy: the potential impact of recombinant technology on clinical practice," *International Journal of Radiation Oncology Biology Physics*, vol. 30, no. 5, pp. 1019–1028, 1994.

[20] K. V. A. Thompson and R. Holliday, "Chromosome changes during the in vitro ageing of MRC 5 human fibroblasts," *Experimental Cell Research*, vol. 96, no. 1, pp. 1–6, 1975.

[21] P. A. Benn, "Specific chromosome aberrations in senescent fibroblast cell lines derived from human embryos," *American Journal of Human Genetics*, vol. 28, no. 5, pp. 465–473, 1976.

[22] A. J. Nakamura, Y. J. Chiang, K. S. Hathcock et al., "Both telomeric and non-telomeric DNA damage are determinants of mammalian cellular senescence," *Epigenetics & Chromatin*, vol. 1, p. 6, 2008.

[23] J. P. Murnane, "Telomere dysfunction and chromosome instability," *Mutation Research*, vol. 730, no. 1-2, pp. 28–36, 2012.

[24] D. A. Alcorta, Y. Xiong, D. Phelps, G. Hannon, D. Beach, and J. C. Barrett, "Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 24, pp. 13742–13747, 1996.

[25] J. N. Sarkaria, R. S. Tibbetts, E. C. Busby, A. P. Kennedy, D. E. Hill, and R. T. Abraham, "Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin," *Cancer Research*, vol. 58, no. 19, pp. 4375–4382, 1998.

[26] R. Araki, R. Fukumura, A. Fujimori et al., "Enhanced phosphorylation of p53 serine 18 following DNA damage in DNA-dependent protein kinase catalytic subunit-deficient cells," *Cancer Research*, vol. 59, no. 15, pp. 3543–3546, 1999.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 728547, 6 pages
doi:10.1155/2012/728547

Review Article

Is Physical Activity Able to Modify Oxidative Damage in Cardiovascular Aging?

Graziamaria Corbi,[1] Valeria Conti,[2] Giusy Russomanno,[2] Giuseppe Rengo,[3] Piergiusto Vitulli,[1] Anna Linda Ciccarelli,[1] Amelia Filippelli,[2] and Nicola Ferrara[1,3]

[1] *Department of Medicine and Health Sciences, University of Molise, Via Giovanni Paolo II, Località Tappino, 86100 Campobasso, Italy*
[2] *Department of Medicine and Sugery, University of Salerno, Via Salvador Allende, 84081 Baronissi (SA), Italy*
[3] *Istituto Scientifico di Campoli/Telese, Fondazione Salvatore Maugeri, IRCCS, Via Bagni Vecchi 1, 82037 Telese Terme (BN), Italy*

Correspondence should be addressed to Graziamaria Corbi, graziamaria.corbi@unimol.it

Received 22 June 2012; Accepted 13 August 2012

Academic Editor: William C. Burhans

Aging is a multifactorial process resulting in damage of molecules, cells, and tissues. It has been demonstrated that the expression and activity of antioxidant systems (SOD, HSPs) are modified in aging, with reduced cell ability to counteract the oxidant molecules, and consequent weak resistance to ROS accumulation. An important mechanism involved is represented by sirtuins, the activity of which is reduced by aging. Physical activity increases the expression and the activity of antioxidant enzymes, with consequent reduction of ROS. Positive effects of physical exercise in terms of antioxidant activity could be ascribable to a greater expression and activity of SOD enzymes, HSPs and SIRT1 activity. The antioxidant effects could increase, decrease, or not change in relation to the exercise protocol. Therefore, some authors by using a new approach based on the in vivo/vitro technique demonstrated that the highest survival and proliferation and the lowest senescence were obtained by performing an aerobic training. Therefore, the in vivo/vitro technique described could represent a good tool to better understand how the exercise training mediates its effects on aging-related diseases, as elderly with heart failure that represents a special population in which the exercise plays an important role in the improvement of cardiovascular function, quality of life, and survival.

1. Aging and Oxidative Stress

Aging is a multifactorial process resulting in damage of molecules, cells, and tissues, leading to a reduced efficacy of functions with different pathophysiological consequences and a variety of clinical phenotypes. It is characterized by altered regulation of many genes implicated in stress resistance and processes of tissue regeneration and repair. In particular, old animals are intrinsically less resistant to oxidative stress [1, 2].

Several studies have shown that advanced age is associated with abnormalities in cardiac performance and structure, such as a decline in early diastolic left ventricular filling [3] and increases in wall thickness, but also by changes in vascular and cardiac beta-receptor function [4]. Abete et al. [5] demonstrated that changes in response to stress, induced by intracoronary infusion of hydrogen peroxide were pronounced in old rats. In fact they found that cardiac release of oxidized glutathione (an index of the ability of the heart to inactivate oxygen metabolites) was significantly lower in old hearts than in younger animals, and that this finding was associated with decreased cardiac concentrations of the scavenger enzymes glutathione peroxidase and Mn-superoxide dismutase. The authors concluded that in rat heart metabolic and functional tolerance toward oxidative stress decreases with age. These observations indicate that the heart undergoes anatomic and functional changes over aging, the interaction of which may eventually result in excessive risk for cardiovascular diseases.

In elderly patients, in addition to age-related changes in function and anatomy of the cardiovascular system, a progression of coronary artery disease and organ damage

associated with hypertension, diabetes, and often complicated by heart failure are found.

Abnormalities in mitochondrial function, calcium handling, electrolytes alterations, hormones, oxidative stress, and cardioprotective signaling have all been proposed as potentially implicated [6]. Effects of high levels of insulin, involved in many age-related diseases, on the cardiovascular function are well studied, even if conflicting data are reported in literature about the effects of insulin on myocardial contractility [7]. In a model of isolated rats papillary muscles, it was demonstrated that insulin-induced modulation of contractility is calcium independent and that insulin leads to a supersensitization on the beta1-adrenoceptors [8], involved in pathogenesis of age-related cardiac diseases, such as heart failure [9]. It has been demonstrated that one of the pathophysiological mechanisms involved in the genesis of heart failure is represented by a persistent beta1-adrenoceptors stimulation, that evokes a multitude of cardiac toxic effects, including myocyte apoptosis and hypertrophy, as showed in vivo on rodent hearts and in vitro on cultured cardiomyocytes [10–13].

Regarding the effects of electrolytes changes implicated in the regulation of myocardial function, for instance, it has been demonstrated that magnesium interferes on failed cardiac contractility [14], by modifying sarcoplasmic reticular Ca^{2+} transport systems with a calcium antagonism mechanism based on competition between Mg^{2+} and Ca^{2+} for the same binding sites on key myocardial contractile proteins, such as troponin C, myosin, and actin [15] that could explain the opposite effects of Mg^{2+} and Ca^{2+} on myocardial contractility [16].

Furthermore, the aging is characterized by an impaired responsiveness to stress and by a reduced efficiency of endogenous protective mechanisms (i.e., ischemic preconditioning and postconditioning), which results in increased vulnerability to injury [17, 18].

A greater myocardial dysfunction induced by ischemia-reperfusion injury [19] represents another feature of aging process, then reinforcing the association with oxidative stress. The oxidative stress has been suggested to be also responsible for some metabolic changes.

In particular, old animals show increased release of glutathione and decreased release of oxidized glutathione, suggesting that cardiac oxidative tolerance (considered as the ability of heart to suffer oxidative insults without crucial effects) decreases with age [5]. Ferrara et al. [20] also demonstrated that impairment of systolic and diastolic functions, induction of arrhythmias, release of glutathione, and other abnormalities caused by oxidant exposure can be prevented through antioxidants administration. The oxidative stress that is responsible for these changes, represents an imbalance between production of reactive oxygen species (ROS) (such as nitrogen, superoxide anion, and hydrogen peroxide) and antioxidants cellular systems unable to cope with the excess of oxidants. ROS are continuously formed during life as result of the metabolism of oxygen, and their production is increased during some pathological processes. Mitochondrial respiration is a physiological source of ROS and impairment in this system has been related to dysfunction of several homeostatic processes, such as membrane lipid peroxidation, enzymes inactivation, and nucleic acid bases damage. The protective systems involved in antioxidant cellular defense are principally represented by peroxidase, superoxide dismutases (SOD), and heat shock proteins (HSPs). The SOD catalyze the dismutation of superoxide into oxygen and hydrogen peroxide during physiological and pathological conditions, including aging. It has been demonstrated that the expression and activity of the SOD system are modified in aging, with reduced cell ability to counteract the oxidant molecules, and consequent weak resistance to ROS accumulation [21]. Obviously, cytotypes with limited replication ability, such as brain and heart, are particularly vulnerable to this phenomenon, suggesting that it could explain, at least in part, high prevalence of cardiovascular and neurological disorders in old people [22]. In fact it is widely known that oxidative stress and reduced antioxidant defense have negative effects on cardiac structure and function [23], and they are also involved in lipid membrane oxidation and other heart age-related conditions. Rinaldi et al. [21] showed that high levels of cardiac lipid peroxidation in sedentary old rats were not paired by increased SOD expression. These findings suggested that aging could be associated with increased oxidants without significant changes in antioxidant activity [24]. HSPs are another system of cellular defense against oxidative stress. These "stress-induced proteins" are ubiquitous and highly conserved chaperones, important in the folding of new synthesized or damaged proteins. Moreover HSPs mediate mitochondrial protection against oxidative stress and some of those such as HSP70 have been associated with myocardial protection. Martin et al. [25] showed an increased survival in HSP70-transfected cardiomyocytes and consequent increased expression of the HSP70 enzyme against ischemic cardiac damage. The Rinaldi's study [21] suggested that aging adversely affects HSP70 protein expression with increased levels of HSP27.

Snoeckx et al. [26] showed doubled HSP27 levels during the first 16 hours after heat shock. It is probable that many different aspects of the aged heart are at least partially caused by accumulation of oxidative damage. It has been found that cardiac fibrosis and size of myocytes increase with aging, while the number of myocytes decreases and ventricular hypertrophy is almost a constant finding in the aging rat heart [27–29]. All these findings may be related to oxidative stress, change of calcium homeostasis, with reference to the prolonged calcium transient and contraction, and decreased calcium sequestration by sarcoplasmic reticulum, characteristics of senescent heart. Another important mechanism involved in cellular aging process is represented by family of sirtuins, a cluster of proteins composed by seven homologues that regulate cellular biology and metabolism through deacetylation of histones and other cellular factors such as NFkB, HSF1, p53, FOXOs, and PGC-1. SIRT1, the human homologous of the family, is involved in many functions of human physiology, including DNA repair, cell cycle regulation, apoptosis, gene expression, and aging [30]. By FOXO3 acetylation and/or phosphorylation oxidative stress induces arrangement of SIRT1-FOXO3a,

complex indispensable for cell cycle arrest and induction of DNA repair [31]. SIRT1 can modulate the cellular stress response directly deacetylating some proteins and regulating their expression [32]. In fact SIRT1 inhibition determines transcription suppression of genes activated by exposure to heat shock. On the contrary, SIRT1 activation byagents such as resveratrol, or SIRT1 overexpression enhances the heat shock response [33]. Furthermore, SIRT1 modulates the threshold of cell death in the setting of exogenous stress, including oxidative damage, interacting with p53, inhibits Bax-induced apoptosis by deacetylation of Ku70, and regulation of other targets linked to cell death [34]. Then the ability of SIRT1 to modulate stress resistance is multifaceted and it is not only linked to oxidative stress, but also to other stressful stimuli that play a role in the cellular aging, such as telomere shortening and other types of genotoxic stress.

2. Physical Activity and Oxidative Stress

The physical activity increases the expression and the activity of antioxidant enzymes, with consequent reduction of ROS. In part, it seems that positive effects of the physical exercise on senile heart in terms of antioxidant activity could be ascribable to a greater expression and activity of SOD and HSPs.

Rinaldi et al. [21] showed that a physical training program induced high levels of SOD and increased HSP70 and of HSP27 expression in trained old rats compared to sedentary old and young rats. This finding was in accordance with other studies where training protocols were able to increase SOD activity in sedentary old rats [24].

Physical activity has been demonstrated able to reduce generation of oxidants during ischemia-reperfusion damage and to have a calcium-protective role via activation of the ROS scavenger MnSOD. This better oxidative status consequent to a correct program of physical activity is partially responsible for some benefits (such as decreased arterial stiffness, improved endothelial function and metabolic and clotting setting, and reduced body weight).

The relation between aging and accumulation of oxidatively damaged proteins, lipids, and nucleic acids explains how a high resistance to the oxidative stress is associated with increased lifespan, as suggested by many studies in various model organisms [35, 36]. An 8-week training period has been shown to reduce body weight and cardiac abnormalities characteristic of senescence heart, such as structural changes, ventricular systolic pressure, left ventricle weight/body weight ratio, and heart rate [37], suggesting that exercise training antioxidant effects might be also mediated by mechanisms involving metabolic pathways.

Other findings derived from clinical studies show that regular physical activity decreases cardiovascular comorbidity and mortality in adult and in elderly by restoring the protective effect of ischemic preconditioning [38] and partially contrasting loss of antioxidant defense in the aged heart [37]. In particular, it has been demonstrated that the physical activity in the heart failure works at cardiac level increasing angiogenesis and at levels of adrenal medulla restoring the GRK2-alpha 2 adrenergic receptor axis which regulates the production of catecholamines [39, 40], and therefore it is able to increase norepinephrine release in response to a preconditioning stimulus [41]. This phenomenon has been suggested to be also involved in the cardioprotective effects of exercise training and caloric restriction. In fact, it has been demonstrated that exercise training and food restriction produce together total preservation of ischemic preconditioning in the aging heart, and that one of the mechanisms responsible for early ischemic preconditioning conservation in the aging heart maybe the restoration of norepinephrine release in response to preconditioning stimulus, as confirmed by the absence of the protection by reserpine administration [42, 43]

Thus physical activity delays the damage accumulation and consequent physiological dysfunction characteristic of aging process.

An interesting observation is that both exercise training and caloric restriction improve the antioxidant system, and this could also explain the analogy between benefits derived by these tools.

By observation that caloric restriction induces increased lifespan by induction of sirtuins activity and that both exercise training and caloric restriction share many benefits it has been hypothesized that SIRT1 is also involved in the physical activity effects, whereas only caloric-restricted subjects showed an increased lifespan [1].

SIRT1 has been implicated in many different processes, such as glucose metabolism and fat mobilization/metabolism. It is possible that SIRT1 mediates skeletal muscle adaptations to endurance exercise by regulating cytosolic NAD^+/NADH ratio changes during muscular contraction. It has been shown that physical activity is able to increase activity of SIRT1 in aged old rats [22], and this finding could be linked to increased NOS expression in skeletal muscle after contraction induced by electrical stimulation in vivo [44].

3. Cellular Models of Physical Activity and Aging

Physical activity is one of the best methods in treatment of age-related chronic-degenerative diseases and in prevention of changes associated with aging.

The selection of exercise program is also important, in fact it has been noticed that antioxidant effects could increase, decrease, or not change in relation with the exercise protocol [37]. Recommendable physical activity should be aerobic, with constant and regular exercise training, not vigorous or intense and planned adjusting the physical activity to the characteristics of subject in order to avoid injuries and to represent useful means of prevention in the elderly. Although the beneficial effects of the exercise are well known in terms of pathophysiology and hemodynamics, very little is known about the molecular bases that are responsible for the effectiveness of physical activity in humans, as previously mentioned. Moreover all studies carried out to investigate this topic have been performed "in vivo" on humans or by

using "in vitro/in vivo" animal models. In particular, study on human sera is not able to show the cellular changes induced by physical activity, such as in vivo experiments in animal model could not be translated to human experience. A demonstration of the controversial debate on this topic is provided by the recent review by Lee et al. [45] that reported that in-human oxidative stress represents a key feature in all stages of cardiovascular disease. In particular, the authors underline the importance of in-human measurement of oxidative stress circulating biomarkers, some of them with predictive value in cardiovascular disease, but Lee et al. also concluded that these biomarkers do not necessarily reflect intravascular oxidative stress and therefore cannot be used as therapeutic targets or markers to monitor pharmacological treatments in clinical settings.

On the other hand, in animal models several studies demonstrated the molecular basis underlining the physical activity effects. Yang et al. [46] showed that training induces a significant enlargement of collateral vessels in striated ischemic muscle and that these effects were lost upon inhibition of NO production. Eksakulkla et al. [47] suggested that physical activity could improve endothelial dysfunction in aged rats by increasing NO bioavailability. Moreover, Ferrara et al. [37] demonstrated that exercise training, which significantly increases SIRT1 activity, could counteract age-related systems impairment, by activation of antioxidant systems and DNA repair and disability [48], suggesting therefore a possible role of exercise training in conditioning lifespan (Figure 1).

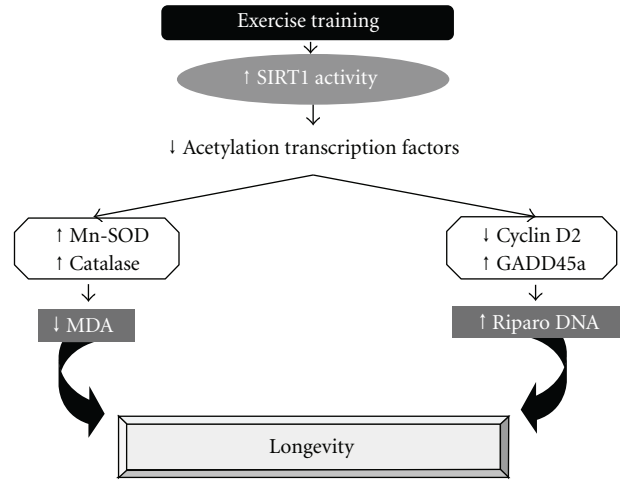


Figure 1: Mechanism of exercise training through SIRT1 activity on antioxidant enzymes and factors involved in cell cycle. Exercise training induces increased SIRT1 activity that is responsible both of raised activation of antioxidants system (by MnSOD and Catalase) and cell cycle arrest to promote DNA repair. These phenomena could induce extended lifespan.

Although studies in animal models help to examine the changes induced by exercise both at the molecular and functional level, they may not accurately represent what happens in humans in their entirety and complexity during aging.

Obviously, in order to better understand the changes induced by exercise in the elderly, it is necessary to first define "normality" of such modifications, by studying what physical activity induces on blood and cells in healthy young subjects.

An interesting approach is represented by that one used by Conti [49]. Based on Allard's paper [50] in which the authors examined the effects of sera from calorie-restricted subjects on cultured cells, Conti et al. investigated the effects of sera collected from three groups of athletes characterized by different types of sports (aerobic, anaerobic, or mixed) on human endothelial cells [49]. After determination of some antioxidant enzymes activities and levels of oxidants in the sera, they used the same sera to treat cellular lines. In particular human endothelial cell lines EA.hy 926 (ECs) and primary human umbilical vein (HUVEC) were used for the in vitro experiments. The method consisted in growing EA.hy 926 and HUVEC in an endothelial growth medium supplemented with the athletes' sera and exposed or not to oxidative stress. On these cultures the levels of different enzymes and oxidants molecules, and parameters of survival, senescence, and viability were determined. By these experiments the authors demonstrated that survival and proliferation rates were higher in ECs supplemented with serum of athletes performing an aerobic training than in ECs treated with sera of a athletes performing an anaerobic or mixed sport. Moreover, by the evidence that changes in many functional aspects of cell behavior, including proliferation, are closely related to NO bioavailability [51, 52] and that NO production is strongly limited in senescent ECs [53]. In Conti's paper [49] the highest survival and proliferation rates and the lowest senescence observed in endothelial cells conditioned with aerobic serum, both at baseline and after stress induction, suggest that these changes could depend, at least in part, on high NO bioavailability in the aerobic exercise serum. Therefore, the high NO bioavailability in serum from athletes undergoing aerobic training produces favorable effects on human endothelial cells. Finally they concluded that different types of exercise training induced different molecular effects in terms of survival, morphology, and antioxidant system efficiency [49].

The in vivo/vitro technique used in this paper is interesting because for the first time connects the physiological effects of physical activity on the blood cells to molecular changes induced by the same blood cells on endothelial cells, by helping to shed light on the molecular basis of effects of long-term physical activity in humans. The issue of how exercise may favorably affect the modifications related to aging is even more complex. In fact, in aging investigation it is difficult to discern the effects of senescence "per se" from those produced by conditions highly prevalent, such as hypertension, diabetes, and atherosclerosis [54, 55]. Based on these data, that represent the basis to understand the "normal" response of different sera in these "in vitro/vivo" experiments we have planned a similar study.

Cardiac rehabilitation, including physical exercise as an integral part, has been recognized as a fundamental component in the continuum of care for patients with heart failure. Advancements in the understanding of oxidative metabolism, the effects of physical activity on oxidative stress

[56], the intracellular energy transfer in both skeletal and cardiac muscle, and mechanisms of endothelial dysfunction provide possible explanations of the pathophysiologic factors involved in the development of exercise intolerance in chronic heart failure patients [57], but again they are not sufficient to represent a molecular target for treatment. Exercise training promotes SIRT1 activity [22] in animal models and it improves cardiovascular function and enhances endothelial homeostasis in patients with cardiovascular diseases. However, also the study on aging and heart failure and their connections are addressed only on pathophysiology or cellular aspects, because the limitation in the available tools. By all of the evidence, the elderly with heart failure may represent the population that may derive more benefits from an understanding of these processes. From this point of view the technique used by Conti et al. [49] could represent a good tool to better understand how the exercise training mediates its effects on aging-related diseases, in particular in elderly with heart failure.

References

[1] G. Corbi, V. Conti, G. Scapagnini, A. Filippelli, and N. Ferrara, "Role of sirtuins, calorie restriction and physical activity in aging," *Frontiers in Bioscience (Elite Edition)*, vol. 4, pp. 768–778, 2012.

[2] S. Vasto, G. Scapagnini, M. Bulati et al., "Biomarkes of aging," *Frontiers in Bioscience (Scholar Edition)*, vol. 2, pp. 392–402, 2010.

[3] M. Galderisi, V. S. Lomoriello, A. Santoro et al., "Differences of myocardial systolic deformation and correlates of diastolic function in competitive rowers and young hypertensives: a speckle-tracking echocardiography study," *Journal of the American Society of Echocardiography*, vol. 23, no. 11, pp. 1190–1198, 2010.

[4] C. H. Davies, N. Ferrara, and S. E. Harding, "β-Adrenoceptor function changes with age of subject in myocytes from non-failing human ventricle," *Cardiovascular Research*, vol. 31, no. 1, pp. 152–156, 1996.

[5] P. Abete, C. Napoli, G. Santoro et al., "Age-related decrease in cardiac tolerance to oxidative stress," *Journal of Molecular and Cellular Cardiology*, vol. 31, no. 1, pp. 227–236, 1999.

[6] S. Besse, C. Delcayre, B. Chevalier et al., "Is the senescent heart overloaded and already failing?" *Cardiovascular Drugs and Therapy*, vol. 8, no. 4, pp. 581–587, 1994.

[7] G. Vitale, M. Galderisi, A. Colao et al., "Circulating IGF-I levels are associated with increased biventricular contractility in top-level rowers," *Clinical Endocrinology (Oxford)*, vol. 69, no. 2, pp. 231–236, 2008.

[8] N. Ferrara, P. Abete, G. Corbi et al., "Insulin-induced changes in β-adrenergic response: an experimental study in the isolated rat papillary muscle," *American Journal of Hypertension*, vol. 18, no. 3, pp. 348–353, 2005.

[9] L. Amato, G. Paolisso, F. Cacciatore et al., "Congestive heart failure predicts the development of non-insulin-dependent diabetes mellitus in the elderly. The Osservatorio Geriatrico Regione Campania Group," *Diabetes and Metabolism*, vol. 23, no. 3, pp. 213–218, 1997.

[10] M. Zheng, W. Zhu, Q. Han, and R. P. Xiao, "Emerging concepts and therapeutic implications of β-adrenergic receptor subtype signaling," *Pharmacology and Therapeutics*, vol. 108, no. 3, pp. 257–268, 2005.

[11] N. Ferrara, K. Davia, P. Abete, F. Rengo, and S. E. Harding, "Alterations in β-adrenoceptor mechanisms in the aging heart. Relationship with heart failure," *Aging—Clinical and Experimental Research*, vol. 9, no. 6, pp. 391–403, 1997.

[12] G. Rengo, C. Zincarelli, G. D. Femminella et al., "Myocardial β_2-adrenoceptor gene delivery promotes coordinated cardiac adaptive remodelling and angiogenesis in heart failure," *British Journal of Pharmacology*, vol. 166, no. 8, pp. 2348–2361, 2012.

[13] G. Rengo, A. Lymperopoulos, C. Zincarelli et al., "Blockade of β-adrenoceptors restores the GRK2-mediated adrenal α_2-adrenoceptor-catecholamine production axis in heart failure," *British Journal of Pharmacology*, vol. 166, no. 8, pp. 2430–2440, 2012.

[14] G. Corbi, D. Acanfora, G. L. Iannuzzi et al., "Hypermagnesemia predicts mortality in elderly with congestive heart disease: relationship with laxative and antacid use," *Rejuvenation Research*, vol. 11, no. 1, pp. 129–138, 2008.

[15] K. L. Koss and R. D. Grubbs, "Elevated extracellular Mg^{2+} increases Mg^{2+} buffering through a Ca-dependent mechanism in cardiomyocytes," *American Journal of Physiology*, vol. 267, no. 2, pp. C633–C641, 1994.

[16] S. Kawano, "Dual mechanisms of Mg^{2+} block of ryanodine receptor Ca^{2+} release channel from cardiac sarcoplasmic reticulum," *Receptors and Channels*, vol. 5, no. 6, pp. 405–416, 1998.

[17] G. Longobardi, P. Abete, N. Ferrara et al., "'Warm-up' phenomenon in adult and elderly patients with coronary artery disease: further evidence of the loss of "ischemic preconditioning" in the aging heart," *Journals of Gerontology—Series A*, vol. 55, no. 3, pp. M124–M129, 2000.

[18] P. Abete, A. Cioppa, C. Calabrese et al., "Ischemic threshold and myocardial stunning in the aging heart," *Experimental Gerontology*, vol. 34, no. 7, pp. 875–884, 1999.

[19] C. P. Campobasso, A. S. Dell'Erba, A. Addante, F. Zotti, A. Marzullo, and M. F. Colonna, "Sudden cardiac death and myocardial ischemia indicators: a comparative study of four immunohistochemical markers," *American Journal of Forensic Medicine and Pathology*, vol. 29, no. 2, pp. 154–161, 2008.

[20] N. Ferrara, P. Abete, G. Ambrosio et al., "Protective role of chronic ubiquinone administration on acute cardiac oxidative stress," *Journal of Pharmacology and Experimental Therapeutics*, vol. 274, no. 2, pp. 858–865, 1995.

[21] B. Rinaldi, G. Corbi, S. Boccuti et al., "Exercise training affects age-induced changes in SOD and heat shock protein expression in rat heart," *Experimental Gerontology*, vol. 41, no. 8, pp. 764–770, 2006.

[22] A. Navarro-Arévalo, C. Cañavate, and M. J. Sánchez-del-Pino, "Myocardial and skeletal muscle aging and changes in oxidative stress in relationship to rigorous exercise training," *Mechanisms of Ageing and Development*, vol. 108, no. 3, pp. 207–217, 1999.

[23] P. K. Singal, A. Petkau, J. M. Gerrard, S. Hrushovetz, and J. Foerster, "Free radicals in health and disease," *Molecular and Cellular Biochemistry*, vol. 84, no. 2, pp. 121–122, 1988.

[24] F. Gündüz, U. K. Sentürk, O. Kuru, B. Aktekin, and M. R. Aktekin, "The effect of one year's swimming exercise on oxidant stress and antioxidant capacity in aged rats," *Physiological Research*, vol. 53, no. 2, pp. 171–176, 2004.

[25] J. L. Martin, R. Mestril, R. Hilal-Dandan, L. L. Brunton, and W. H. Dillmann, "Small heat shock proteins and protection against ischemic injury in cardiac myocytes," *Circulation*, vol. 96, no. 12, pp. 4343–4348, 1997.

[26] L. H. E. H. Snoeckx, R. N. Cornelussen, F. A. Van Nieuwenhoven, R. S. Reneman, and G. J. Van Der Vusse, "Heat shock

proteins and cardiovascular pathophysiology," *Physiological Reviews*, vol. 81, no. 4, pp. 1461–1497, 2001.

[27] P. Anversa, B. Hiler, R. Ricci, G. Guideri, and G. Olivetti, "Myocyte cell loss and myocyte hypertrophy in the aging rat heart," *Journal of the American College of Cardiology*, vol. 8, no. 6, pp. 1441–1448, 1986.

[28] S. Besse, V. Robert, P. Assayag, C. Delcayre, and B. Swynghedauw, "Nonsynchronous changes in myocardial collagen mRNA and protein during aging: effect of DOCA-salt hypertension," *American Journal of Physiology*, vol. 267, no. 6, pp. H2237–H2244, 1994.

[29] M. Klima, T. R. Burns, and A. Chopra, "Mycardial fibrosis in the elderly," *Archives of Pathology and Laboratory Medicine*, vol. 114, no. 9, pp. 938–942, 1990.

[30] O. Grubisha, B. C. Smith, and J. M. Denu, "Small molecule regulation of Sir2 protein deacetylases," *FEBS Journal*, vol. 272, no. 18, pp. 4607–4616, 2005.

[31] A. Brunet, L. B. Sweeney, J. F. Sturgill et al., "Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase," *Science*, vol. 303, no. 5666, pp. 2011–2015, 2004.

[32] M. Porcu and A. Chiarugi, "The emerging therapeutic potential of sirtuin-interacting drugs: from cell death to lifespan extension," *Trends in Pharmacological Sciences*, vol. 26, no. 2, pp. 94–103, 2005.

[33] S. D. Westerheide, J. Anckar, S. M. Stevens Jr., L. Sistonen, and R. I. Morimoto, "Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT," *Science*, vol. 323, no. 5917, pp. 1063–1066, 2009.

[34] H. Y. Cohen, C. Miller, K. J. Bitterman et al., "Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase," *Science*, vol. 305, no. 5682, pp. 390–392, 2004.

[35] Z. Ungvari, R. Buffenstein, S. N. Austad, A. Podlutsky, G. Kaley, and A. Csiszar, "Oxidative stress in vascular senescence: lessons from successfully aging species," *Frontiers in Bioscience*, vol. 13, no. 13, pp. 5056–5070, 2008.

[36] Heart Protection Study Collaborative Group, "MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20 536 high-risk individuals: a randomised placebo-controlled trial," *The Lancet*, vol. 360, no. 9326, pp. 23–33, 2002.

[37] N. Ferrara, B. Rinaldi, G. Corbi et al., "Exercise training promotes SIRT1 activity in aged rats," *Rejuvenation Research*, vol. 11, no. 1, pp. 139–150, 2008.

[38] P. Abete, N. Ferrara, F. Cacciatore et al., "High level of physical activity preserves the cardioprotective effect of preinfarction angina in elderly patients," *Journal of the American College of Cardiology*, vol. 38, no. 5, pp. 1357–1365, 2001.

[39] D. Leosco, G. Rengo, G. Iaccarino et al., "Exercise promotes angiogenesis and improves β-adrenergic receptor signalling in the post-ischaemic failing rat heart," *Cardiovascular Research*, vol. 78, no. 2, pp. 385–394, 2008.

[40] G. Rengo, D. Leosco, C. Zincarelli et al., "Adrenal GRK2 lowering is an underlying mechanism for the beneficial sympathetic effects of exercise training in heart failure," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 298, no. 6, pp. H2032–H2038, 2010.

[41] P. Abete, C. Calabrese, N. Ferrara et al., "Exercise training restores ischemic preconditioning in the aging heart," *Journal of the American College of Cardiology*, vol. 36, no. 2, pp. 643–650, 2000.

[42] P. Abete, G. Testa, G. Galizia et al., "Tandem action of exercise training and food restriction completely preserves ischemic preconditioning in the aging heart," *Experimental Gerontology*, vol. 40, no. 1-2, pp. 43–50, 2005.

[43] P. Abete, G. Testa, N. Ferrara et al., "Cardioprotective effect of ischemic preconditioning is preserved in food-restricted senescent rats," *American Journal of Physiology*, vol. 282, no. 6, pp. H1978–H1987, 2002.

[44] E. Nisoli, C. Tonello, L. Briscini, and M. O. Carruba, "Inducible nitric oxide synthase in rat brown adipocytes: implications for blood flow to brown adipose tissue," *Endocrinology*, vol. 138, no. 2, pp. 676–682, 1997.

[45] R. Lee, M. Margaritis, K. M. Channon, and C. Antoniades, "Evaluating oxidative stress in human cardiovascular disease: methodological aspects and considerations," *Current Medicinal Chemistry*, vol. 19, no. 16, pp. 2504–2520, 2012.

[46] H. T. Yang, B. M. Prior, P. G. Lloyd et al., "Training-induced vascular adaptations to ischemic muscle," *Journal of Physiology and Pharmacology*, vol. 59, no. 7, pp. 57–70, 2008.

[47] S. Eksakulkla, D. Suksom, P. Siriviriyakul, and S. Patumraj, "Increased NO bioavailability in aging male rats by genistein and exercise training: using 4, 5-diaminofluorescein diacetate," *Reproductive Biology and Endocrinology*, vol. 7, article 93, 2009.

[48] F. Cacciatore, P. Abete, S. Maggi et al., "Disability and 6-year mortality in elderly population. Role of visual impairment," *Aging—Clinical and Experimental Research*, vol. 16, no. 5, pp. 382–388, 2004.

[49] V. Conti, G. Corbi, G. Russomanno et al., "Oxidative stress effects on endothelial cells treated with different athletes' sera," *Medicine & Science in Sports & Exercise*, vol. 44, no. 1, pp. 39–49, 2012.

[50] J. S. Allard, L. K. Heilbronn, C. Smith et al., "In vitro cellular adaptations of indicators of longevity in response to treatment with serum collected from humans on calorie restricted diets," *PLoS ONE*, vol. 3, no. 9, Article ID e3211, 2008.

[51] A. Hassid, J. Yao, and S. Huang, "NO alters cell shape and motility in aortic smooth muscle cells via protein tyrosine phosphatase 1B activation," *American Journal of Physiology*, vol. 277, no. 3, pp. H1014–H1026, 1999.

[52] F. Fazio, L. Lionetto, G. Molinaro et al., "Cinnabarinic acid, an endogenous metabolite of the kynurenine pathway, activates type 4 metabotropic glutamate receptors," *Molecular Pharmacology*, vol. 81, no. 5, pp. 643–656, 2012.

[53] I. Sato, I. Morita, K. Kaji, M. Ikeda, M. Nagao, and S. Murota, "Reduction of nitric oxide producing activity associated with in vitro aging in cultured human umbilical vein endothelial cell," *Biochemical and Biophysical Research Communications*, vol. 195, no. 2, pp. 1070–1076, 1993.

[54] D. F. Dai and P. S. Rabinovitch, "Cardiac aging in mice and humans: the role of mitochondrial oxidative stress," *Trends in Cardiovascular Medicine*, vol. 19, no. 7, pp. 213–220, 2009.

[55] D. Dutta, R. Calvani, R. Bernabei, C. Leeuwenburgh, and E. Marzetti, "Contribution of impaired mitochondrial autophagy to cardiac aging: mechanisms and therapeutic opportunities," *Circulation Research*, vol. 110, no. 8, pp. 1125–1138, 2012.

[56] Z. Ungvári, S. A. Gupte, F. A. Recchia, S. Bátkai, and P. Pacher, "Role of oxidative-nitrosative stress and downstream pathways in various forms of cardiomyopathy and heart failure," *Current Vascular Pharmacology*, vol. 3, no. 3, pp. 221–229, 2005.

[57] J. Downing and G. J. Balady, "The role of exercise training in heart failure," *Journal of the American College of Cardiology*, vol. 58, no. 6, pp. 561–569, 2011.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 854502, 8 pages
doi:10.1155/2012/854502

Research Article

Overexpression of Fatty-Acid-β-Oxidation-Related Genes Extends the Lifespan of *Drosophila melanogaster*

Shin-Hae Lee,[1] Su-Kyung Lee,[1] Donggi Paik,[2] and Kyung-Jin Min[1]

[1] *Department of Biological Sciences, Inha University, 100 Inha-ro, Nam-gu, Incheon 402-751, Republic of Korea*
[2] *Department of Biological Sciences, Korea Advanced Institute of Science & Technology, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea*

Correspondence should be addressed to Shin-Hae Lee, lmjinee@inha.ac.kr and Kyung-Jin Min, minkj@inha.ac.kr

Received 14 May 2012; Revised 23 July 2012; Accepted 3 August 2012

Academic Editor: Heinz D. Osiewacz

A better understanding of the aging process is necessary to ensure that the healthcare needs of an aging population are met. With the trend toward increased human life expectancies, identification of candidate genes affecting the regulation of lifespan and its relationship to environmental factors is essential. Through misexpression screening of EP mutant lines, we previously isolated several genes extending lifespan when ubiquitously overexpressed, including the two genes encoding the fatty-acid-binding protein and dodecenoyl-CoA delta-isomerase involved in fatty-acid β-oxidation, which is the main energy resource pathway in eukaryotic cells. In this study, we analyzed flies overexpressing the two main components of fatty-acid β-oxidation, and found that overexpression of fatty-acid-β-oxidation-related genes extended the *Drosophila* lifespan. Furthermore, we found that the ability of dietary restriction to extend lifespan was reduced by the overexpression of fatty-acid-β-oxidation-related genes. Moreover, the overexpression of fatty-acid-β-oxidation-related genes enhanced stress tolerance to oxidative and starvation stresses and activated the dFOXO signal, indicating translocation to the nucleus and transcriptional activation of the dFOXO target genes. Overall, the results of this study suggest that overexpression of fatty-acid-β-oxidation-related genes extends lifespan in a dietary-restriction-related manner, and that the mechanism of this process may be related to FOXO activation.

1. Introduction

The trend towards increased life expectancy demands a greater understanding of the aging process to ensure that healthcare needs of an aging population are met. This goal requires identification of the so-called "longevity candidate genes," which are potential genes important to the regulation of lifespan, as well as appropriate understanding of how the effects of these genes are modulated by environmental factors such as diet. Numerous longevity candidate genes have been identified in model systems using extended longevity mutant phenotypes, offering important insights into the mechanisms of aging and lifespan determination [1–5]. Insulin/insulin-like growth factor (IGF) signaling (IIS), a major nutrient-sensing pathway, is a well-characterized age-related pathway. The loss of IIS function by mutations affecting insulin/IGF receptor, phosphatidylinositol-3 kinase (PI3K), Akt, and forkhead box (FOXO) has been found to extend the lifespan of *C. elegans*, *Drosophila,* and mammals [3, 6–11]. In addition, energy-sensing pathways such as those associated with sirtuins, target of rapamycin (TOR) and AMP-activated protein kinase (AMPK) signaling are well known to be linked to the aging process [3–5, 12]. As nutrient-sensing pathways are linked to aging, the reduction of dietary intake, namely dietary restriction, also extends the lifespan of various model systems [13–16]. Furthermore, the ecdysteroid hormone pathway is known to modulate organismal lifespan [17, 18].

While investigating longevity candidate genes, we previously conducted misexpression screening of EP lines containing 14 copies of upstream activator sequence (UAS) to which Gal4 binds, allowing conditional overexpression or knockdown of genes of flanking genomic DNA located downstream of the basal promoter dependent on its insertion orientation [19]. In that study, we preliminary selected 40 EP lines to demonstrate the lifespan extension, including

the two EP lines (EP^{CG6783}, $EP^{CG13890}$) targeting fatty-acid-β-oxidation-related genes (*CG6783*, *CG13890*), but they were excluded from further investigation since they were induced in the absence of Gal4 driver [19]. It has long been suggested that lipid metabolism plays a central role in regulation of the metazoan lifespan. One of the well-known longevity-candidate genes, AMPK, was reported to regulate fatty-acid synthesis and oxidation through the phosphorylation of acetyl-CoA-carboxylase [20]. In addition, calorie restriction and IIS mutation has been reported to promote fatty-acid β-oxidation [21, 22]. However, there has been no direct evidence of lifespan extension through the modulation of fatty-acid β-oxidation to date, except for our previous study [19], in which we did not investigate the relationship with dietary restriction and its underlying mechanisms.

In the current study, we analyzed EP lines that overexpressed two main components of fatty-acid β-oxidation and found that the overexpression of fatty-acid β-oxidation related genes extended their lifespan in a dietary-restriction-related manner, increased their stress resistance, and activated the FOXO transcription factor.

2. Materials and Methods

2.1. Fly Stocks and Food Preparation. *Drosophila melanogaster* were cultured and reared at 25°C. Cantonized *white* (*CS10* [23]) was used as wild-type control. The EP^{CG6783} (GX62810) and $EP^{CG13890}$ (GX4385) lines, which carry the P-element mediated upstream activator sequence (UAS) on the 5′ untranslated region of the *CG6783* or *CG13890* genes, respectively, were obtained from GenExel Inc. (KAIST Bio Medical Research Center, Korea). To generate *UAS-CG6783* flies, the full open reading frame of *fabp*-RA from RH46282 (Drosophila Genomics Resource Center, Bloomington, USA) was cloned into p*UAST* using *Eco*RI/*Bgl*II sites. Standard germline transformation into a w^{1118} background was then performed for transgenic lines. Corn meal-sugar-yeast (CSY) media (5.2% cornmeal, 11% sucrose, 2.4% yeast, 0.8% agar, and 0.2% methyl-4-hydroxybenzoate (Sigma-Aldrich, St. Louis, MO, USA)) was used for larval development and routine culture. In the dietary restriction (DR) experiment, the concentration of yeast in the media fed to separate groups of flies was 2, 4, 8, 12, and 16%.

2.2. Lifespan Assays. Newly eclosed F1 generations were collected over 48 hours and the males were randomly assigned to 500 mL demography cages to achieve a final density of 100 male flies per cage. Food vials containing SY diet (10% sucrose, the indicated concentration of yeast, 0.2% methyl-4-hydroxybenzoate, and 0.8% agar) were affixed to separate cages and changed every two days, at which time the dead flies were removed and recorded. Three replicate cages were established for this experiment.

2.3. Stress-Resistance Assay

Oxidative Stress. Ten-day-old flies (20 males per vial) were fed SY medium supplemented with 18 mM paraquat (methyl viologen dichloride hydrate, Sigma-Aldrich). The flies were transferred into fresh vials containing paraquat solution every six hours, and the dead flies were scored after each transfer. Fifteen replicates were established.

Starvation Stress. Newly eclosed flies were kept in vials (20 males per vial) containing 1% agar and transferred into fresh vials containing agar every six hours. Dead flies were scored after each transfer. Fifteen replicates were established.

2.4. Immunostaining of the Larval Fat Bodies. Dissected third instar larvae were fixed with 4% paraformaldehyde (USB Corp., Cleveland, OH, USA) for 30 min, washed with phosphate-buffered saline/0.1% Triton X-100/2% bovine serum albumin (PBST-BSA), and then incubated overnight with primary antibodies in PBST-BSA at 4°C. Samples were then washed in PBST-BSA, incubated with Alexa488 (Molecular Probes, Eugene, OR, USA) for 1 hour at 25°C, and washed and mounted with Vectashield (Vector Labs, CA, USA). The resulting images were analyzed using a Confocal Laser Scanning Microscope (LSM510 META, Carl Zeiss Inc., Germany). Anti-dFOXO antibody (a gift from O. Puig) was diluted to 1 : 300 in 2% BSA solution. DAPI was used to counterstain the nuclei.

2.5. Real-Time qPCR. Five-day-old adults were frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA from homogenized whole-body lysates was prepared with RNAiso reagent (TAKARA, Japan). Next, total RNA (5 μg) was reverse-transcribed using PrimeScript RT Reagent Kit (TAKARA) and real-time qPCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, USA) using SYBR Premix Ex-Taq II (TAKARA). Mean induction folds were calculated from the values of 3–6 independent experiments and statistically evaluated by a Student *t*-test.

2.6. Statistical Analysis. Data are presented as the mean ± SEM. Statistical analyses for the demographic data were carried out using standard survival models in the JMP statistical package (SAS, Cary, NC, USA).

3. Results

3.1. Overexpression of Fatty-Acid β-Oxidation Components Extended Lifespan in a Dietary-Restriction-Dependent Manner. In the previous study, we selected long-lived EP lines, which extend lifespan when crossed to *da-Gal4* driver [19]. Among them, the two EP lines (GX62810, GX4385) targeting fatty-acid-β-oxidation-related genes (*CG6783*, *CG13890*) were of interest, however, they were excluded to further investigation in the previous study because they were induced in the absence of Gal4 driver [19]. *CG6783* encodes the fatty-acid-binding protein (FABP), which facilitates the intracellular movement of fatty acids, thus permitting the initiation of fatty-acid oxidation [24], while *CG13890* encodes the dodecenoyl-CoA delta-isomerase (DCI) localized in the inner mitochondria where it catalyzes the

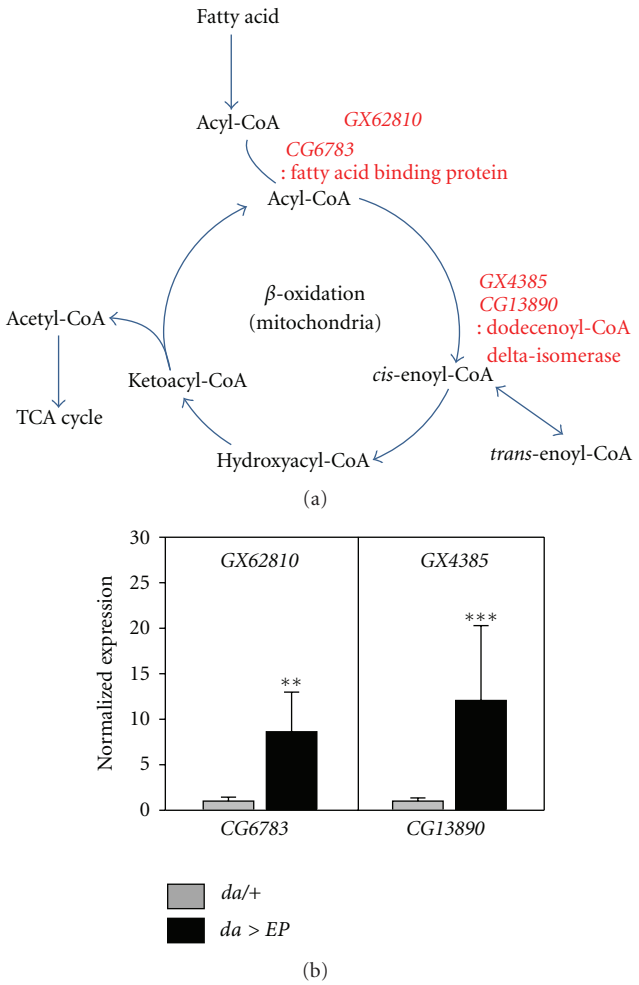


Figure 1: Overexpression of fatty-acid β-oxidation-related genes using EP lines. (a) Schematic representation of fatty-acid β-oxidation. CG6783 encodes the fatty-acid-binding protein, which mediates the transportation of acyl-CoA to cellular organelles. CG13890 encodes dodecenoyl-CoA delta-isomerase, which modifies *cis*-enoyl-CoA to *trans*-enoyl-CoA, a common substrate for enoyl-CoA hydratase in the β-oxidation cycle of saturated fatty acyl-CoA esters. (b) Overexpression of *CG6783* or *CG13890* using EP lines (GX62610, GX4385) and *da-Gal4*. The mRNA of *CG6783* or *CG13890* was analyzed in the whole body RNA extract from *da > EP* (black bars) and *da/+* (gray bars) flies. Significance was determined via a *t*-test ($^{**}P < 0.001$, $^{***}P < 0.0001$).

degradation of long-chain fatty acids during fatty-acid β-oxidation [25, 26] (Figure 1(a)). We confirmed that the EP lines overexpressed *CG6783* or *CG13890* when crossed with the *da-Gal4* driver compared to *da/+* flies via real-time qPCR (Figure 1(b)).

To further assess the EP lines, they were crossed with *da-Gal4* driver or wild-type control stock to produce the *da/EP* and two controls (*EP/+* and *da/+*) after backcrossing eight times to rule out heterosis. Consistent with previous reports [19], the overexpression of these two fatty-acid β-oxidation-related genes using the *da-Gal4* driver increased lifespan. In media containing 16% yeast, the median lifespan of *CG6783*- or *CG13980*-overexpressing flies was extended to nearly 58 or 42 days, respectively, from the 32 days observed for the *da/+* control flies (Figure 2(a)). In addition, the EP line overexpressing *CG6783* also showed extended maximum lifespan (closed-square line in Figure 2(a)), and the two EP lines consistently reduced mortality rate across adult ages (Figure 2(b)). However, it should be noted that the *EP/+* control cohorts without the Gal4 driver showed longer lifespans than the parental cohorts (open-square line and open-triangle line, Figure 2(a)), which could be considered to be the side effect of EP insertion to express the target genes under control of the basal promoter possessed in the EP.

To further confirm the extension of lifespan by the β-oxidation-related gene, we used the *UAS-CG6783* transgenic line, which was generated by standard germ line transformation using a p*UAST-CG6783* construct, and analyzed the lifespan when the transgene was driven in adults with the conditional Gene Switch (GS) driver system [27] to produce cohorts of identical genetic background. Female offspring of the *Act-GS-Gal4 > UAS-CG6783* genotype showed increased median lifespan compared to uninduced control (Figure 2(c)), indicating that the extension of lifespan by the β-oxidation-related gene occurs independently from the insertion site and the genetic background.

To investigate whether or not the mechanistic basis of dietary restriction has an effect on fatty-acid β-oxidation in association with lifespan extension, flies overexpressing fatty-acid oxidation components were fed an SY diet ranging from 2-to-16% yeast. The median lifespan of the control cohorts increased with decreasing yeast concentration [13, 28]. While control flies showed a 31.4% increase in lifespan upon 2% SY compared to 16% SY conditions (circular lines, Figures 2(d) and 2(e)), the flies expressing *CG6783* or *CG13890* showed a reduction in the lifespan extension with dietary restriction (12% or 15%, respectively, Figures 2(d) and 2(e)). These results indicated that the promotion of fatty-acid β-oxidation extends lifespan via a mechanism similar to dietary restriction.

3.2. Overexpression of Fatty-Acid β-Oxidation Components Increased Resistance to Oxidative and/or Starvation Stress. A positive relationship between stress tolerance and longevity has been well defined [29, 30], and long-lived organisms tend to be resistant to various forms of environmental stress [31]. Thus, we investigated the effects of overexpressed fatty-acid β-oxidation-related genes on stress resistance. To induce oxidative stress, flies overexpressing fatty-acid-β-oxidation components were subjected to feed dosed with 18 mM paraquat. We found that both of the flies overexpressing each fatty-acid-β-oxidation component showed substantial resistance to oxidative stress (Figures 3(a) and 3(b)). In addition, when subjected to nutrient deprivation, flies overexpressing *CG13890* showed more resistance to starvation than the control (Figure 3(d)), whereas the survival rate of the flies overexpressing *CG6783* was not significantly altered by starvation (Figure 3(c)). These results indicated that overexpression of fatty-acid β-oxidation-related genes increases lifespan and stress tolerance.

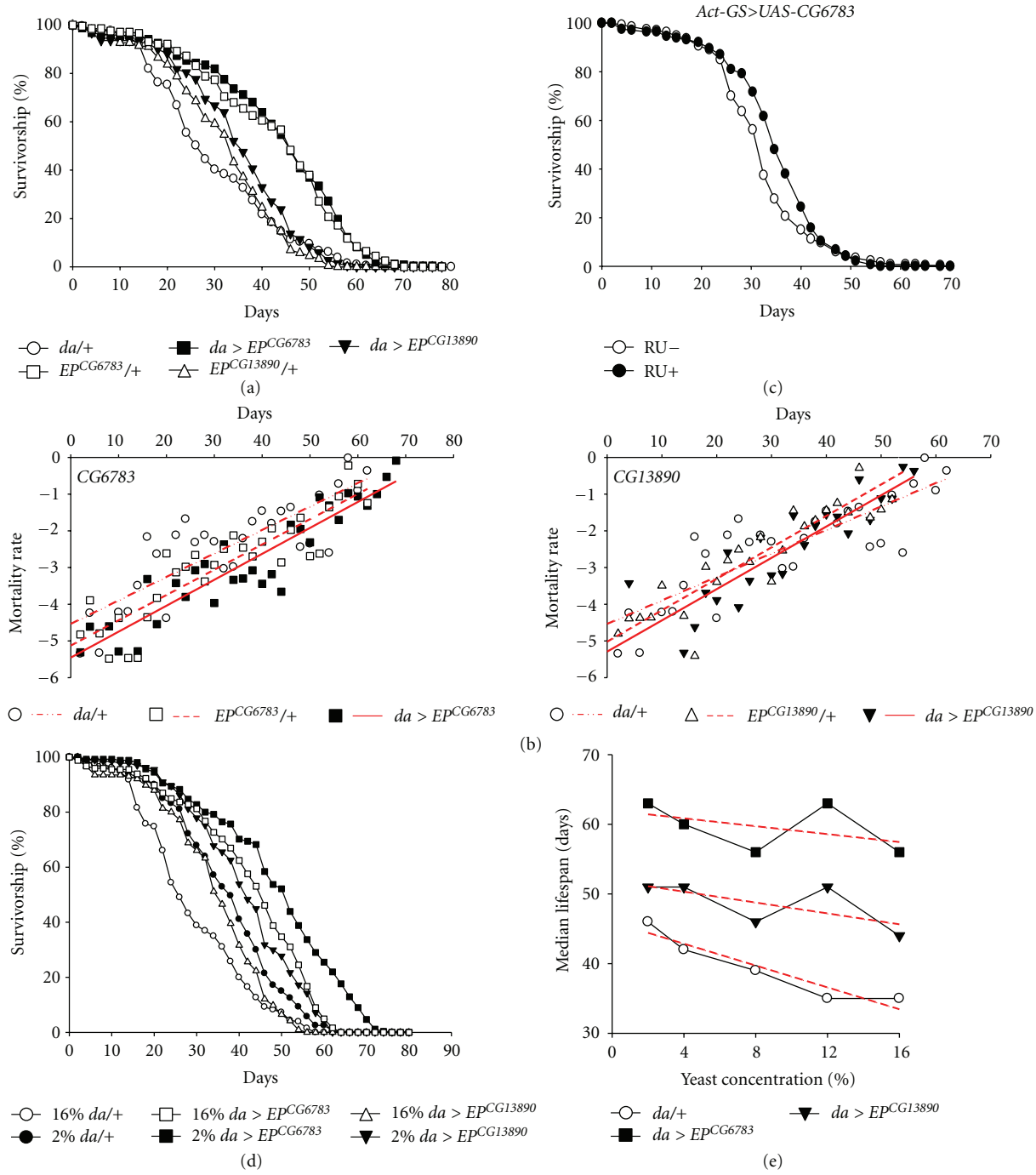


Figure 2: Overexpression of *CG6783* or *CG13890* throughout the whole body extends lifespan, which is associated with diet restriction. (a) Flies exhibiting overexpression of *EP* ^{CG6783} (closed-square line) and *EP* ^{CG13890} (closed-triangle line) fed 16% SY media display increased median and/or maximum lifespan when compared to the *da-Gal4* driver alone (open-circular line). Significance was determined via a log-rank test (CG6783, $P < 0.001$ to *da*/+, $P = 0.6$ to *EP* ^{CG6783}/+; CG13890, $P < 0.1$ to *da*/+, $P < 0.05$ to *EP* ^{CG13890}/+). (b) Flies overexpressing *CG6783* using *UAS-CG6783* and *Act-GS-Gal4* showed increased median lifespan in response to feeding with RU486-containing food from day 3 of adulthood (RU+, closed-symbol line) when compared to the uninduced control (RU−, open-symbol line). Significance was determined via a log-rank test ($P < 0.0001$). (c) Mortality curves of the flies that overexpressed *CG6783* or *CG13890*. The natural log of the mortality rate was plotted using the Gompertz mortality model. Red lines indicate linear regressions for each category. (d) Overexpression of *CG6783* or *CG13890* throughout the whole body reduced the lifespan extension with dietary restriction. Survival curves of the flies that overexpressed *CG6783* (square line) or *CG13890* (triangular line) fed 2% or 16% SY media. (e) Dietary restriction in adult *Drosophila* when *CG6783* or *CG13890* are overexpressed throughout the whole body. The median lifespan was calculated from Kaplan-Meier survival analysis of the EP lines fed a range of yeast concentrations. Red dashed lines indicate linear regressions for each line.

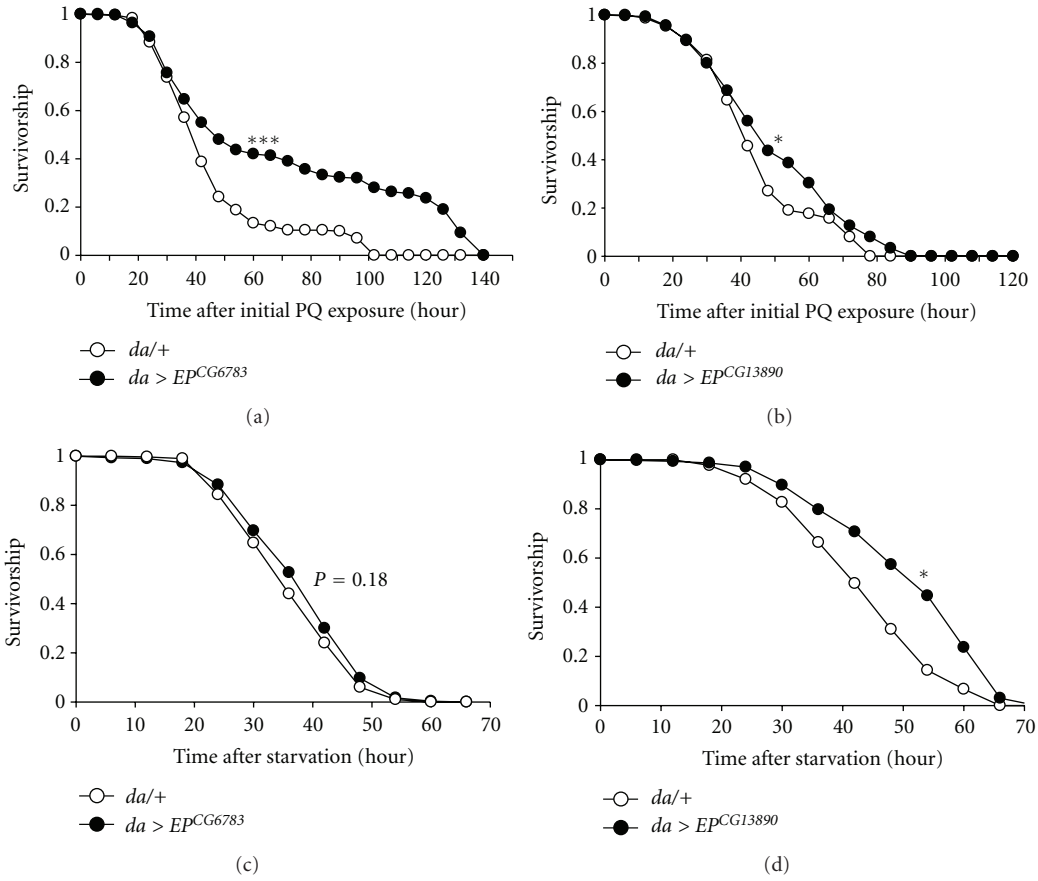


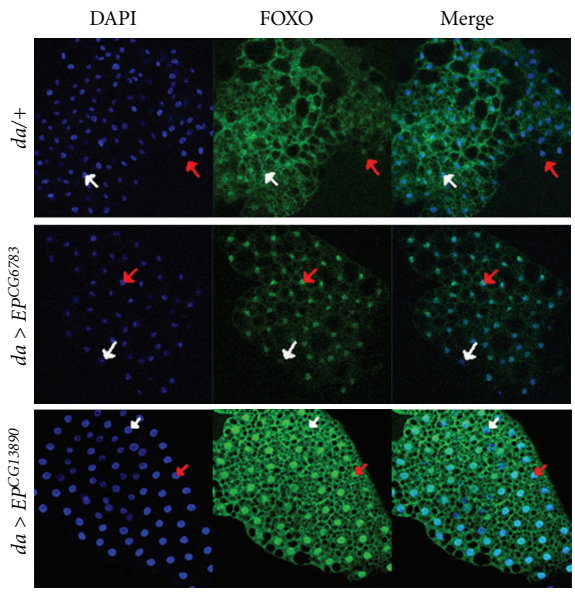
Figure 3: Overexpression of *CG6783* or *CG13890* increases resistance to stresses. Flies expressing *CG6783* or *CG13890* exhibited elevated resistance to oxidative stress (a, b) and starvation (c, d) when compared to the control (*da*/+, open-circule lines). Significance was determined via a log-rank test ($^{*}P < 0.01$, $^{***}P < 0.0001$).

3.3. Overexpression of Fatty-Acid-β-Oxidation Components Activated the dFOXO Signal. Forkhead box (FOXO) is a key mediator of the aging-related pathway that is regulated by signaling pathways including IIS/PI3K/Akt, JNK, AMPK, MST1, CBP, and Sirt1 [12, 32–36]. In addition, dFOXO activation in *Drosophila* fat body is reportedly associated with physiological traits such as aging, stress resistance, and lipid metabolism [15, 37, 38]. To determine whether lifespan extension and stress resistance produced by the overexpression of fatty-acid-β-oxidation components were associated with dFOXO activation, we immunostained larval fat bodies with anti-dFOXO antibody. In the control fat bodies, an endogenous dFOXO signal was detected in the cytoplasm of all cells and the nuclei of some of the cells (Figure 4(a)). However, the dFOXO signal increased in the nuclei of the fat body as a result of overexpression of the fatty-acid-β-oxidation components (Figure 4(a)).

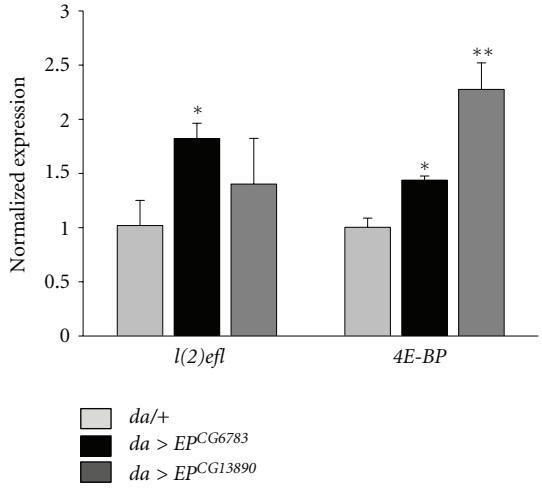
To further assess whether the fatty-acid β-oxidation-related genes activate dFOXO in adults, we analyzed the expression level of the dFOXO transcriptional target gene *l(2)efl* and *4E-BP* in adult whole bodies. The mRNA level of *l(2)efl* and *4E-BP* in the adult whole bodies increased in response to overexpression of the fatty-acid β-oxidation component (Figure 4(b)). These results indicated that increased fatty-acid β-oxidation leads to the activation of FOXO signaling, suggesting that fatty-acid-β-oxidation-induced lifespan extension is linked to FOXO activation.

4. Discussion

In this study, we demonstrated that the overexpression of fatty-acid-β-oxidation-related genes extended median and maximum lifespan and increased stress resistance, suggesting that the level of fatty-acid β-oxidation regulates lifespan. Consistent with our results, many investigations have suggested fatty-acid β-oxidation as a lifespan determinant. One of the well-known longevity-candidate genes, AMPK reportedly regulates fatty-acid synthesis and oxidation [20]. Moreover, calorie restriction and IIS have been reported to promote fatty-acid β-oxidation [21, 22]. In addition, *enigma* mutant, which exhibits oxidative stress resistance and a longevity phenotype, was found to encode a fatty-acid-β-oxidation related enzyme [39]. A mutant of *Withered*, which contains the carnitine palmitoyltransferase activity used to import long-chain fatty acids into the mitochondria, was found to be hypersensitive to oxidative and starvation stresses [40]. Furthermore, the mutant fly for *mitochondria trifunctional protein* containing three kinds of



(a)



(b)

Figure 4: Overexpression of *CG6783* or *CG13890* activates dFOXO. (a) Overexpression of *CG6783* or *CG13890* induced the translocation of dFOXO to the nucleus. The fat bodies of the third instar larvae expressing *CG6783* or *CG13890* under *da-Gal4* were stained with anti-dFOXO (green) and DAPI (blue). White arrows indicate dFOXO-negative cells and red arrows indicate dFOXO-positive cells. Original magnification is 200x. (b) Overexpression of *CG6783* or *CG13890* increased the expression of dFOXO target genes. The mRNA of *l(2)efl* and *4E-BP* was analyzed in the whole body RNA extract from *da > EP* (black or dark gray bars) and *da/+* (gray bars) flies. Significance was determined via a *t*-test ($^*P < 0.01$, $^{**}P < 0.001$).

enzyme activities associated with fatty-acid β-oxidation, was recently reported to have a shortened lifespan and decreased locomotion and fecundity [41]. However, the present study is the first to provide direct evidence that the modulation of fatty-acid-β-oxidation components extends lifespan.

Our data showed that lifespan extension by dietary restriction decreased with the overexpression of fatty-acid β-oxidation-related genes, indicating that lifespan extension by fatty-acid-β-oxidation components is associated with dietary restriction. It was previously reported that calorie restriction increased whole-body-fat oxidation [21]. Energy deprivation subsequent to calorie restriction activates AMPK, which subsequently enables the increase of fatty-acid oxidation necessary to utilize the energy resource. These findings suggested that fatty acid oxidation and dietary restriction are related by same underlying mechanisms. However, it should be noted that flies expressing fatty-acid-β-oxidation-related genes still responded to dietary restriction, especially in the lowest-yeast-feeding group. This result suggests that the flies have gained longevity through changes in the fatty-acid-β-oxidation-related genes and also other mechanism(s) unrelated to fatty-acid-β-oxidation in the dietary restriction condition.

Overexpression of the two fatty-acid-β-oxidation components showed similar effects, such as the extension of lifespan, mortality, stress resistance and dFOXO activation, throughout current study. However, flies overexpressing FABP (*CG6783*) were more resistant to oxidative stress, while DCI (*CG13890*) expressing flies were more resistant to starvation when compared to each other. FABP facilitates the intracellular movement of fatty acids, thus permitting the translocation of fatty acids to the mitochondria for fatty-acid oxidation and to the nucleus for activation of transcription of the FABP target gene via the fatty-acid nuclear receptors [42]. Therefore, the different effects of the two components on stress resistance may be caused by distinct functions of FABP and DCI.

In this study, we showed that the enhancement of fatty-acid oxidation components activates FOXO transcription factor, suggesting that fatty-acid-β-oxidation-induced lifespan extension is associated with FOXO activation. Fasting DCI homozygous mutants have been found to deposit large amounts of triglycerides in their hepatocytes and accumulated unsaturated fatty acyl groups in their ester lipids [25]. Surprisingly, our data showed that overexpression of FABP and DCI genes led to a mild increase in triglycerides levels (data not shown). This finding was likely the result of activation of FOXO by FABP or DCI overexpression, as it was recently reported that constitutively nuclear FOXO1 in mouse liver produces increased triglyceride accumulation [43]. As a mediator of aging-related signaling pathways, dFOXO is known to be regulated by several factors, including AMPK, JNK, MST1, Sir2, and IIS [12, 32–36]. Thus, further investigations are needed to determine whether the FOXO activation is required for longevity and stress resistance in flies overexpressing fatty acid oxidation-related genes and which signaling pathways are associated with fatty-acid-β-oxidation-related FOXO activation.

Authors' Contribution

S.-H. Lee and S.-K. Lee are contributed equally to the paper.

Acknowledgments

The authors thank Dr. O. Puig for providing antibody and the KAIST Bio Medical Research Center and Bloomington Stock Center for the fly stocks. This work was supported by a grant from Inha University.

References

[1] A. B. Paaby and P. S. Schmidt, "Dissecting the genetics of longevity in *Drosophila melanogaster*," *Fly*, vol. 3, no. 1, pp. 29–38, 2009.

[2] E. Ziv and D. Hu, "Genetic variation in insulin/IGF-1 signaling pathways and longevity," *Ageing Research Reviews*, vol. 10, no. 2, pp. 201–204, 2011.

[3] C. J. Kenyon, "The genetics of ageing," *Nature*, vol. 464, no. 7288, pp. 504–512, 2010.

[4] P. Kapahi, D. Chen, A. N. Rogers et al., "With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging," *Cell Metabolism*, vol. 11, no. 6, pp. 453–465, 2010.

[5] W. Mair, I. Morantte, A. P. C. Rodrigues et al., "Lifespan extension induced by AMPK and calcineurin is mediated by CRTC-1 and CREB," *Nature*, vol. 470, no. 7334, pp. 404–408, 2011.

[6] M. Tatar, A. Kopelman, D. Epstein, M. P. Tu, C. M. Yin, and R. S. Garofalo, "A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function," *Science*, vol. 292, no. 5514, pp. 107–110, 2001.

[7] G. A. Walker and G. J. Lithgow, "Lifespan extension in C. elegans by a molecular chaperone dependent upon insulin-like signals," *Aging Cell*, vol. 2, no. 2, pp. 131–139, 2003.

[8] C. S. Carter, M. M. Ramsey, and W. E. Sonntag, "A critical analysis of the role of growth hormone and IGF-1 in aging and lifespan," *Trends in Genetics*, vol. 18, no. 6, pp. 295–301, 2002.

[9] N. Klöting and M. Blüher, "Extended longevity and insulin signaling in adipose tissue," *Experimental Gerontology*, vol. 40, no. 11, pp. 878–883, 2005.

[10] R. Kaletsky and C. T. Murphy, "The role of insulin/IGF-like signaling in C. elegans longevity and aging," *DMM Disease Models and Mechanisms*, vol. 3, no. 7-8, pp. 415–419, 2010.

[11] A. Salminen and K. Kaarniranta, "Insulin/IGF-1 paradox of aging: regulation via AKT/IKK/NF-κB signaling," *Cellular Signalling*, vol. 22, no. 4, pp. 573–577, 2010.

[12] G. Rizki, T. N. Iwata, J. Li et al., "The evolutionarily conserved longevity determinants HCF-1 and SIR-2.1/SIRT1 collaborate to regulate DAF-16/FOXO," *PLoS Genetics*, vol. 7, no. 9, Article ID e1002235, 2011.

[13] M. Tatar, "Diet restriction in *Drosophila melanogaster*: design and analysis," *Interdisciplinary Topics in Gerontology*, vol. 35, pp. 115–136, 2007.

[14] D. M. O'Brien, K. J. Min, T. Larsen, and M. Tatar, "Use of stable isotopes to examine how dietary restriction extends *Drosophila* lifespan," *Current Biology*, vol. 18, no. 4, pp. R155–R156, 2008.

[15] K. J. Min, R. Yamamoto, S. Buch, M. Pankratz, and M. Tatar, "*Drosophila* lifespan control by dietary restriction independent of insulin-like signaling," *Aging Cell*, vol. 7, no. 2, pp. 199–206, 2008.

[16] J. V. Smith, L. K. Heilbronn, and E. Ravussin, "Energy restriction and aging," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 7, no. 6, pp. 615–622, 2004.

[17] M. Gáliková, P. Klepsatel, G. Senti, and T. Flatt, "Steroid hormone regulation of C. elegans and *Drosophila* aging and life history," *Experimental Gerontology*, vol. 46, no. 2-3, pp. 141–147, 2011.

[18] F. Broué, P. Liere, C. Kenyon, and E. E. Baulieu, "A steroid hormone that extends the lifespan of *Caenorhabditis elegans*," *Aging Cell*, vol. 6, no. 1, pp. 87–94, 2007.

[19] D. Paik, Y. G. Jang, Y. E. Lee et al., "Misexpression screen delineates novel genes controlling *Drosophila* lifespan," *Mechanisms of Ageing and Development*, vol. 133, no. 5, pp. 234–245, 2012.

[20] D. G. Hardie and D. A. Pan, "Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase," *Biochemical Society Transactions*, vol. 30, no. 6, pp. 1064–1070, 2002.

[21] M. D. Bruss, C. F. Khambatta, M. A. Ruby, I. Aggarwal, and M. K. Hellerstein, "Calorie restriction increases fatty acid synthesis and whole body fat oxidation rates," *American Journal of Physiology*, vol. 298, no. 1, pp. E108–E116, 2010.

[22] X. Xu, P. Gopalacharyulu, T. Seppanen- et al., "Insulin signaling regulates fatty acid catabolism at the level of CoA activation," *PLoS Genetics*, vol. 8, no. 1, Article ID e1002478, 2012.

[23] J. E. Zimmerman, M. T. Chan, N. Jackson, G. Maislin, and A. I. Pack, "Genetic background has a major impact on differences in sleep resulting from environmental influences in *Drosophila*," *Sleep*, vol. 35, pp. 545–557, 2012.

[24] J. H. Veerkamp and R. G. H. J. Maatman, "Cytoplasmic fatty acid-binding proteins: their structure and genes," *Progress in Lipid Research*, vol. 34, no. 1, pp. 17–52, 1995.

[25] U. Janssen and W. Stoffel, "Disruption of mitochondrial β-oxidation of unsaturated fatty acids in the 3,2-trans-enoyl-CoA isomerase-deficient mouse," *Journal of Biological Chemistry*, vol. 277, no. 22, pp. 19579–19584, 2002.

[26] S. M. Houten and R. J. A. Wanders, "A general introduction to the biochemistry of mitochondrial fatty acid β-oxidation," *Journal of Inherited Metabolic Disease*, vol. 33, no. 5, pp. 469–477, 2010.

[27] G. Roman, K. Endo, L. Zong, and R. L. Davis, "P[switch], a system for spatial and temporal control of gene expression in *Drosophila melanogaster*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 22, pp. 12602–12607, 2001.

[28] M. D. W. Piper and A. Bartke, "Diet and aging," *Cell Metabolism*, vol. 8, no. 2, pp. 99–104, 2008.

[29] M. Ristow and S. Schmeisser, "Extending life span by increasing oxidative stress," *Free Radical Biology and Medicine*, vol. 51, no. 2, pp. 327–336, 2011.

[30] B. P. Yu and H. Y. Chung, "Stress resistance by caloric restriction for longevity," *Annals of the New York Academy of Sciences*, vol. 928, pp. 39–47, 2001.

[31] S. J. Broughton, M. D. W. Piper, T. Ikeya et al., "Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 8, pp. 3105–3110, 2005.

[32] K. S. Lee, K. Iijima-Ando, K. Iijima et al., "JNK/FOXO-mediated neuronal expression of fly homologue of peroxiredoxin II reduces oxidative stress and extends life span," *Journal of Biological Chemistry*, vol. 284, no. 43, pp. 29454–29461, 2009.

[33] E. L. Greer, M. R. Banko, and A. Brunet, "AMP-activated protein kinase and FoxO transcription factors in dietary restriction-induced longevity," *Annals of the New York Academy of Sciences*, vol. 1170, pp. 688–692, 2009.

[34] M. K. Lehtinen, Z. Yuan, P. R. Boag et al., "A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span," *Cell*, vol. 125, no. 5, pp. 987–1001, 2006.

[35] T. B. Dansen, L. M. M. Smits, M. H. Van Triest et al., "Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity," *Nature Chemical Biology*, vol. 5, no. 9, pp. 664–672, 2009.

[36] D. H. Kim, J. Y. Kim, B. P. Yu, and H. Y. Chung, "The activation of NF-κB through Akt-induced FOXO1 phosphorylation during aging and its modulation by calorie restriction," *Biogerontology*, vol. 9, no. 1, pp. 33–47, 2008.

[37] D. S. Hwangbo, B. Gershman, M. P. Tu, M. Palmer, and M. Tatar, "Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body," *Nature*, vol. 429, no. 6991, pp. 562–566, 2004.

[38] M. E. Giannakou, M. Goss, J. Jacobson, G. Vinti, S. J. Leevers, and L. Partridge, "Dynamics of the action of dFOXO on adult mortality in *Drosophila*," *Aging Cell*, vol. 6, no. 4, pp. 429–438, 2007.

[39] P. Mourikis, G. D. Hurlbut, and S. Artavanis-Tsakonas, "Enigma, a mitochondrial protein affecting lifespan and oxidative stress response in *Drosophila*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 5, pp. 1307–1312, 2006.

[40] B. R. Strub, T. L. Parkes, S. T. Mukai et al., "Mutations of the withered (whd) gene in *Drosophila melanogaster* confer hypersensitivity to oxidative stress and are lesions of the carnitine palmitoyltransferase I (CPT I) gene," *Genome*, vol. 51, no. 6, pp. 409–420, 2008.

[41] Y. Kishita, M. Tsuda, and T. Aigaki, "Impaired fatty acid oxidation in a *Drosophila* model of mitochondrial trifunctional protein (MTP) deficiency," *Biochemical and Biophysical Research Communications*, vol. 419, pp. 344–349, 2012.

[42] J. R. Gerstner, W. M. Vanderheyden, P. J. Shaw, C. F. Landry, and J. C. Yin, "Cytoplasmic to nuclear localization of fatty-acid binding protein correlates with specific forms of long-term memory in *Drosophila*," *Communicative & Integrative Biology*, vol. 4, pp. 623–626, 2011.

[43] M. Matsumoto, S. Han, T. Kitamura, and D. Accili, "Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism," *Journal of Clinical Investigation*, vol. 116, no. 9, pp. 2464–2472, 2006.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 718491, 10 pages
doi:10.1155/2012/718491

Review Article

Nutraceutical Interventions for Promoting Healthy Aging in Invertebrate Models

Yuqing Dong,[1,2] Sujay Guha,[1] Xiaoping Sun,[3] Min Cao,[1,2] Xiaoxia Wang,[1] and Sige Zou[3]

[1] *Department of Biological Sciences, Clemson University, 132 Long Hall, Clemson, SC 29634, USA*
[2] *Institute for Engaged Aging, Clemson University, 535A Edwards Hall, Clemson, SC 29634, USA*
[3] *Laboratory of Experimental Gerontology, National Institute on Aging, 251 Bayview Boulevard, Baltimore, MD 21224, USA*

Correspondence should be addressed to Yuqing Dong, ydong@clemson.edu and Sige Zou, zous@grc.nia.nih.gov

Received 22 June 2012; Revised 8 August 2012; Accepted 10 August 2012

Academic Editor: Heinz D. Osiewacz

Aging is a complex and inevitable biological process that is associated with numerous chronically debilitating health effects. Development of effective interventions for promoting healthy aging is an active but challenging area of research. Mechanistic studies in various model organisms, noticeably two invertebrates, *Caenorhabditis elegans* and *Drosophila melanogaster*, have identified many genes and pathways as well as dietary interventions that modulate lifespan and healthspan. These studies have shed light on some of the mechanisms involved in aging processes and provide valuable guidance for developing efficacious aging interventions. Nutraceuticals made from various plants contain a significant amount of phytochemicals with diverse biological activities. Phytochemicals can modulate many signaling pathways that exert numerous health benefits, such as reducing cancer incidence and inflammation, and promoting healthy aging. In this paper, we outline the current progress in aging intervention studies using nutraceuticals from an evolutionary perspective in invertebrate models.

1. Introduction

Aging is associated with a gradual decline of physiological and cognitive functions [1]. Age is a major risk factor for numerous diseases, such as diabetes, cancer, and various degenerative diseases, including Alzheimer's, Huntington's, and Parkinson's diseases [2, 3]. A major challenge to health care systems around the world is how to encourage and maintain a healthy lifespan in large and increasing populations of elderly individuals [1]. Significant progress has been made over the past two decades in elucidating the molecular mechanisms of aging [4–7]. Hundreds of genetic factors, called longevity-related genes, have been identified to modulate lifespan and healthspan in model organisms ranging from yeast, worms, flies, and rodents. A number of the longevity-related genes fall into three conserved nutrient sensing pathways: target-of-rapamycin (TOR), insulin/IGF-1-like signaling (IIS), and sirtuin pathways [7–9]. These pathways primarily sense cellular amino acid, glucose, and NAD^+ or NAD^+/NADH levels, respectively, (Figure 1). Genome-wide association studies have linked some longevity-related genes discovered in model organisms to human longevity, such as insulin-like growth factor receptor (IGF1R) and forkhead transcription factor (FOXO3a) in the IIS pathway [10–14]. Mechanistic studies of aging have led to the proposal of a number of hypotheses of aging. Perhaps the most prominent is the free radical hypothesis of aging, which states that free radicals, such as reactive oxygen species, generated from metabolism inflict oxidative damage to macromolecules, including protein, DNA, and lipid. Accumulation of such oxidative damage with age causes biological aging and eventually results in death [15, 16]. However, numerous studies have now shown that this simplified version of free radical hypothesis of aging is not necessarily sufficient to explain the mechanisms underlying aging processes. Another is the hormesis hypothesis of aging, which has been frequently used to interpret the prolongevity effects induced by nutraceuticals. Hormesis theory states that mild stress-induced stimulation of defense response at the organismal level results in biologically beneficial effects and extends

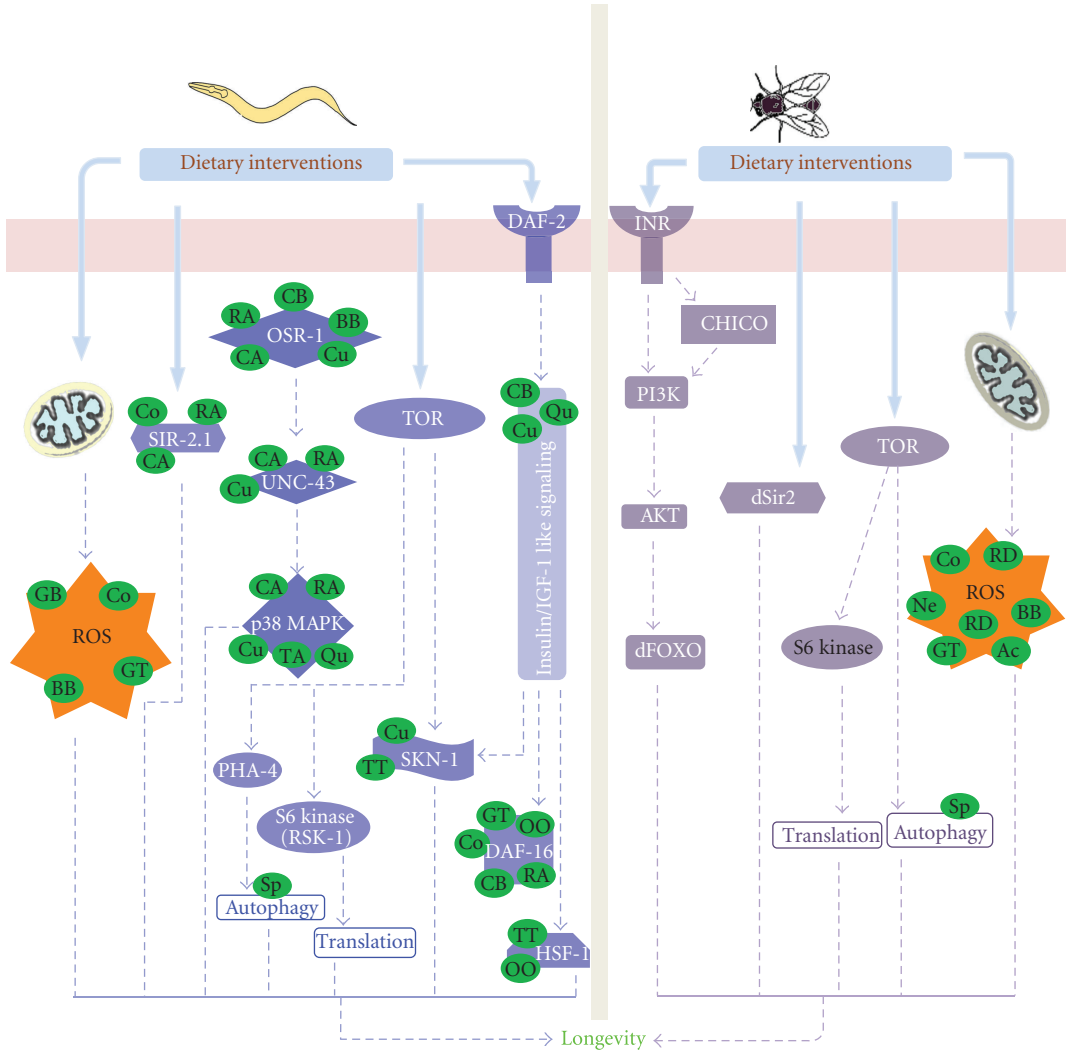


Figure 1: Nutrient sensing signaling and stress response pathways in *C. elegans* and *D. melanogaster*. Dietary interventions promote healthy lifespan and stress resistance through at least three conserved nutrient sensing pathways, IIS, TOR, and sirtuins. Many nutraceuticals improve healthspan by regulating ROS/redox state, some are known to interact with the IIS and osmotic resistant pathways to extend lifespan, and some modulate lifespan extension via autophagy mediated by TOR signaling. Each nutraceutical is represented by a green circle and the location of the green circle indicates the possible target of a nutraceutical action*. Ac: açai, BB: blueberry, CA: caffeic acid, CB: cranberry, Co: cocoa, Cu: curcumin, GB: ginkgo biloba, GT: green tea, Ne: nectarine, OO: olive oil, Qu: quercetin, RD: rosa damascene, RA: rosmarinic acid, Sp: spermidine, TA: tannic acid, and TT: thioflavin T. *ROS presented in the figure indicates the regulation of ROS/redox state.

lifespan and healthspan. The defense response involves many protective mechanisms and influences with gene expression and metabolism [16–18]. Therefore, significant efforts in developing interventions for promoting healthy aging have been devoted to identify effective ways to modulate metabolism and stress [8].

Dietary restriction (DR) by modest reduction of all macronutrients or protein content is one of the most effective environmental interventions for promoting healthy aging [19–25]. However, it is challenging to implement dietary restriction in humans. A number of genes, including components in the conserved nutrient sensing pathways, have been identified to mediate lifespan extension induced by DR [9, 26]. As an alternative to DR, many studies have been devoted to the identification of pharmacological and nutraceutical reagents that extend lifespan and healthspan, some of which may mimic the effect of DR [27, 28]. Due to their short lifespan and rich genetic resource, *Drosophila melanogaster* and *Caenorhabditis elegans*, have been in the forefront of research on aging interventions. In this paper, we focus on aging interventions in invertebrate models using nutraceuticals, which refer to extracts made out of edible fruits, vegetables, and herbs. Interventions with pharmacological reagents, such as resveratrol, metformin, and rapamycin, have been discussed in numerous comprehensive reviews elsewhere [28–33], and will not be described in this

paper. Here we attempt to integrate the findings from studies on the mechanisms of aging and nutraceutical interventions with an evolutionary perspective.

2. Dietary Botanicals and Phytochemicals

Numerous studies have demonstrated the effects of nutraceuticals from fruit or plant extracts in reducing oxidative damage and promoting healthy aging in invertebrate models. The active ingredients in nutraceuticals that are generally produced by plants as "secondary compounds" appear to help plants overcome stressful conditions [34–36]. The beneficial properties of nutraceuticals can be attributed to the varieties of phytochemicals, such as flavonoids, anthocyanin glycosides, triterpenoids, and proanthocyanidin oligomers [37–39]. In this section, we will describe the antiaging properties of several nutraceuticals made from fruits, spices, and teas, which are commonly consumed by humans in daily life.

2.1. Ginkgo biloba Extract (EGb 761). Ginkgo is widely used as an ancient Chinese medicine due to its benefits to the health of the elderly [40]. The ginkgo biloba extract has been proposed to help prevent Alzheimer's disease (AD). The herbal extract EGb761 made from Ginkgo biloba is rich in flavonoids and terpenoids, which improves the functions of platelet and nerve cells and the blood flow to the nervous system and brain, probably due to its antioxidant properties [41, 42]. In wild type worms, EGb761 extends lifespan by ~10%, reduces accumulation of oxidative damage, and represses expression of thermal stress-response protein hsp-16.2 [43–46]. In addition, EGb 761 inhibits Aβ oligomerization and alleviates Aβ-induced paralysis in the *C. elegans* model of AD [47]. Aβ oligomerization is a hallmark of AD [48]. However, it remains to be determined whether ginkgo biloba can promote longevity in other models, such as *Drosophila*. Nevertheless, combined with studies demonstrating the neuroprotective function of EGb 761 in mammalian cells [49, 50], findings in worms suggest that the beneficial effect of EGb761 in prevention and treatment of AD could at least partially be due to its capacity to resist oxidative stress and reduce detrimental protein aggregation.

2.2. Blueberry. Blueberry contains a wide array of polyphenols and offers a host of health benefits [51]. Supplementation of blueberry preserves learning and memory in aged F344 rats by improving neuronal function [52]. Polyphenols from blueberry significantly increase the lifespan of *C. elegans* [53]. In *C. elegans*, blueberry supplementation decreases age-related accumulation of the intracellular level of lipofuscin, a biomarker for age-related cellular damage, and reduces the level of 4-Hydroxynonenal level, a biomarker for lipid peroxidation. In addition, blueberry polyphenols improve the pharyngeal pumping rates of the aged worms and increase thermotolerance, suggesting that blueberry improves worms' healthspan. Mechanistic studies indicate that blueberry supplementation reduces mRNA levels of heat shock proteins and requires OSR-1/UNC-43/SEK-1, components of the osmotic stress resistance pathway [53], to promote longevity in *C. elegans*. A recent study has demonstrated that blueberry extracts extends mean lifespan by approximately 10% in *Drosophila* [54]. Associated with lifespan extension, blueberry increased expression of several antioxidant genes, including superoxide dismutase 1 (SOD1), SOD2, and catalase (CAT) in *Drosophila*. Further genetic studies are needed to establish whether any of the antioxidant genes are required for lifespan extension induced by blueberry in *Drosophila*. These findings in invertebrates suggest that antioxidant machinery and/or osmotic stress pathway may play a pivotal and common role in mediating lifespan extension induced by blueberry.

2.3. Oregano and Cranberry. Cranberry and oregano possess multiple medicinal properties, such as antimicrobial, antiviral, antimutagenic, antiangiogenic, and antioxidation functions [55, 56]. A mixture of oregano and cranberry (OC) extract increased lifespan in Mexican fruit flies (mexfly) in a diet composition dependent manner [57]. OC extended lifespan in mexflies fed diets with a relatively higher sugar to protein ratio, but not in mexflies fed diets with a lower sugar to protein ratio. OC supplementation did not compromise the lifetime reproductive output, a parameter of healthspan. In addition, OC supplementation in middle age was sufficient to promote longevity [58]. However, lifespan was not increased when OC was supplemented only in young age or old age. These findings point out the importance of considering diet composition and implementing time in developing an efficacious aging intervention.

We have recently assessed the effect of cranberry extract alone on lifespan and healthspan in *C. elegans*. Our data indicate that the cranberry extract alone is sufficient to prolong lifespan in *C. elegans* [59]. The cranberry-mediated lifespan extension was suppressed almost completely by the absence of DAF-16, a forkhead transcription factor in the IIS. Cranberry supplementation did not further extend the lifespan of DAF-2 or AGE-1 mutants, either. DAF-2 and AGE-1 are insulin-like receptor and phosphatidylinositol 3 kinase (PI3 K) in the IIS pathway of *C. elegans* [60, 61]. Cranberry is high in antioxidants and phytochemicals, including proanthocyanidins and vitamin C, which may neutralize free radicals and reduce oxidative damage, and more importantly modulating signaling transduction pathways [51, 62]. Our findings suggest that IIS and DAF-16 play an important role in cranberry mediated prolongevity in *C. elegans*. Further studies are warranted to determine to what extent the prolongevity effect of cranberry attributes to its antioxidant properties.

2.4. Nectarine and Açai. Nectarine is a globally consumed fruit [63], and açai is a fruit indigenous to the Amazon River area [64]. Both fruits contain various kinds of bioactive phytochemicals [2, 3, 65]. Nectarine supplementation can extend lifespan in flies fed standard, DR, and high-fat diets. The lifespan extension induced by nectarine is associated with increased lifetime reproductive output and reduced lipid oxidation [66]. In contrast, supplementation of açai pulp promotes survival in *Drosophila* fed a high-fat but not a standard diet [67]. The diet composition dependent effect

of açai is also evident in the mexfly. Açai supplementation promotes the survival of the mexfly fed a high-fat and high-sugar diet but not other nonfat diets [68]. Along with the OC study described above, the importance of diet composition is also evident in aging intervention studies using pharmacological agents, such as resveratrol. Studies in *Drosophila*, mexfly, and mice have shown that the prolongevity effects of resveratrol depend on diet composition [69–71]. These studies again stress the importance of diet composition in modulating the health benefits of nutraceuticals. Moreover, both nectarine and açai can promote the survival of flies with *sod1* deficiency. *sod1* deficient flies have a short lifespan and experience high levels of oxidative damage. These findings suggest that nectarine and açai possess antioxidant activities at the organismal level [26, 67, 72]. However, it remains to be determined the mechanisms underlying the prosurvival effects of nectarine and açai.

2.5. Rosa damascena. A hybrid rose species, *Rosa damascena*, is traditionally used to make rose oil and water in cosmetic and food industries [73, 74]. Extracts from *R. damascena* contains numerous volatile organic compounds including various terpenes such as citronellol, heneicosane, and disiloxane, and also polyphenols, such as quercetin, myricetin, kaempferol, and gallic acid [75]. *R. damascena* extracts have been shown to possess biological properties that are protective against microbial infection, seizures in rats, and toxicity of amyloid beta in neurons, a biomarker of AD [76–79]. An *R. damascena* extract has been found to increase both mean and maximum lifespan in *Drosophila*. This extract also enhances flies' resistance to oxidative stress and low iron stress, but paradoxically increases flies' sensitivity to heat stress. Molecular studies indicate that the rose extract reduces the heat-induced expression of a major heat shock protein HSP70 and a small mitochondrial heat shock protein HSP22 [80]. Therefore, it has been proposed that *R. damascena* extract extends lifespan by protecting flies against iron-induced stress.

2.6. Cocoa Polyphenols. Numerous polyphenols with high antioxidant activities, such as flavonoids, have been isolated from cocoa [81]. Flavonoid-enriched cocoa powder reduces oxidative stress in *C. elegans* [82]. Moreover, the flavonoid-enriched cocoa powder also significantly increases the lifespan of wild type N2 worms [82]. This lifespan extension was dependent on both SIR-2.1 and DAF-16. In addition, the flavonoid-enriched cocoa powder failed to exert any longevity effect on *daf-2* mutants, indicating that cocoa powder-mediated lifespan extension may also act through the IIS. Considering the mechanism by which SIR-2.1 and IIS modulates lifespan, cocoa polyphenols may promote longevity by reducing oxidative stress, influencing metabolism, and altering chromatin structure.

2.7. Green Tea. Green tea contains polyphenolic catechins that have been reported to have a number of health benefits, including prevention of AD, Parkinson's disease, and heart disease [83]. Green tea can protect against angiogenesis and tumor formation [84]. The health benefits of green tea are mainly attributed to bioactive properties of its phytochemical constituents [85, 86]. A number of polyphenolic catechins, including epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG), catechin, and gallocatechin (GC), are abundant in green tea [83]. Among these, EGCG is perhaps the most abundant catechin in green tea, and has been reported to induce antioxidant enzymes, including glutathione peroxidase, catalase, and glutathione *S*-transferase, in mice [87]. Similar results were obtained in worms and flies. Supplementation with green tea catechins increases the antioxidant enzymatic activity of superoxide dismutase and catalase in *Drosophila* [88, 89]. Green tea extends median lifespan by 36% and mean lifespan by 16% in flies [89]. In *C. elegans*, EGCG treatment significantly reduces ROS levels under both normal culture and oxidative stress conditions, and increases the expression of *sod-3* and *hsp-16.2* [90, 91]. Administration of EGCG significantly extends the lifespan of *C. elegans* under heat stress and oxidative stress [90, 91]. In addition, a recent study demonstrated that L-theanine, a unique amino acid particularly enriched in green tea, promoted the survival of *C. elegans* in the presence of paraquat [92]. L-theanine has been reported to provide broad health benefits, such as antitumor, AD prevention, and blood pressure reduction [93–96]. Together, these findings suggest that green tea increases lifespan and stress resistance partially through its antioxidant properties.

2.8. Olive Oil. Numerous studies have demonstrated that consumption of olive oil has multiple beneficial effects on health and longevity in humans [97–101]. The abundance of phenolic compounds present in olive oil is considered to play an important role in exerting these healthy effects. Lastest studies have revealed that the tyrosol, one of the most abundant phenols in olive oil [102], significantly promotes the longevity of *C. elegans*, as well as the resistance to thermal and oxidative stress [103]. IIS/DAF-16 and HSF-1 are required in tyrosol-mediated prolongevity in *C. elegans*. *hsp-12.6*, a coregulated target gene of DAF-16, and HSF-1 [104], is significantly upregulated in adult worms after the tyrosol treatment. It has been reported that small heat shock proteins, including HSP-12.6, can extend lifespan and delay polyglutamine protein aggregation in *C. elegans* [104]. HSF-1 is critical for maintaining protein homeostasis. Together, these studies suggest that tyrosol in olive oil extends lifespan by increasing oxidative stress resistance and thermotolerance as well as improving protein homeostasis [103].

2.9. Quercetin and Tannic Acid. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the most important dietary flavonoids present in a wide array of foods. Numerous health benefits are linked to consumption of fruits and vegetables containing a high content of quercetin [105]. Quercetin has been shown to dramatically increase the lifespan of *C. elegans* [106–109]. However, the studies on the molecular basis yielded conflicting results. One study indicates that lifespan extension induced by quercetin supplementation requires DAF-2 and AGE-1, the components of *C. elegans* insulin/IGF signaling [106]. Quecertin induces nuclear translocation of

DAF-16 [110]. This study suggests that the IIS pathway mediates the prolongevity effect of quercetin. However, this hypothesis has been challenged by another study, which indicates that quercetin mediated lifespan extension does not depend on DAF-16 [107]. Additional studies are warranted to resolve the conflicting observation and understand the mechanisms underlying the prolongevity effect of quercetin.

Tannic acid (TA) belongs to tannins, which are secondary metabolites of plants with many health effects. TA has the biological activities to prevent neurodegeneration [111], pathogen infection [112, 113], carcinogenesis [114, 115], and oxidative damage [116, 117]. In addition, TA significantly increases the lifespan of worms [108]. The IIS pathway and DAF-16 are not essential for the prolongevity effects of TA. Only SEK-1, one component of the p38 MAPK pathway, has been shown to be essential for TA-induced lifespan extension.

Transcriptome studies indicate that quercetin affects expression of genes in the TGF-β signaling, insulin-like signaling, and p38 MAPK pathways, while TA changes expressions of genes in the TGF-β and the p38 MAPK pathways as well as the amino acid metabolism. Together, these studies suggest that TGF-β and p38 MAPK pathways play crucial roles in mediating the prolongevity effects of quercetin and TA [118].

2.10. Caffeic Acid and Rosmarinic Acid. Caffeic acid (3-(3,4-dihydroxyphenyl)-2-propenoic acid; CA) and Rosmarinic acid (α-o-caffeoyl-3, 4-dihydroxyphenyl lactic acid; RA) are abundantly present in a variety of fruits, vegetables, and herbs. CA and RA have anticarcinogenesis, antioxidant, antimicrobial, anti-inflammatory, and antirheumatic properties [119–123]. CA and RA can prolong the healthy lifespan of *C. elegans* [124]. DAF-16, SIR-2.1, OSR-1, UNK-43, and SEK-1 are required for RA triggered lifespan extension. Similar results have been obtained for CA mediated lifespan extension except that DAF-16 does not appear to play a pivotal role in this lifespan extension. Together, these findings suggest that CA and RA promote lifespan extension through overlapping pathways involved in metabolism and stress response.

2.11. Spermidine. Spermidine is an important polyamine presented in citrus fruit and soybean, and has effects on epigenetic modifications, autophagy, and necrosis [125, 126]. Polyamine concentrations and autophagy have been shown to decline in various organisms, including humans [127, 128]. Supplementation of spermidine prolongs the lifespan of *C. elegans* and *Drosophila* by 15% and 30%, respectively [125]. A growing number of evidence shows that autophagy plays an essential role in the regulation of lifespan mediated by the TOR pathway [129–131]. Autophagy is required for spermidine-mediated lifespan extension in both worms and flies. It will be interesting to see if spermidine and its derivatives can confer lifespan extension in humans by, at least in part, enhancing the autophagy.

2.12. Curcumin and Thioflavin T. Curcumin (diferuloylmethane) is the pharmacologically active substance in turmeric (*Curcuma longa*), and has been widely used as a herbal medicine in Asia. A large body of evidence indicates that curcumin possesses many biological activities, such as antioxidative, anti-inflammatory, anticancer, chemopreventive, and antineurodegenerative properties [132–136]. With its pleiotropic activities, curcumin has been considered as a potential aging intervention compound. Studies in *Drosophila* and *C. elegans* have demonstrated that curcumin can delay aging and prolong the lifespan [137, 138]. Curcumin-treated flies exhibited enhanced resistance against to oxidative stress, improved locomotor activity, and higher tolerance to chemotherapy drugs. Curcumin reduces expression of *Methuselah*, a longevity gene, and genes in the IIS and JNK pathways. Curcumin-induced lifespan extension has been shown to be mediated by the OSR-1/UNC-43/SEK-1 pathway and possible the members of the IIS and SKN-1 pathways in *C. elegans*.

Protein homeostasis is an essential lifespan modulator of animals [139]. Dysfunctional protein homeostasis results in protein misfolding and aggregation, which is a hallmark of aging and age-related diseases [140]. ThT is a compound known to bind and inhibit aggregation of amyloids, which leads to various neurodegenerative disorders [141]. Flavonoid thioflavin T (4-(3,6-dimethyl-1,3-benzothiazole-3-ium-2-yl)-N,N-dimethylaniline chloride, ThT) has been shown to promote longevity in *C. elegans* [142]. Treatment with 50 μM ThT, a concentration conferring a largest increase of lifespan, significantly suppresses the protein aggregation mediated paralysis in worms. The prolongevity effect of ThT depends on HSF-1 and SKN-1, but independent of the IIS. Importantly, the beneficial effects of ThT require an intact machinery for maintaining protein homeostasis. Like ThT, curcumin also has the ability to bind and inhibit protein aggregates [142]. Treatment of both ThT and curcumin does not yield synergistic effects on longevity. ThT and curcumin may act through similar mechanisms, for example, improving protein homeostasis, to modulate aging and age-related disorders.

3. Concluding Remarks

Nutraceuticals made from widely-consumed plant products represent both opportunities and challenges in aging interventions. On the one hand, nutraceuticals from plant extracts can promote longevity and improve healthspan through multiple mechanisms, such as reducing oxidative stress, altering signaling pathways, influencing metabolism, and maintaining protein homeostasis (Figure 1). Nutraceuticals that promote longevity in invertebrate models are often made from edible and relatively easily accessible fruits, vegetables, spices, and other plant products. Thus, when appropriately implemented nutraceutical interventions can be efficient and cost-effective ways for promoting healthy aging in humans.

On the other hand, major challenges remain to be addressed in implementing nutraceutical interventions. First, nutraceuticals contain numerous bioactive phytochemicals. It is a daunting task to determine which phytochemicals are the active ingredients for promoting health benefits, or determine how the beneficial effects are mediated through

synergistic actions among multiple ingredients. Second, more research is needed to elucidate the molecular mechanisms underlying the actions of nutraceuticals, in order to better assess the effects of nutraceutical supplementation on the health of animals. For instance, more recent studies have revealed that ROS plays a physiologically vital role in signal transduction, gene regulation, and redox regulation [143]. The previous concept of the "free radical hypothesis of aging" has been modified and diversified ROS a messenger role in addition to its toxicity. Thus, it would be harmful to eliminate ROS completely. In the context, although many nutraceuticals have antioxidant properties, we need more careful exams to unravel the molecular basis rather than simply attribute to their ability to scavenge free radicals. It is in line with this notion that some nutraceuticals, such as quercetin and blueberry polyphenols, modulate lifespan by activating molecular pathways independent to their direct antioxidant capacity [53, 107]. Third, it is essential to investigate the impact of both short-term and long-term nutraceutical interventions on healthspan. A recently developed behavioral monitoring system capable of recording lifetime behavioral changes at a high resolution represents an example of a useful tool for assessing healthspan in invertebrates [144]. Lastly, an important and challenging issue is to further understand how the effects of nutraceuticals depend on diet composition and genetic background. The prolongevity effects of several nutraceuticals have been shown to depend on the timing of supplementation and the composition of diet [143, 145–148]. The diet composition-dependent effects will have a significant impact on the increasing demand for personalized nutritional intervention. The individual and synergistic effects of nutraceuticals as a component of dietary composition will require further study and scientific scrutiny, particularly since little regulatory oversight regarding their sale for human consumption currently exists.

We have highlighted how nutraceuticals prevent and protect against aging and stress in invertebrate models. Some of nutraceuticals and their synthetic derivatives are being tested for their therapeutic potential [149]. Numerous promising results have been obtained in model organisms that suggest evolutionarily conserved mechanisms are involved in their beneficial effects. Much progress has been made to decipher the molecular mechanisms of aging shared among multiple species, which provide valuable guidance for aging interventions. However, further extensive studies will be required to demonstrate whether any nutraceuticals or pharmaceuticals can effectively delay aging or age-related disease in humans. A wider range of additional assays should be considered to help us better understand aging processes and improve the quality and quantity of human life in the foreseeable future.

Acknowledgments

The authors thank Edward Spangler and Robert Kosinski for critical reading of the paper. They also thank Cole Murbach for editorial assistance. This work was supported by the Creative Inquiry fund at Clemson University and the Yamada Research Grant to Y. Dong and M. Cao, an American Federation of Aging Research (AFAR) grant to Y. Dong and the Intramural Research Program at the National Institute on Aging, NIH to S. Zou.

References

[1] E. De Luca d'Alessandro, S. Bonacci, and G. Giraldi, "Aging populations: the health and quality of life of the elderly," *La Clinica Terapeutica*, vol. 162, no. 1, pp. e13–e18, 2011.

[2] V. Pizza, A. Agresta, C. W. ĎAcunto, M. Festa, and A. Capasso, "Neuroinflamm-aging and neurodegenerative diseases: an overview," *CNS and Neurological Disorders*, vol. 10, no. 5, pp. 621–634, 2011.

[3] L. Fontana, "Modulating human aging and age-associated diseases," *Biochimica et Biophysica Acta*, vol. 1790, no. 10, pp. 1133–1138, 2009.

[4] F. Peysselon and S. Ricard-Blum, "Understanding the biology of aging with interaction networks," *Maturitas*, vol. 69, no. 2, pp. 126–130, 2011.

[5] G. M. Martin, "The biology of aging: 1985–2010 and beyond," *The FASEB Journal*, vol. 25, no. 11, pp. 3756–3762, 2011.

[6] C. J. Kenyon, "The genetics of ageing," *Nature*, vol. 464, no. 7288, pp. 504–512, 2010.

[7] L. Fontana, L. Partridge, and V. D. Longo, "Extending healthy life span-from yeast to humans," *Science*, vol. 328, no. 5976, pp. 321–326, 2010.

[8] M. C. Haigis and B. A. Yankner, "The aging stress response," *Molecular Cell*, vol. 40, no. 2, pp. 333–344, 2010.

[9] N. Alic and L. Partridge, "Death and dessert: nutrient signalling pathways and ageing," *Current Opinion in Cell Biology*, vol. 23, no. 6, pp. 738–743, 2011.

[10] E. Ziv and D. Hu, "Genetic variation in insulin/IGF-1 signaling pathways and longevity," *Ageing Research Reviews*, vol. 10, no. 2, pp. 201–204, 2011.

[11] M. Soerensen, S. Dato, K. Christensen et al., "Replication of an association of variation in the *FOXO3A* gene with human longevity using both case-control and longitudinal data," *Aging Cell*, vol. 9, no. 6, pp. 1010–1017, 2010.

[12] L. Pawlikowska, D. Hu, S. Huntsman et al., "Association of common genetic variation in the insulin/IGF1 signaling pathway with human longevity," *Aging Cell*, vol. 8, no. 4, pp. 460–472, 2009.

[13] F. Flachsbart, A. Caliebe, R. Kleindorp et al., "Association of *FOXO3A* variation with human longevity confirmed in German centenarians," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 8, pp. 2700–2705, 2009.

[14] B. J. Willcox, T. A. Donlon, Q. He et al., "*FOXO3A* genotype is strongly associated with human longevity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 37, pp. 13987–13992, 2008.

[15] D. Harman, "Aging: a theory based on free radical and radiation chemistry," *Journal of Gerontology*, vol. 11, no. 3, pp. 298–300, 1956.

[16] A. B. Salmon, A. Richardson, and V. I. Pérez, "Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging?" *Free Radical Biology and Medicine*, vol. 48, no. 5, pp. 642–655, 2010.

[17] M. Ristow and K. Zarse, "How increased oxidative stress promotes longevity and metabolic health: the concept of mitochondrial hormesis (mitohormesis)," *Experimental Gerontology*, vol. 45, no. 6, pp. 410–418, 2010.

[18] S. I. S. Rattan, H. Sejersen, R. A. Fernandes, and W. Luo, "Stress-mediated hormetic modulation of aging, wound healing, and angiogenesis in human cells," *Annals of the New York Academy of Sciences*, vol. 1119, no. 1, pp. 112–121, 2007.

[19] C. M. McCay, M. F. Crowell, and L. A. Maynard, "The effect of retarded growth upon the length of life span and upon the ultimate body size," *Journal of Nutrition*, vol. 10, pp. 63–79, 1935.

[20] R. Weindruch, R. L. Walford, S. Fligiel, and D. Guthrie, "The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake," *Journal of Nutrition*, vol. 116, no. 4, pp. 641–654, 1986.

[21] J. C. Jiang, E. Jaruga, M. V. Repnevskaya, and S. M. Jazwinski, "An intervention resembling caloric restriction prolongs life span and retards aging in yeast," *The FASEB Journal*, vol. 14, no. 14, pp. 2135–2137, 2000.

[22] D. J. Clancy, D. Gems, E. Hafen, S. J. Leevers, and L. Partridge, "Dietary restriction in long-lived dwarf flies," *Science*, vol. 296, no. 5566, p. 319, 2002.

[23] W. Mair and A. Dillin, "Aging and survival: the genetics of life span extension by dietary restriction," *Annual Review of Biochemistry*, vol. 77, pp. 727–754, 2008.

[24] R. J. Colman, R. M. Anderson, S. C. Johnson et al., "Caloric restriction delays disease onset and mortality in rhesus monkeys," *Science*, vol. 325, no. 5937, pp. 201–204, 2009.

[25] E. L. Greer and A. Brunet, "Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*," *Aging Cell*, vol. 8, no. 2, pp. 113–127, 2009.

[26] M. D. W. Piper and A. Bartke, "Diet and aging," *Cell Metabolism*, vol. 8, no. 2, pp. 99–104, 2008.

[27] E. Morselli, M. C. Maiuri, M. Markaki et al., "Caloric restriction and resveratrol promote longevity through the Sirtuin-1-dependent induction of autophagy," *Cell Death and Disease*, vol. 1, no. 1, article e10, 2010.

[28] L. Mouchiroud, L. Molin, N. Dallière, and F. Solari, "Life span extension by resveratrol, rapamycin, and metformin: the promise of dietary restriction mimetics for an healthy aging," *BioFactors*, vol. 36, no. 5, pp. 377–382, 2010.

[29] A. R. Mendelsohn and J. W. Larrick, "Rapamycin as an anti-aging therapeutic? Targeting mammalian target of rapamycin to treat Hutchinson-Gilford progeria and neurodegenerative diseases," *Rejuvenation Research*, vol. 14, no. 4, pp. 437–441, 2011.

[30] G. E. Mullin, "Red wine, grapes, and better health—resveratrol," *Nutrition in Clinical Practice*, vol. 26, no. 6, pp. 722–723, 2011.

[31] O. Vang, N. Ahmad, C. A. Baile et al., "What is new for an old molecule? systematic review and recommendations on the use of resveratrol," *PLoS ONE*, vol. 6, no. 6, Article ID e19881, 2011.

[32] S. Bulterijs, "Metformin as a geroprotector," *Rejuvenation Research*, vol. 14, no. 5, pp. 469–482, 2011.

[33] F. Madeo, T. Eisenberg, S. Büttner, C. Ruckenstuhl, and G. Kroemer, "Spermidine: a novel autophagy inducer and longevity elixir," *Autophagy*, vol. 6, no. 1, pp. 160–162, 2010.

[34] B. Winkel-Shirley, "Biosynthesis of flavonoids and effects of stress," *Current Opinion in Plant Biology*, vol. 5, no. 3, pp. 218–223, 2002.

[35] P. Reymond, H. Weber, M. Damond, and E. E. Farmer, "Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis," *Plant Cell*, vol. 12, no. 5, pp. 707–719, 2000.

[36] D. Hermsmeier, U. Schittko, and I. T. Baldwin, "Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs," *Plant Physiology*, vol. 125, no. 2, pp. 683–700, 2001.

[37] R. Tsao, "Chemistry and biochemistry of dietary polyphenols," *Nutrients*, vol. 2, no. 12, pp. 1231–1246, 2010.

[38] D. O. Kennedy and E. L. Wightman, "Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function," *Advances in Nutrition*, vol. 2, no. 1, pp. 32–50, 2011.

[39] A. Salminen, A. Kauppinen, and K. Kaarniranta, "Phytochemicals suppress nuclear factor-kappaB signaling: impact on health span and the aging process," *Current Opinion in Clinical Nutrition & Metabolic Care*, vol. 15, no. 1, pp. 23–28, 2012.

[40] T. Y. Wu, C. P. Chen, and T. R. Jinn, "Traditional Chinese medicines and Alzheimer's disease," *Taiwanese Journal of Obstetrics and Gynecology*, vol. 50, no. 2, pp. 131–135, 2011.

[41] B. A. Abdel-Wahab and S. M. Abd El-Aziz, "Ginkgo biloba protects against intermittent hypoxia-induced memory deficits and hippocampal DNA damage in rats," *Phytomedicine*, vol. 19, no. 5, pp. 444–450, 2012.

[42] F. V. DeFeudis and K. Drieu, "Ginkgo biloba extract (EGb 761) and CNS functions: basic studies and clinical applications," *Current Drug Targets*, vol. 1, no. 1, pp. 25–58, 2000.

[43] A. Kampkötter, T. Pielarski, R. Rohrig et al., "The Ginkgo biloba extract EGb761 reduces stress sensitivity, ROS accumulation and expression of catalase and glutathione S-transferase 4 in *Caenorhabditis elegans*," *Pharmacological Research*, vol. 55, no. 2, pp. 139–147, 2007.

[44] J. Vining Smith and Y. Luo, "Elevation of oxidative free radicals in Alzheimer's disease models can be attenuated by Ginkgo biloba extract EGb 761," *Journal of Alzheimer's Disease*, vol. 5, no. 4, pp. 287–300, 2003.

[45] A. Strayer, Z. Wu, Y. Christen, C. D. Link, and Y. Luo, "Expression of the small heat-shock protein Hsp16-2 in *Caenorhabditis elegans* is suppressed by Ginkgo biloba extract EGb 761," *The FASEB Journal*, vol. 17, no. 15, pp. 2305–2307, 2003.

[46] Z. Wu, J. V. Smith, V. Paramasivam et al., "Ginkgo biloba extract EGb 761 increases stress resistance and extends life span of *Caenorhabditis elegans*," *Cellular and Molecular Biology*, vol. 48, no. 6, pp. 725–731, 2002.

[47] Y. Wu, Z. Wu, P. Butko et al., "Amyloid-β-induced pathological behaviors are suppressed by Ginkgo biloba extract EGB 761 and ginkgolides in transgenic *Caenorhabditis elegans*," *Journal of Neuroscience*, vol. 26, no. 50, pp. 13102–13113, 2006.

[48] V. H. Finder and R. Glockshuber, "Amyloid-β aggregation," *Neurodegenerative Diseases*, vol. 4, no. 1, pp. 13–27, 2007.

[49] Y. Luo, J. V. Smith, V. Paramasivam et al., "Inhibition of amyloid-β aggregation and caspase-3 activation by the Ginkgo biloba extract EGb761," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 19, pp. 12197–12202, 2002.

[50] J. V. Smith, A. J. Burdick, P. Golik, I. Khan, D. Wallace, and Y. Luo, "Anti-apoptotic properties of Ginkgo biloba extract EGb 761 in differentiated PC12 cells," *Cellular and Molecular Biology*, vol. 48, no. 6, pp. 699–707, 2002.

[51] C. C. Neto, "Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases," *Molecular*

Nutrition and Food Research, vol. 51, no. 6, pp. 652–664, 2007.

[52] J. A. Joseph, B. Shukitt-Hale, N. A. Denisova et al., "Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation," *Journal of Neuroscience*, vol. 19, no. 18, pp. 8114–8121, 1999.

[53] M. A. Wilson, B. Shukitt-Hale, W. Kalt, D. K. Ingram, J. A. Joseph, and C. A. Wolkow, "Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*," *Aging Cell*, vol. 5, no. 1, pp. 59–68, 2006.

[54] C. Peng, Y. Zuo, K. M. Kwan et al., "Blueberry extract prolongs lifespan of *Drosophila melanogaster*," *Experimental Gerontology*, vol. 47, no. 2, pp. 170–178, 2012.

[55] E. Pappas and K. M. Schaich, "Phytochemicals of cranberries and cranberry products: characterization, potential health effects, and processing stability," *Critical Reviews in Food Science and Nutrition*, vol. 49, no. 9, pp. 741–781, 2009.

[56] F. E. Babili, J. Bouajila, J. P. Souchard et al., "Oregano: chemical analysis and evaluation of its antimalarial, antioxidant, and cytotoxic activities," *Journal of Food Science*, vol. 76, no. 3, pp. C512–C518, 2011.

[57] S. Zou, J. R. Carey, P. Liedo, D. K. Ingram, B. Yu, and R. Ghaedian, "Prolongevity effects of an oregano and cranberry extract are diet dependent in the mexican fruit fly (*Anastrepha ludens*)," *Journals of Gerontology*, vol. 65, no. 1, pp. 41–50, 2010.

[58] S. Zou, J. R. Carey, P. Liedo, D. K. Ingram, and B. Yu, "Prolongevity effects of a botanical with oregano and cranberry extracts in Mexican fruit flies: examining interactions of diet restriction and age," *Age*, vol. 34, no. 2, pp. 269–279, 2012.

[59] S. Guha, M. Cao, R. M. Kane, A. M. Savino, S. Zou, and Y. Dong, "The longevity effect of cranberry extract in *Caenorhabditis elegans* is modulated by *daf*-16 and *osr*-1," *Age*. In press.

[60] E. Cohen and A. Dillin, "The insulin paradox: aging, proteotoxicity and neurodegeneration," *Nature Reviews Neuroscience*, vol. 9, no. 10, pp. 759–767, 2008.

[61] J. Z. Morris, H. A. Tissenbaum, and G. Ruvkun, "A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*," *Nature*, vol. 382, no. 6591, pp. 536–539, 1996.

[62] X. He and H. L. Rui, "Cranberry phytochemicals: isolation, structure elucidation, and their antiproliferative and antioxidant activities," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 19, pp. 7069–7074, 2006.

[63] D. Konopacka, K. Jesionkowska, D. Kruczyńska et al., "Apple and peach consumption habits across European countries," *Appetite*, vol. 55, no. 3, pp. 478–483, 2010.

[64] A. G. Schauss, X. Wu, R. L. Prior et al., "Phytochemical and nutrient composition of the freeze-dried amazonian palm berry, *Euterpe oleraceae* Mart. (Acai)," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 22, pp. 8598–8603, 2006.

[65] A. G. Schauss, X. Wu, R. L. Prior et al., "Antioxidant capacity and other bioactivities of the freeze-dried Amazonian palm berry, *Euterpe oleraceae* Mart. (Acai)," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 22, pp. 8604–8610, 2006.

[66] O. Boyd, P. Weng, X. Sun et al., "Nectarine promotes longevity in *Drosophila melanogaster*," *Free Radical Biology and Medicine*, vol. 50, no. 11, pp. 1669–1678, 2011.

[67] X. Sun, J. Seeberger, T. Alberico et al., "Açai palm fruit (Euterpe oleracea Mart.) pulp improves survival of flies on a high fat diet," *Experimental Gerontology*, vol. 45, no. 3, pp. 243–251, 2010.

[68] P. Liedo, J. R. Carey, D. K. Ingram, and S. Zou, "The interplay among dietary fat, sugar, protein and acai (*Euterpe oleracea* Mart.) pulp in modulating lifespan and reproduction in a Tephritid fruit fly," *Experimental Gerontology*, vol. 47, no. 7, pp. 536–539, 2012.

[69] K. J. Pearson, J. A. Baur, K. N. Lewis et al., "Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span," *Cell Metabolism*, vol. 8, no. 2, pp. 157–168, 2008.

[70] S. Zou, J. R. Carey, P. Liedo et al., "The prolongevity effect of resveratrol depends on dietary composition and calorie intake in a tephritid fruit fly," *Experimental Gerontology*, vol. 44, no. 6-7, pp. 472–476, 2009.

[71] C. Wang, C. T. Wheeler, T. Alberico et al., "The effect of resveratrol on lifespan depends on both gender and dietary nutrient composition in *Drosophila melanogaster*," *Age*. In press.

[72] P. Liedo, J. R. Carey, D. K. Ingram, and S. Zou, "The interplay among dietary fat, sugar, protein and acai (*Euterpe oleracea* Mart.) pulp in modulating lifespan and reproduction in a Tephritid fruit fly," *Experimental Gerontology*, vol. 47, no. 7, pp. 536–539, 2012.

[73] N. G. Baydar, H. Baydar, and T. Debener, "Analysis of genetic relationships among *Rosa damascena* plants grown in Turkey by using AFLP and microsatellite markers," *Journal of Biotechnology*, vol. 111, no. 3, pp. 263–267, 2004.

[74] T. Hongratanaworakit, "Relaxing effect of rose oil on humans," *Natural Product Communications*, vol. 4, no. 2, pp. 291–296, 2009.

[75] N. Kumar, P. Bhandari, B. Singh, A. P. Gupta, and V. K. Kaul, "Reversed phase-HPLC for rapid determination of polyphenols in flowers of rose species," *Journal of Separation Science*, vol. 31, no. 2, pp. 262–267, 2008.

[76] E. Basim and H. Basim, "Antibacterial activity of *Rosa damascena* essential oil," *Fitoterapia*, vol. 74, no. 4, pp. 394–396, 2003.

[77] R. Ramezani, A. Moghimi, H. Rakhshandeh, H. Ejtehadi, and M. Kheirabadi, "The effect of *Rosa damascena* essential oil on the amygdala electrical kindling seizures in rat," *Pakistan Journal of Biological Sciences*, vol. 11, no. 5, pp. 746–751, 2008.

[78] N. Shokouhinejad, M. Emaneini, M. Aligholi, and F. Jabalameli, "Antimicrobial effect of *Rosa damascena* extract on selected endodontic pathogens," *Journal of the California Dental Association*, vol. 38, no. 2, pp. 123–126, 2010.

[79] S. Awale, C. Tohda, Y. Tezuka et al., "Protective effects of *Rosa damascena* and its active constituent on abeta(25–35)-induced neuritic atrophy," *Evidence-based Complementary and Alternative Medicine*, vol. 2011, Article ID 131042, 2011.

[80] S. E. Schriner, N. S. Katoozi, K. Q. Pham, M. Gazarian, A. Zarban, and M. Jafari, "Extension of *Drosophila* lifespan by *Rosa damascena* associated with an increased sensitivity to heat," *Biogerontology*, vol. 13, no. 2, pp. 105–117, 2012.

[81] J. H. Weisburger, "Chemopreventive effects of cocoa polyphenols on chronic diseases," *Experimental Biology and Medicine*, vol. 226, no. 10, pp. 891–897, 2001.

[82] P. Martorell, J. V. Forment, R. De Llanos et al., "Use of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* as model organisms to study the effect of cocoa polyphenols in the resistance to oxidative stress," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 5, pp. 2077–2085, 2011.

[83] N. T. Zaveri, "Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications," *Life Sciences*, vol. 78, no. 18, pp. 2073–2080, 2006.

[84] S. M. Sagar, D. Yance, and R. K. Wong, "Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer-part 1," *Current Oncology*, vol. 13, no. 1, pp. 14–26, 2006.

[85] J. A. Yang, J. H. Choi, and S. J. Rhee, "Effects of green tea catechin on phospholipase A2 activity and antithrombus in streptozotocin diabetic rats," *Journal of Nutritional Science and Vitaminology*, vol. 45, no. 3, pp. 337–346, 1999.

[86] P. G. Pietta, "Flavonoids as antioxidants," *Journal of Natural Products*, vol. 63, no. 7, pp. 1035–1042, 2000.

[87] S. G. Khan, S. K. Katiyar, R. Agarwal, and H. Mukhtar, "Enhancement of antioxidant and phase II enzymes by oral feeding of green tea polyphenols in drinking water to SKH-1 hairless mice: possible role in cancer chemoprevention," *Cancer Research*, vol. 52, no. 14, pp. 4050–4052, 1992.

[88] Y. M. Li, H. Y. E. Chan, Y. Huang, and Z. Y. Chen, "Green tea catechins upregulate superoxide dismutase and catalase in fruit flies," *Molecular Nutrition and Food Research*, vol. 51, no. 5, pp. 546–554, 2007.

[89] Y. M. Li, H. Y. E. Chan, X. Q. Yao, Y. Huang, and Z. Y. Chen, "Green tea catechins and broccoli reduce fat-induced mortality in *Drosophila melanogaster*," *Journal of Nutritional Biochemistry*, vol. 19, no. 6, pp. 376–383, 2008.

[90] S. Abbas and M. Wink, "Epigallocatechin gallate from green tea (*Camellia sinensis*) increases lifespan and stress resistance in *Caenorhabditis elegans*," *Planta Medica*, vol. 75, no. 3, pp. 216–221, 2009.

[91] L. Zhang, G. Jie, J. Zhang, and B. Zhao, "Significant longevity-extending effects of EGCG on *Caenorhabditis elegans* under stress," *Free Radical Biology and Medicine*, vol. 46, no. 3, pp. 414–421, 2009.

[92] K. Zarse, S. Jabin, and M. Ristow, "L: -Theanine extends lifespan of adult *Caenorhabditis elegans*," *European Journal of Nutrition*, vol. 51, no. 6, pp. 765–768, 2012.

[93] T. I. Kim, Y. K. Lee, S. G. Park et al., "l-Theanine, an amino acid in green tea, attenuates β-amyloid-induced cognitive dysfunction and neurotoxicity: reduction in oxidative damage and inactivation of ERK/p38 kinase and NF-κB pathways," *Free Radical Biology and Medicine*, vol. 47, no. 11, pp. 1601–1610, 2009.

[94] J. Bryan, "Psychological effects of dietary components of tea: caffeine and L-theanine," *Nutrition Reviews*, vol. 66, no. 2, pp. 82–90, 2008.

[95] Y. Sadzuka, T. Sugiyama, A. Miyagishima, Y. Nozawa, and S. Hirota, "The effects of theanine, as a novel biochemical modulator, on the antitumor activity of adriamycin," *Cancer Letters*, vol. 105, no. 2, pp. 203–209, 1996.

[96] H. Yokogoshi, Y. Kato, Y. M. Sagesaka, T. Takihara-Matsuura, T. Kakuda, and N. Takeuchi, "Reduction effect of theanine on blood pressure and brain 5-hydroxyindoles in spontaneously hypertensive rats," *Bioscience, Biotechnology, and Biochemistry*, vol. 59, no. 4, pp. 615–618, 1995.

[97] G. Buckland, A. Agudo, N. Travier et al., "Adherence to the Mediterranean diet reduces mortality in the Spanish cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC-Spain)," *British Journal of Nutrition*, vol. 106, no. 10, pp. 1581–1591, 2011.

[98] M. Gerber, "Biofactors in the Mediterranean diet," *Clinical Chemistry and Laboratory Medicine*, vol. 41, no. 8, pp. 999–1004, 2003.

[99] P. Lagiou, D. Trichopoulos, S. Sandin et al., "Mediterranean dietary pattern and mortality among young women: a cohort study in Sweden," *British Journal of Nutrition*, vol. 96, no. 2, pp. 384–392, 2006.

[100] J. C. Stoclet, T. Chataigneau, M. Ndiaye et al., "Vascular protection by dietary polyphenols," *European Journal of Pharmacology*, vol. 500, no. 1–3, pp. 299–313, 2004.

[101] A. Trichopoulou, "Modified Mediterranean diet and survival: EPIC-elderly prospective cohort study," *British Medical Journal*, vol. 330, no. 7498, pp. 991–995, 2005.

[102] A. Bendini, L. Cerretani, A. Carrasco-Pancorbo et al., "Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade," *Molecules*, vol. 12, no. 8, pp. 1679–1719, 2007.

[103] A. Cañuelo, B. Gilbert-López, P. Pacheco-Liñán, E. Martínez-Lara, E. Siles, and A. Miranda-Vizuete, "Tyrosol, a main phenol present in extra virgin olive oil, increases lifespan and stress resistance in *Caenorhabditis elegans*," *Mechanisms of Ageing and Development*, vol. 133, no. 8, pp. 563–574, 2012.

[104] A. L. Hsu, C. T. Murphy, and C. Kenyon, "Regulation of aging and age-related disease by DAF-16 and heat-shock factor," *Science*, vol. 300, no. 5622, pp. 1142–1145, 2003.

[105] A. W. Boots, G. R. M. M. Haenen, and A. Bast, "Health effects of quercetin: from antioxidant to nutraceutical," *European Journal of Pharmacology*, vol. 585, no. 2-3, pp. 325–337, 2008.

[106] K. Pietsch, N. Saul, R. Menzel, S. R. Stürzenbaum, and C. E. W. Steinberg, "Quercetin mediated lifespan extension in *Caenorhabditis elegans* is modulated by *age*-1, *daf*-2, *sek*-1 and *unc*-43," *Biogerontology*, vol. 10, no. 5, pp. 565–578, 2009.

[107] N. Saul, K. Pietsch, R. Menzel, and C. E. W. Steinberg, "Quercetin-mediated longevity in *Caenorhabditis elegans*: is DAF-16 involved?" *Mechanisms of Ageing and Development*, vol. 129, no. 10, pp. 611–613, 2008.

[108] N. Saul, K. Pietsch, R. Menzel, S. R. Stürzenbaum, and C. E. W. Steinberg, "The longevity effect of tannic acid in *Caenorhabditis elegans*: disposable soma meets hormesis," *Journals of Gerontology*, vol. 65, no. 6, pp. 626–635, 2010.

[109] F. Surco-Laos, J. Cabello, E. Gómez-Orte et al., "Effects of O-methylated metabolites of quercetin on oxidative stress, thermotolerance, lifespan and bioavailability on *Caenorhabditis elegans*," *Food & Function*, vol. 2, no. 8, pp. 445–456, 2011.

[110] A. Kampkötter, C. Timpel, R. F. Zurawski et al., "Increase of stress resistance and lifespan of *Caenorhabditis elegans* by quercetin," *Comparative Biochemistry and Physiology Part B*, vol. 149, no. 2, pp. 314–323, 2008.

[111] K. Yazawa, T. Kihara, H. Shen, Y. Shimmyo, T. Niidome, and H. Sugimoto, "Distinct mechanisms underlie distinct polyphenol-induced neuroprotection," *FEBS Letters*, vol. 580, no. 28-29, pp. 6623–6628, 2006.

[112] T. J. Kim, J. L. Silva, and Y. S. Jung, "Antibacterial activity of fresh and processed red muscadine juice and the role of their polar compounds on *Escherichia coli* O157:H7," *Journal of Applied Microbiology*, vol. 107, no. 2, pp. 533–539, 2009.

[113] T. Taguri, T. Tanaka, and I. Kouno, "Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease," *Biological and Pharmaceutical Bulletin*, vol. 27, no. 12, pp. 1965–1969, 2004.

[114] M. T. Huang, R. L. Chang, and A. W. Wood, "Inhibition of the mutagenicity of bay-region diol-epoxides of polycyclic aromatic hydrocarbons by tannic acid, hydroxylated anthraquinones and hydroxylated cinnamic acid derivatives," *Carcinogenesis*, vol. 6, no. 2, pp. 237–242, 1985.

[115] S. Taffetani, Y. Ueno, F. Meng et al., "Tannic acid inhibits cholangiocyte proliferation after bile duct ligation via a cyclic adenosine 5′,3′-monophosphate-dependent pathway," *American Journal of Pathology*, vol. 166, no. 6, pp. 1671–1679, 2005.

[116] R. G. Andrade Jr., J. S. Ginani, G. K. B. Lopes, F. Dutra, A. Alonso, and M. Hermes-Lima, "Tannic acid inhibits in vitro iron-dependent free radical formation," *Biochimie*, vol. 88, no. 9, pp. 1287–1296, 2006.

[117] G. K. B. Lopes, H. M. Schulman, and M. Hermes-Lima, "Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions," *Biochimica et Biophysica Acta*, vol. 1472, no. 1-2, pp. 142–152, 1999.

[118] K. Pietsch, N. Saul, S. C. Swain, R. Menzel, C. E. Steinberg, and S. R. Stürzenbaum, "Meta-analysis of global transcriptomics suggests that conserved genetic pathways are responsible for quercetin and tannic acid mediated longevity in *C. elegans*," *Frontiers in Genetics*, vol. 3, article 48, 2012.

[119] J. L. Johannes and V. L. S. Cilliers, "Characterization of the products of nonenzymic autoxidative phenolic reactions in a caffeic acid model system," *Journal of Agricultural and Food Chemistry*, vol. 39, no. 7, pp. 1298–1303, 1991.

[120] M. R. Al-Sereiti, K. M. Abu-Amer, and P. Sen, "Pharmacology of rosemary (*Rosmarinus officinalis* Linn.) and its therapeutic potentials," *Indian Journal of Experimental Biology*, vol. 37, no. 2, pp. 124–130, 1999.

[121] A. Sahu, N. Rawal, and M. K. Pangburn, "Inhibition of complement by covalent attachment of rosmarinic acid to activated C3b," *Biochemical Pharmacology*, vol. 57, no. 12, pp. 1439–1446, 1999.

[122] M. A. Kelm, M. G. Nair, G. M. Strasburg, and D. L. DeWitt, "Antioxidant and cyclooxygenase inhibitory phenolic compounds from *Ocimum sanctum* Linn," *Phytomedicine*, vol. 7, no. 1, pp. 7–13, 2000.

[123] W. Zheng and S. Y. Wang, "Antioxidant activity and phenolic compounds in selected herbs," *Journal of Agricultural and Food Chemistry*, vol. 49, no. 11, pp. 5165–5170, 2001.

[124] K. Pietsch, N. Saul, S. Chakrabarti, S. R. Stürzenbaum, R. Menzel, and C. E. W. Steinberg, "Hormetins, antioxidants and prooxidants: defining quercetin-, caffeic acid- and rosmarinic acid-mediated life extension in *C. elegans*," *Biogerontology*, vol. 12, no. 4, pp. 329–347, 2011.

[125] T. Eisenberg, H. Knauer, A. Schauer et al., "Induction of autophagy by spermidine promotes longevity," *Nature Cell Biology*, vol. 11, no. 11, pp. 1305–1314, 2009.

[126] E. Morselli, G. Mariño, M. V. Bennetzen et al., "Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome," *Journal of Cell Biology*, vol. 192, no. 4, pp. 615–629, 2011.

[127] B. Levine and G. Kroemer, "Autophagy in the pathogenesis of disease," *Cell*, vol. 132, no. 1, pp. 27–42, 2008.

[128] G. Scalabrino and M. E. Ferioli, "Polyamines in mammalian aging: an oncological problem, too? A review," *Mechanisms of Ageing and Development*, vol. 26, no. 2-3, pp. 149–164, 1984.

[129] M. Braunstein, R. E. Sobel, C. D. Allis, B. M. Turner, and J. R. Broach, "Efficient transcriptional silencing in *Saccharomyces cerevisiae* requires a heterochromatin histone acetylation pattern," *Molecular and Cellular Biology*, vol. 16, no. 8, pp. 4349–4356, 1996.

[130] E. Ford, R. Voit, G. Liszt, C. Magin, I. Grummt, and L. Guarente, "Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription," *Genes and Development*, vol. 20, no. 9, pp. 1075–1080, 2006.

[131] R. Mostoslavsky, K. F. Chua, D. B. Lombard et al., "Genomic instability and aging-like phenotype in the absence of mammalian SIRT6," *Cell*, vol. 124, no. 2, pp. 315–329, 2006.

[132] B. B. Aggarwal and K. B. Harikumar, "Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases," *International Journal of Biochemistry and Cell Biology*, vol. 41, no. 1, pp. 40–59, 2009.

[133] B. B. Aggarwal and B. Sung, "Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets," *Trends in Pharmacological Sciences*, vol. 30, no. 2, pp. 85–94, 2009.

[134] S. Bengmark, "Curcumin, an atoxic antioxidant and natural NFκB, cyclooxygenase-2, lipooxygenase, and inducible nitric oxide synthase inhibitor: a shield against acute and chronic diseases," *Journal of Parenteral and Enteral Nutrition*, vol. 30, no. 1, pp. 45–51, 2006.

[135] R. K. Maheshwari, A. K. Singh, J. Gaddipati, and R. C. Srimal, "Multiple biological activities of curcumin: a short review," *Life Sciences*, vol. 78, no. 18, pp. 2081–2087, 2006.

[136] H. Nishino, H. Tokuda, Y. Satomi et al., "Cancer prevention by antioxidants," *BioFactors* , vol. 22, no. 1–4, pp. 57–61, 2004.

[137] K. S. Lee, B. S. Lee, S. Semnani et al., "Curcumin extends life span, improves health span, and modulates the expression of age-associated aging genes in *Drosophila melanogaster*," *Rejuvenation Research*, vol. 13, no. 5, pp. 561–570, 2010.

[138] V. H. Liao, C. W. Yu, Y. J. Chu, W. H. Li, Y. C. Hsieh, and T. T. Wang, "Curcumin-mediated lifespan extension in *Caenorhabditis elegans*," *Mechanisms of Ageing and Development*, vol. 132, no. 10, pp. 480–487, 2011.

[139] R. I. Morimoto, "Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging," *Genes and Development*, vol. 22, no. 11, pp. 1427–1438, 2008.

[140] R. R. Kopito and D. Ron, "Conformational disease," *Nature Cell Biology*, vol. 2, no. 11, pp. E207–E209, 2000.

[141] D. Eisenberg and M. Jucker, "The amyloid state of proteins in human diseases," *Cell*, vol. 148, no. 6, pp. 1188–1203, 2012.

[142] S. Alavez, M. C. Vantipalli, D. J. S. Zucker, I. M. Klang, and G. J. Lithgow, "Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan," *Nature*, vol. 472, no. 7342, pp. 226–230, 2011.

[143] D. A. Skorupa, A. Dervisefendic, J. Zwiener, and S. D. Pletcher, "Dietary composition specifies consumption, obesity, and lifespan in *Drosophila melanogaster*," *Aging Cell*, vol. 7, no. 4, pp. 478–490, 2008.

[144] S. Zou, P. Liedo, L. Altamirano-Robles et al., "Recording lifetime behavior and movement in an invertebrate model," *PLoS ONE*, vol. 6, no. 4, Article ID e18151, 2011.

[145] J. R. Carey, L. G. Harshman, P. Liedo, H. G. Müller, J. L. Wang, and Z. Zhang, "Longevity-fertility trade-offs in the tephritid fruit fly, *Anastrepha ludens*, across dietary-restriction gradients," *Aging Cell*, vol. 7, no. 4, pp. 470–477, 2008.

[146] G. D. Lee, M. A. Wilson, M. Zhu et al., "Dietary deprivation extends lifespan in *Caenorhabditis elegans*," *Aging Cell*, vol. 5, no. 6, pp. 515–524, 2006.

[147] M. D. W. Piper, L. Partridge, D. Raubenheimer, and S. J. Simpson, "Dietary restriction and aging: a unifying perspective," *Cell Metabolism*, vol. 14, no. 2, pp. 154–160, 2011.

[148] K. J. Vinknes, S. De Vogel, A. K. Elshorbagy et al., "Dietary intake of protein is positively associated with percent body fat in middle-aged and older adults," *Journal of Nutrition*, vol. 141, no. 3, pp. 440–446, 2011.

[149] M. Meydani and S. T. Hasan, "Dietary polyphenols and obesity," *Nutrients*, vol. 2, no. 7, pp. 737–751, 2010.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 491759, 8 pages
doi:10.1155/2012/491759

Research Article

Apple Can Act as Anti-Aging on Yeast Cells

Vanessa Palermo,[1] Fulvio Mattivi,[2] Romano Silvestri,[3] Giuseppe La Regina,[3] Claudio Falcone,[1] and Cristina Mazzoni[1]

[1] *Dipartimento di Biologia e Biotecnologie Charles Darwin, Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza Università di Roma, Piazzale Aldo Moro 5, 00185 Roma, Italy*
[2] *Food Quality and Nutrition Department, Research and Innovation Centre, Fondazione Edmund Mach, Via E. Mach 1, 38010 San Michele all'Adige, Italy*
[3] *Dipartimento di Chimica e Tecnologie del Farmaco, Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza Università di Roma, Piazzale Aldo Moro 5, 00185 Roma, Italy*

Correspondence should be addressed to Cristina Mazzoni, cristina.mazzoni@uniroma1.it

Received 31 May 2012; Revised 7 July 2012; Accepted 14 July 2012

Academic Editor: Paula Ludovico

In recent years, epidemiological and biochemical studies have shown that eating apples is associated with reduction of occurrence of cancer, degenerative, and cardiovascular diseases. This association is often attributed to the presence of antioxidants such as ascorbic acid (vitamin C) and polyphenols. The substances that hinder the presence of free radicals are also able to protect cells from aging. In our laboratory we used yeast, a unicellular eukaryotic organism, to determine *in vivo* efficacy of entire apples and their components, such as flesh, skin and polyphenolic fraction, to influence aging and oxidative stress. Our results indicate that all the apple components increase lifespan, with the best result given by the whole fruit, indicating a cooperative role of all apple components.

1. Introduction

During the last years, consumption of fruits and vegetables has received growing interest because many epidemiological and biochemical studies have demonstrated that they possess beneficial effects on human health. It is assumed that the cultivation of the apple tree goes back to prehistoric times and has developed with the formation of the *M.* × *domestica* gene pool from *M. sieversii*, originary of Thien Shan region, in Central Asia [1].

Apples can be eaten in countless ways, such as fresh and as juice that can be processed into cider, apple vinegar, or distilled.

Apples also contain nutrients and other compounds of interest, including high levels of polyphenols [2]. These compounds have biological activity and they can influence different mechanisms of fundamental relevance in cancer prevention [3]. The peel also contains high levels of triterpenoids, whereas red apples contain anthocyanins [4, 5]. The most studied molecules of this fruit are phenolic compounds that are distributed in the skin, in the core, and in the flesh. The composition of these compounds is quite variable depending on the environment of growth, period, and year of harvest. Generally, the average total polyphenol content of the most commonly consumed apples varies between 0.66 g/kg and 2.1 g/kg of fresh weight [6]. Apple polyphenols, and in particular the oligomeric proanthocyanidins, give the largest contribution to the antioxidant activity of apple extracts [7].

Numerous studies undertaken in recent years have shown that apples and their derivatives may have a wide range of biological activities, particularly useful in cases of asthma, cardiovascular disorders, polmonar disfunction and cancer. Studies in human models have shown that apples extracts may have a role in preventing several types of cancer. It has also been amply demonstrated beneficial effects on aging in mammalian skin [8].

Apple (sp. *Malus domestica*), is also rich in minerals, vitamin B, citric, and malic acid that can contribute to the welfare of the body, promoting digestion and maintaining the acidity of the digestive system.

It has been proposed that anticancer action of apple is due to the presence of vitamin C and pectin, which, during its fermentation, produce butyric acid, a substance used in some experimental drugs to treat cancer [9].

It was also shown that polyphenolic-rich apple extracts may inhibit the activity of cytosolic PKC and have a role in the suppression of human cancer cell growth *in vitro* [8] indicating that apple's polyphenols could be of interest in cancer prevention [2, 3].

Yeast has been extensively used as a model organism for the study of complex phenomena that occur in higher eukaryotes. The use of yeast compared to mammalian cell culture, lies in the ease of manipulation and the large degree of homology between yeast and human genes.

Yeast is also used as a model to study aging as the principal pathways are conserved during evolution [10]. In recent years, several homologues of classical cell-death regulators have also been identified and characterized and yeast mutants that show an accelerated/delayed aging associated with early/late cell death are available to study the effect of molecules [11–13] on cell proliferation.

To test the effect of a substance on aging, cells are maintained in the presence of the substance to be tested and their ability to form colonies over the time is measured.

An increase in cell viability in cultures containing a substance, compared to the control, is a clear indicator of the effectiveness of such substance on the aging process.

To make a more sensitive analysis, in this work we used a peculiar mutant (*Kllsm4*Δ) that shows premature aging and cell death [14–16]. We cultivated such strain in the presence of apple extracts from skin, flesh, the entire fruit and the partially purified polyphenols fraction and we found that all of these components were able to increase lifespan at different extents, with the best results given by the whole fruit, indicating that the latter contains additional important substances. In view of future practical applications, we also verified the effectiveness of apple solutions included in ointment, pointing to this kind of preparation as a base for the prevention of skin aging.

2. Results

2.1. The Antiaging Power of Apples. To better understand the antiaging effects of apples, we followed the chronological aging of a *Saccharomyces cerevisiae* mutant that shows a very premature aging [16] in the presence of apple raw extracts from the entire fruit, flesh, skin and also in the presence of a purified polyphenols fraction. This mutant, named *Kllsm4*Δ, is mutated in the *LSM4*, a key gene of mRNA degradation, and shows a marked stabilization of mRNA accompanied by rapid loss of viability during aging [14, 17, 18].

The comparison of the viability of the cells treated with different apple's extracts with the control cultures, can furnish a clear measure of their effectiveness on the aging process.

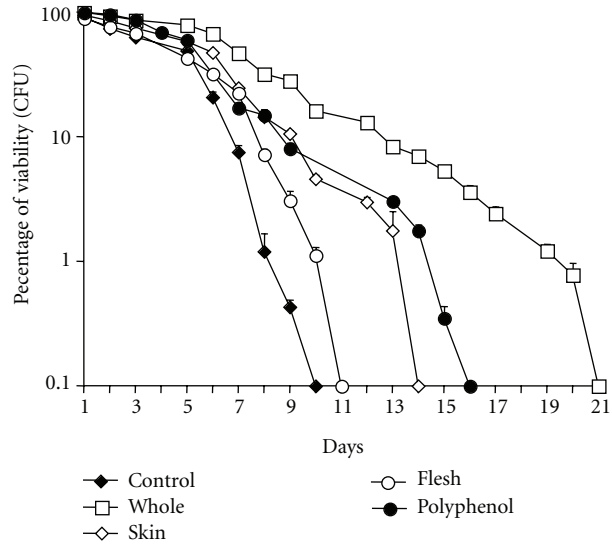


Figure 1: Apple components can recover premature aging of a yeast early-aging mutant. Cells of strain MCY4/Kllsm4::KanMX4 were grown in synthetic dextrose medium (SD) in the presence of extracts from whole apple, skin, flesh, and polyphenolic fractions. Acetone 7% was used as a control. Viability was monitored as percentage of microcolony forming units (CFU). Standard deviation was obtained from three independent experiments.

Apple's extracts consisting of freshly prepared, centrifuged and sterile filtered apple juice resuspended into 70% acetone (See Section 4) were added, prior ten time dilution, to *Kllsm4*Δ*1* cultures at the concentration of about 26 mg/mL, and the chronological aging during stationary phase of yeast samples was followed for 21 days.

As shown in Figure 1, the death kinetics of the *Kllsm4*Δ*1* strain grown in synthetic medium containing only 7% acetone is much faster compared to the cultures containing apple extracts. This difference, clearly evident since the early days, increases with time and we observed the complete loss of viability of control cultures after ten days of treatment.

In contrast, cultures containing the extracts from flesh and peel completely lost viability after 11 and 14 days, respectively, while in the presence of the whole apple extract viability ceased after twenty-one days, with an extension of more than 100% compared to control cultures. Also polyphenols extracts exerted a marked effect in prolonging lifespan, conferring an extension of viability of 60%.

It has been suggested that reactive oxygen species (ROS) are the main cause of cellular aging as they can damage and cross-link DNA, proteins, and lipids.

For this reason, we looked by dihydrorhodamine (DHR) 123 staining at the accumulation of ROS in yeast exponential and stationary (aged) cells grown in the presence of apple components.

As previously demonstrated [19] and shown in Figure 2, DHR staining revealed that in the absence of external oxidative stress, *Kllsm4*Δ*1* cells accumulated ROS during exponential phase (a) which increased with aging (b). The presence of apple components prevented ROS accumulation

in both exponential and stationary phase cells, with the exception of the apple flesh.

This component, unexpectedly, seems to increase the production of ROS since the percentage of cells showing ROS was higher than in control cultures both in exponential and stationary phase (Figure 2(c)).

We then checked the effect of apple extracts on cell survival following an oxidative stress such as the treatment of cultures with hydrogen peroxide. The presence of flesh or skin extracts could not protect cells from such a stress in that viability was comparable to that of control cultures (Figure 3), and, at the concentration of 0,8 M H_2O_2, viability was unclearly even lower.

On the contrary, both the polyphenols and the whole extracts conferred to the cells higher resistance to hydrogen peroxide treatment. As shown in Figure 3, at the highest H_2O_2 concentration tested (3 mM) cells of control cultures showed a viability of 15%, while in the presence of whole apple and polyphenolic extracts showed a viability of 28% and 20%, respectively, suggesting that these extracts contained antioxidant activity having an important role in preventing damage from oxidative stress.

Then we wanted to confirm the results obtained with the purified extracts using formulation containing a solution from homogenates of flesh, skin, or the whole apple added to ointment base. This kind of ointment might represent the basis for a cosmetic use of apple for skin care.

We first evaluated the chronological aging of 10 mL yeast cultures grown in the presence of 1 gr of formulation containing the apple solutions. As can be seen in Figure 4(a), compared to the control, flesh and peel creams extended lifespan of about 7% and 28,5%, respectively. The most striking result was obtained with the whole apple homogenate that extended cell viability of about 78,5%.

We also performed an additional test (viability spot assay) based on serial dilutions of treated cultures followed by cell plating on rich medium containing glucose as carbon source. We incubated the plates at 28°C and we then followed the growth of strain *Kllsm4Δ1* for 15 days on plates containing rich medium (YPD) at intervals of three days. As shown in Figure 4(b) after 20 days of growth at 28°C, only the culture containing the whole apple survived better until the third dilution, confirming the data on chronological aging.

We also evaluated the capacity of the creams containing the different apple components to contrast the oxidative stress induced by treatment with hydrogen peroxide.

As shown in Figure 4(c), the cream added with the whole apple solution showed the best protective effect against oxidative stress in the presence of 0.8 mM H_2O_2 while, at increasing concentrations the compound, cell grown with whole apple and skin containing creams showed the highest protection.

The apple extracts, which were prepared containing a similar concentration of apple (see Section 4), clearly differ in their concentration of total polyphenols, ranging from a minimum (ca. 138 mg/l) in apple flesh and in the partially purified polyphenols, to intermediate values (189 mg/l) in the whole apple extract, up to much higher concentration (584 mg/l) in the apple skin extract. In conclusion, the total polyphenols ranged in our extracts as follows: skin ≫ whole apple > flesh.

The apple extracts were screened also with an untargeted LCMS method, in order to provide an unbiased, semiquantitative fingerprint of their composition. These data can be useful to estimate the relative concentration of known compounds, as well as for extracting additional information useful for the design of future metabolomics experiment. The major apple phenolics according to the literature [6] were assessed and their presence in the extracts is reported in Figure 5. The extracts were screened for the presence of the most representative flavanols (catechin and epicatechin), procyanidin (procyanidin B2), dihydrochalcones (phloridzin), cynnamic acids (chlorogenic acid), and flavonols (quercetin glycosides).

The qualitative composition of these extracts was expected to vary in light of the specific localization of apple polyphenols in the different tissues [20]. In Golden Delicious apple the quercetin glycosides, and in particular the quercetin hexosides, are more concentrated in the skin cell layers lying just below the cuticle, while in the pericarp they are located mainly in correspondence with the vascular system, and the core is relatively rich in quercetin-rhamnoside [21]. Once the not edible part (the core) is removed, the apple flesh is almost devoid of flavonols (Figure 5). Also the procyanidins B2, are more concentrated in the cell layers, ca. 150 micron thick, just below the cuticle while its concentration decreases inside the fruit [21] and this is reflected in the composition of the extract in flavonols (Figure 5).

In summary and in agreement with the literature, apple flavonols were almost absent in the flesh extract, and ca. 3 times more concentrated in skin extract in respect of both whole apple and purified polyphenols extracts. The major flavonols (epicatechin and procyanidin B2), and phloridzin were 2 times higher in skin versus the other extracts. Both catechin and chlorogenic acid were contained in similar concentrations in all the extracts, including the flesh.

3. Discussion

Besides health care, more and more products prolonging youth and acting against aging come to market each year. For most of them, clear scientific demonstration of effectiveness is insufficient or even absent.

In vivo tests with cell cultures are expensive, as well those with animals that, in addition, are rejected by an increasing part of the society for ethical aspects.

For many years yeast has been used as a simple model for the study of basic mechanism underlying the complex phenomena occurring in higher eukaryotes.

Among many other advantages, the knowledge of the entire genome and the availability of mutants in each of the near six thousand genes render this organism a powerful tool for basal as well as for applicative studies.

In this respect, we successfully used yeast to evaluate the toxicity of new arylthioindoles (ATIs), which are potent tubulin assembly inhibitors [22, 23].

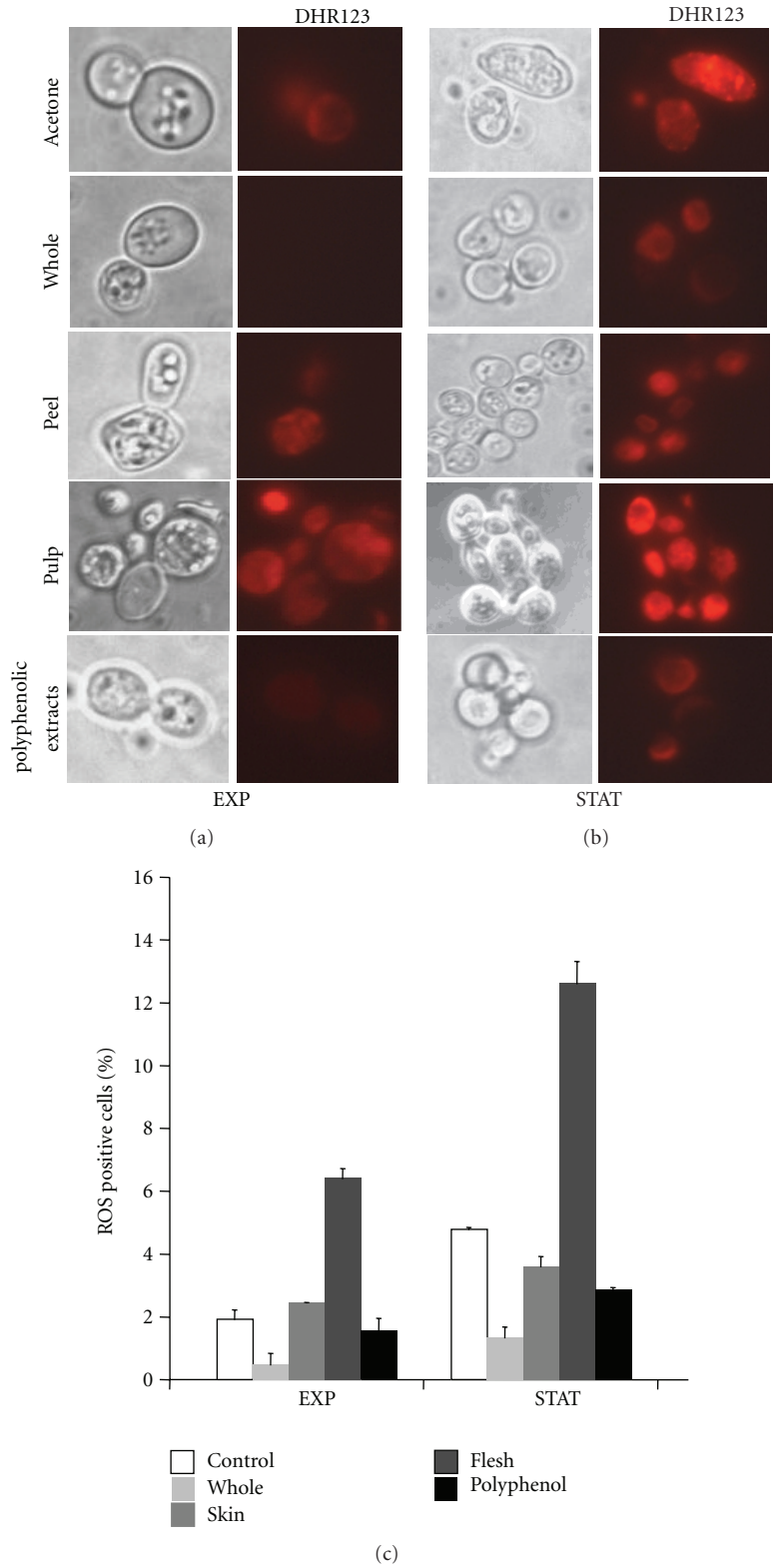


Figure 2: Effect of apple components on the production of intracellular ROS during aging. MCY4/Kllsm4::KanMX4 yeast cells were grown in the presence of extracts from the peel, flesh, or entire apple and the polyphenolic fraction. Acetone was used as a control. Cells from exponential (EXP; (a)) and stationary (STAT; (b)) cultures were incubated with DHR123 and after 3 h observed at the fluorescence microscope. In (c) is reported the percentage of ROS positive cells. Average and standard deviations, obtained from three independent experiments, are also indicated.

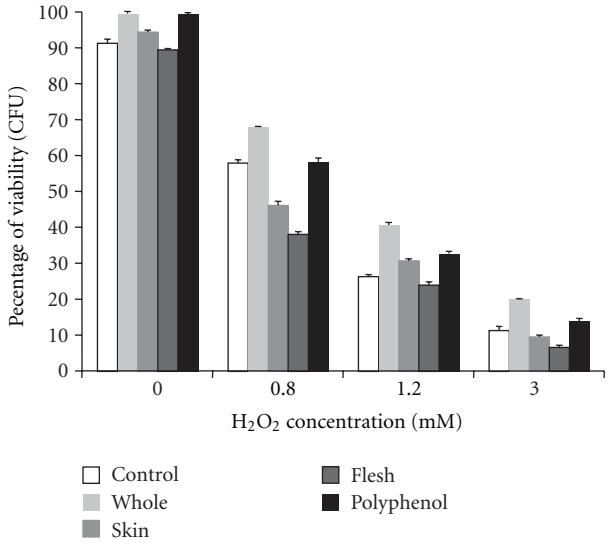


Figure 3: Effect of apple components on the oxidative stress resistance. Viability of the MCY4/Kllsm4Δ1 strain was measured after exposure of cells to H_2O_2 at the indicated concentration for 4 h in the presence of acetone (control) or extracts from whole apple, skin, flesh, and polyphenolic fractions. Average and standard deviations, obtained from three independent experiments, are indicated.

In another work, by the use of a particular strain of *S. cerevisiae*, mutated in the *LSM4* gene and showing premature aging and loss of viability, we demonstrated that carnitines, in particular acetyl-L-carnitine (ALC), are able to prolong lifespan, to protect cells from oxidative stress and prevent mitochondrial fragmentation [13].

LSM4 is an essential gene encoding a subunit of the Lsm complex involved in mRNA decapping and splicing [17]. In previous works we demonstrated that *S. cerevisiae* strains not expressing the endogenous Lsm4p recovered viability when transformed with *Kllsm4*, the hortolog gene from *Kluyveromyces lactis*, as well with a truncated form of this gene (*Kllsm4Δ1*) still containing the Sm-like domains The *Kllsm4Δ1* mutants showed accumulation of mRNA degradation intermediates, the increase of intracellular ROS, undergo cell death prematurely during stationary phase and show the typical markers of apoptosis. A quite normal cell viability observed in one of these mutants (*lsm4*) was recovered following the deletion of the yeastmeta-caspase *YCA1* gene, suggesting that caspase activity is required for apoptosis induced by increased mRNA stability [16, 24, 25].

The short lifespan and the high sensitivity to oxidative stress of *Kllsm4Δ1* mutants, compared to wild-type yeast strains, render them good candidates to test the ability of substances to prevent aging and cell death.

In this paper, we used this particular mutant to study the capability of apple's solution and derived fractions to increase viability and oxidative stress resistance in yeast cells.

Our result show that solutions of entire apples can prolong cellular life span by 100%, more than any single apple fractions. In fact, while flesh showed a modest effect, skin increased life span by 40%, probably due to its content of antioxidant polyphenols, as also suggested by the 60% increase of viability observed with cultures treated with purified polyphenol fractions. The fact that the purified polyphenols had a quite high activity, speaks in favour of an important role of apple polyphenols. However, since the whole apple extract had superior activity in spite of a much lower content of polyphenols in respect of the skin extract suggested that compounds other than polyphenols are contributing to the observed extension of the cellular life span.

Similarly, more than skin and polyphenols, entire apples strongly reduced ROS production in both exponential and stationary aging cells. In contrast, apple flesh strongly increased ROS production, especially in stationary phase cells.

Since flesh shows a very low positive effect on cell viability, one can conclude that the potential toxicity of the observed increase of ROS is in some way neutralized by other components present in the entire apple.

Whole apple extracts, closely followed by the polyphenol fraction, can also protect cells efficiently from hydrogen peroxide treatments while skin and pulp have a minor effect slightly varying with H_2O_2 concentration.

These results, in their substance, were confirmed when the same fractions were included into a creamy matrix, with the entire apple homogenate showing the best protection.

These findings are in agreement with Vanzani et al. [7] who reported that the antioxidant efficiency of the pure apple compounds was lower than that of the apple extracts, and that the higher efficiency of apples appears to be strictly related to the overwhelming presence of oligomeric proanthocyanidins.

In conclusion, the wide knowledge of yeast and the availability of genetic, biochemical and molecular biology tools make this organism a simple system useful for assaying *in vivo* the effects, among others, of anti-aging molecules. The promising results of this preliminary study provide the basis to plan further researches aimed to precisely identifying the apple compounds capable to modulate the life-extending properties of apple on yeasts.

4. Materials and Methods

4.1. Yeast Strains and Growth Conditions. We used the *S. cerevisiae* strains MCY4/KlLSM4::kanMX4 (MAT *α*, *ade1-101, his3-Δ1, trp1-289, ura3, LEU-GAL1-SDB23, pRS416/Kllsm4Δ1*) [14] and MCY4/313Kllsm4Δ1 (Mat *α*, *ade1-101, his3-Δ1, trp1-289, ura3, LEU2-GAL1-SDB23, pRS313/Kllsm4Δ1*) [16].

Cells were grown in YP (1% yeast extract, 2% peptone) supplemented with 2% glucose (YPD) at 28°C with the addition of or in SD (yeast nitrogen base without amino acids and auxotrophic requirement as needed). Solid media were supplemented with 2% Bactoagar (Difco, Detroit, MI, USA).

4.2. Cell Viability. The determination of chronological lifespan was done as described in Palermo et al., 2007 [26]. Briefly, cell suspensions (5 μL) containing approximately

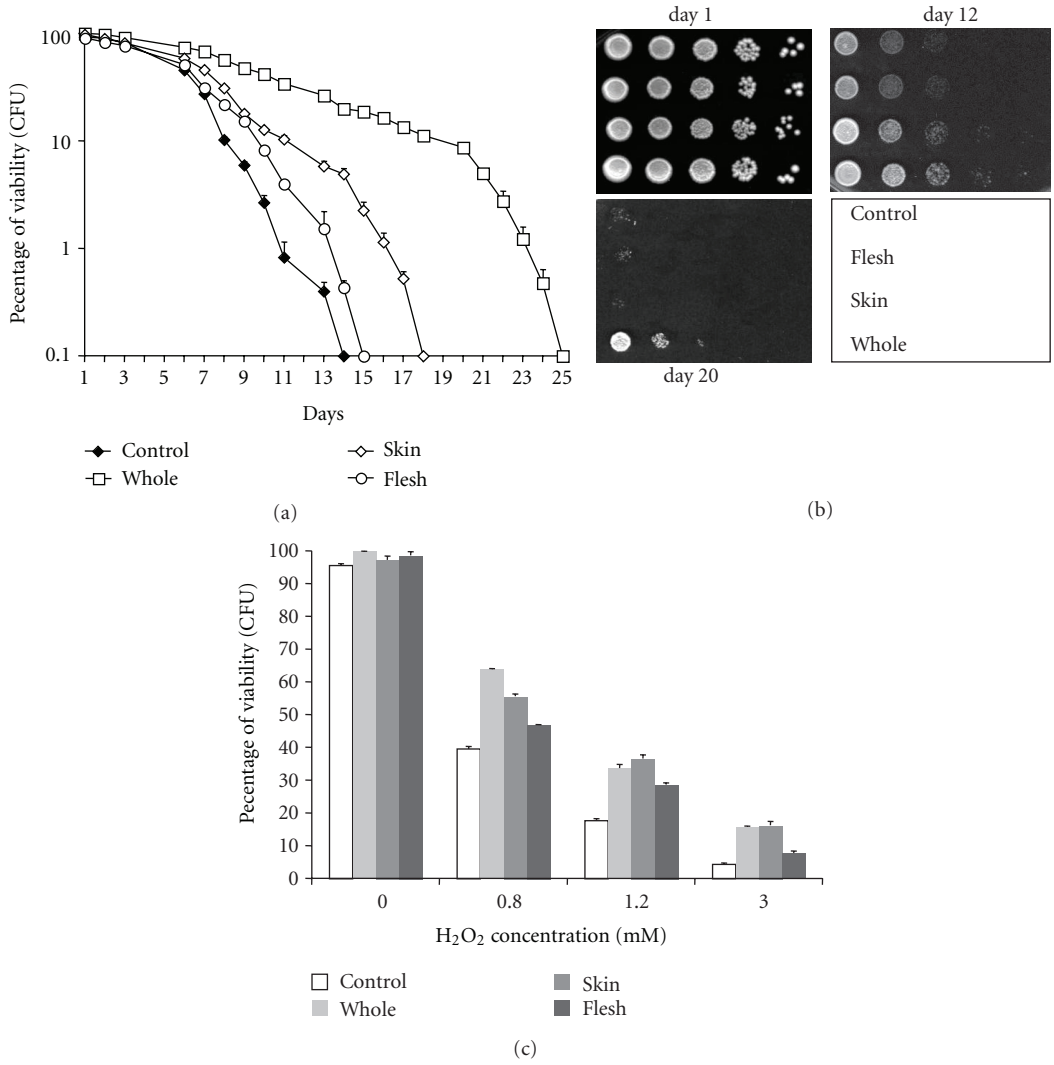


Figure 4: Effect of apple solutions included in ointment. (a) Cells of strain MCY4/Kllsm4::KanMX4 were grown in YPD+G418 antibiotic to prevent contaminations. Ointment base Essex was used as a control. Viability was monitored as percentage of microcolony forming units. Standard deviation was obtained from three independent experiments. (b) 10-fold serial dilutions of each cell culture described above were spotted onto YPD plates (1% bactopeptone, 1% yeast extract, 2% glucose, and 2% bacto agar) and incubated 2 days before recording. (c) Cells of strain MCY4/Kllsm4::KanMX4 were grown in YPD + G418 antibiotic in the presence of apple solutions in ointment and exposed to H_2O_2 at the indicated concentration for 4 h. Average and standard deviations, obtained from three independent experiments are indicated.

$6 \cdot 10^6$ cells mL^{-1} were poured on a thin layer of YPD agar on a microscope slide. A cover slip was placed over the samples and, after 24 h, viable and unviable cells were scored on the basis of their ability to form microcolonies.

Cells were grown at 28°C in minimal medium (0,67% yeast nitrogen base without amino acids) containing 2% glucose (SD) supplemented with 20 microg/mL of the appropriate nutritional requirements according to the genotype of the strains, or in YPD + G418.

For the viability spot assay, cell suspensions at the concentration of 10^7 cells/mL were transferred in microtiter plates, serially ten-fold diluted and spotted onto YP plates (1% yeast extract, 2% peptone) supplemented with 2% glucose (YPD). Plates were incubated at 28°C for five days before recording.

4.3. H_2O_2 Sensitivity. To determine the sensitivity to oxygen peroxide, cells growing exponentially were exposed to 0.8, 1.2, and 3 mM H_2O_2 at 28°C for 4 h. Cell viability was determined by counting the formation of microcolonies.

4.4. ROS Detection. Dihydrorhodamine 123 (Sigma) was added at a concentration of 5 μg/mL of cell culture from a 2.5 mg/mL stock solution in ethanol and cells were viewed without further processing through a rhodamine optical filter after 3 h incubation [27].

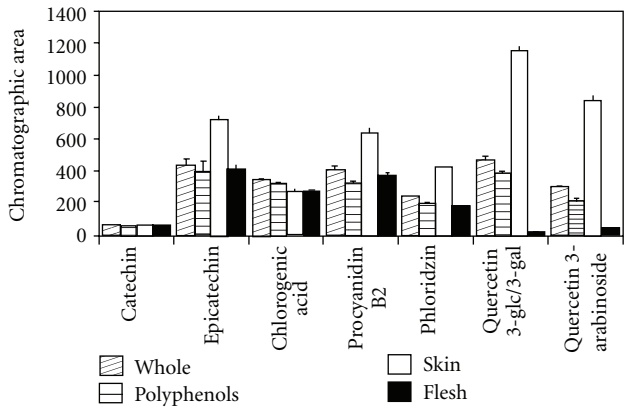


Figure 5: Comparison of the average concentrations (expressed as chromatographic area) of some characteristic apple polyphenols in the extracts used for the viability tests. The vertical bars represent the standard deviation of the measure.

4.5. Sample Preparation

Extracts. Apples, from the cv. Golden Delicious, produced in Trentino, Italy, were purchased directly from the producers in June 2009, and extracted as described in Vrhovsek [6]. To limit the enzymatic oxidation and chemical reactions, both the apples and the extraction solution were cooled to 4°C. Each sample was prepared out of three fruits. The core was removed with a corer, in order to study only the edible part.

Whole Apple Extract. Each apple was cut into equal slices. Three slices (cortex + skin) from the opposite side of each fruit (total weight 400 g) were used for the preparation of aqueous acetone extracts. The sample was homogenized in a blender Osterizer mod. 847–86 at speed one in 1500 mL of mixture acetone/water (70/30 w/w), to produce a raw extract corresponding to 26.7 g of fresh apple in 100 mL.

Whole Apple Polyphenols. An aliquot of 750 mL of the above extract, corresponding to 200 g of apple, was used to prepare a partially purified extract, containing most apple polyphenols. Acetone was removed by evaporation under reduced pressure at 35°C, from a volume of 200 mL of apple polyphenols extract, which was brought to 100 mL with water. This extract was loaded on a preparative column containing 20 g of Isolute ENV + resin (Biotage, Uppsala, Sweden), previously activated with 300 mL MeOH and 400 mL water. All the hydrophilic compounds were washed with 300 mL of water, then the polyphenols were eluted with 400 mL of methanol. After the whole extract was processed, the methanol was removed by evaporation under reduced pressure, and the purified fraction was redissolved into 750 mL of acetone/water (70/30 w/w).

Skin Extract. Each apple was manually peeled. An aliquot of 40 g of apple skins was extracted with 166 mL of the mixture acetone/water (70/30 w/w), to produce a raw extract corresponding to 24.0 g of apple skins in 100 mL.

Flesh Extract. Each apple was manually peeled. An aliquot of 200 g of apple flesh (cortex) was extracted with 750 mL of the mixture acetone/water (70/30 w/w), to produce a raw extract corresponding to 26.7 g of apple cortex in 100 mL.

The centrifuged extracts were stored at −20°C for the cell viability trials and analysis of total polyphenols.

Ointments. Fresh apples were crushed and filtered. The clear liquid (20 mL) was added to ointment base Essex (80 g) while stirring.

4.6. Chemical Analyses

Spectrophotometric Analysis of Total Polyphenols. The total amount of polyphenols was quantified with an optimized Folin-Ciocalteu method [28] according to which interfering compounds such as sugars, amino acids, and ascorbic acid were removed by cleanup on a C-18 cartridge (0.5 g, Sep pak, Waters) from the sample reconstituted in water as described above. The results are expressed as equivalent of (+)-catechin, mg/kg of FW.

Fingerprinting of the Extracts by LCMS. Analysis was carried out using a Waters Acquity UPLC, coupled to a Synapt HDMS QTOF-MS (Waters, Manchester, UK) via an electrospray interface (ESI), operating in W-mode. The software used was Masslynx 4.1. The reverse phase method was performed on a ACQUITY UPLC 1.8 μm 2.1 × 150 mm HSS T3 (Waters) column, maintained at 30°C for 30 min using 0.1% formic acid in water as solvent A and 0.1% formic acid in methanol as solvent B with the following gradient: until 6 min isocratic at 100% A, then increasing linearly to 100% B at 26 min and held isocratic at 100% B till 30 min. After each run, the column was brought the initial conditions (30% of solvent A) in 3 min.

Spectra were collected in negative ESI mode over a mass range 50–3000 amu with a scan duration of 0.3 s in centroid mode. The transfer collision energy and trap collision energy were set at 4 and 6 V, respectively. The source parameters were: capillary 2.5 kV, sampling cone 25 V, extraction cone 3 V, source temperature 150°C, desolvation temperature 500°C, desolvation gas flow 1000 l/h, and nebulizer gas flow 50 l/h. External calibration of the instrument was performed at the beginning of each batch of analysis by direct infusion of a sodium formate solution (10% formic acid/0.1 M NaOH/Acetonitrile at a ratio of 1/1/8) by controlling the mass accuracy (less than 5 ppm) and mass resolution (over 14000 FWHM). LockMass calibration was applied using a solution of leucine enkephaline (0.5 mg/L, *m/z* 556.2771 for positive and 554.2620 for negative ion mode) at 0.1 mL/min.

Each extract was analysed 10 times. The relative concentration of major apple polyphenols in the extracts are given in Figure 5.

Acknowledgments

Domenico Masuero is acknowledged for excellent technical

support in the spectrophotometric and LCMS analysis. This work was supported by PRIN 2009.

References

[1] R. Velasco, A. Zharkikh, J. Affourtit et al., "The genome of the domesticated apple (Malus × domestica Borkh.)," *Nature Genetics*, vol. 42, no. 10, pp. 833–839, 2010.

[2] S. Reagan-Shaw, D. Eggert, H. Mukhtar, and N. Ahmad, "Antiproliferative effects of apple peel extract against cancer cells," *Nutrition and Cancer*, vol. 62, no. 4, pp. 517–524, 2010.

[3] C. Gerhauser, "Cancer chemopreventive potential of apples, apple juice, and apple components," *Planta Medica*, vol. 74, no. 13, pp. 1608–1624, 2008.

[4] J. Boyer and R. H. Liu, "Apple phytochemicals and their health benefits," *Nutrition Journal*, vol. 3, article no. 1, pp. 1–45, 2004.

[5] T. K. McGhie, S. Hudault, R. C. M. Lunken, and J. T. Christeller, "Apple peels, from seven cultivars, have lipase-inhibitory activity and contain numerous ursenoic acids as identified by LC-ESI-QTOF-HRMS," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 1, pp. 482–491, 2012.

[6] U. Vrhovsek, A. Rigo, D. Tonon, and F. Mattivi, "Quantitation of polyphenols in different apple varieties," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 21, pp. 6532–6538, 2004.

[7] P. Vanzani, M. Rossetto, A. Rigo et al., "Major phytochemicals in apple cultivars: contribution to peroxyl radical trapping efficiency," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 9, pp. 3377–3382, 2005.

[8] R. H. Liu and J. Sun, "Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 6, pp. 1718–1723, 2003.

[9] M. Waldecker, T. Kautenburger, H. Daumann et al., "Histone-deacetylase inhibition and butyrate formation: fecal slurry incubations with apple pectin and apple juice extracts," *Nutrition*, vol. 24, no. 4, pp. 366–374, 2008.

[10] M. G. Mirisola and V. D. Longo, "Conserved role of Ras-GEFs in promoting aging: from yeast to mice," *Aging*, vol. 3, pp. 340–343, 2011.

[11] D. Carmona-Gutierrez, T. Eisenberg, S. Büttner, C. Meisinger, G. Kroemer, and F. Madeo, "Apoptosis in yeast: triggers, pathways, subroutines," *Cell Death and Differentiation*, vol. 17, no. 5, pp. 763–773, 2010.

[12] A. Cassidy-Stone, J. E. Chipuk, E. Ingerman et al., "Chemical inhibition of the mitochondrial division dynamin reveals its role in bax/bak-dependent mitochondrial outer membrane permeabilization," *Developmental Cell*, vol. 14, no. 2, pp. 193–204, 2008.

[13] V. Palermo, C. Falcone, M. Calvani, and C. Mazzoni, "Acetyl-L-carnitine protects yeast cells from apoptosis and aging and inhibits mitochondrial fission.," *Aging cell*, vol. 9, no. 4, pp. 570–579, 2010.

[14] C. Mazzoni and C. Falcone, "Isolation and study of KILSM4, a Kluyveromyces lactis gene homologous to the essential gene LSM4 of Saccharomyces cerevisiae," *Yeast*, vol. 18, no. 13, pp. 1249–1256, 2001.

[15] C. Mazzoni, P. Mancini, F. Madeo, V. Palermo, and C. Falcone, "A Kluyveromyces lactis mutant in the essential gene KlLSM4 shows phenotypic markers of apoptosis," *FEMS Yeast Research*, vol. 4, no. 1, pp. 29–35, 2003.

[16] C. Mazzoni, E. Herker, V. Palermo et al., "Yeast caspase 1 links messenger RNA stability to apoptosis in yeast," *EMBO Reports*, vol. 6, no. 11, pp. 1076–1081, 2005.

[17] M. Cooper, L. H. Johnston, and J. D. Beggs, "Identification and characterization of Uss1p (Sdb23p): a novel U6 snRNA-associated protein with significant similarity to core proteins of small nuclear ribonucleoproteins," *EMBO Journal*, vol. 14, no. 9, pp. 2066–2075, 1995.

[18] A. E. Mayes, L. Verdone, P. Legrain, and J. D. Beggs, "Characterization of Sm-like proteins in yeast and their association with U6 snRNA," *EMBO Journal*, vol. 18, no. 15, pp. 4321–4331, 1999.

[19] C. Mazzoni, P. Mancini, L. Verdone et al., "A truncated form of K1Lsm4p and the absence of factors involved in mRNA decapping trigger apoptosis in yeast," *Molecular Biology of the Cell*, vol. 14, no. 2, pp. 721–729, 2003.

[20] F. Chinnici, A. Bendini, A. Gaiani, and C. Riponi, "Radical scavenging activities of peels and pulps from cv. golden delicious apples as related to their phenolic composition," *Journal of Agricultural and Food Chemistry*, vol. 52, no. 15, pp. 4684–4689, 2004.

[21] P. Franceschi, Y. Dong, K. Strupat, U. Vrhovsek, and F. Mattivi, "Combining intensity correlation analysis and MALDI imaging to study the distribution of flavonols and dihydrochalcones in Golden Delicious apples," *Journal of Experimental Botany*, vol. 63, no. 3, pp. 1123–1133, 2012.

[22] G. La Regina, T. Sarkar, R. Bai et al., "New arylthioindoles and related bioisosteres at the sulfur bridging group. 4. Synthesis, tubulin polymerization, cell growth inhibition, and molecular modeling studies," *Journal of Medicinal Chemistry*, vol. 52, no. 23, pp. 7512–7527, 2009.

[23] V. Palermo, L. Pieri, R. Silvestri, G. La Regina, C. Falcone, and C. Mazzoni, "Drug-induced inhibition of tubulin polymerization induces mitochondrion-mediated apoptosis in yeast," *Cell Cycle*, vol. 10, no. 18, pp. 3208–3209, 2011.

[24] C. Mazzoni and C. Falcone, "mRNA stability and control of cell proliferation," *Biochemical Society Transactions*, vol. 39, no. 5, pp. 1461–1465, 2011.

[25] C. Mazzoni and C. Falcone, "Caspase-dependent apoptosis in yeast," *Biochimica et Biophysica Acta*, vol. 1783, no. 7, pp. 1320–1327, 2008.

[26] V. Palermo, C. Falcone, and C. Mazzoni, "Apoptosis and aging in mitochondrial morphology mutants of S. cerevisiae," *Folia Microbiologica*, vol. 52, no. 5, pp. 479–483, 2007.

[27] F. Madeo, E. Fröhlich, M. Ligr et al., "Oxygen stress: a regulator of apoptosis in yeast," *Journal of Cell Biology*, vol. 145, no. 4, pp. 757–767, 1999.

[28] A. Rigo, F. Vianello, G. Clementi et al., "Contribution of proanthocyanidins to the peroxy radical scavenging capacity of some Italian red wines," *Journal of Agricultural and Food Chemistry*, vol. 48, no. 6, pp. 1996–2002, 2000.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 608478, 14 pages
doi:10.1155/2012/608478

Review Article

ROS in Aging *Caenorhabditis elegans*: Damage or Signaling?

Patricia Back, Bart P. Braeckman, and Filip Matthijssens

Laboratory for Aging Physiology and Molecular Evolution, Department of Biology, Ghent University, K. L. Ledeganckstraat 35, 9000 Ghent, Belgium

Correspondence should be addressed to Bart P. Braeckman, bart.braeckman@ugent.be

Received 16 May 2012; Accepted 3 July 2012

Academic Editor: Vitor Costa

Many insights into the mechanisms and signaling pathways underlying aging have resulted from research on the nematode *Caenorhabditis elegans*. In this paper, we discuss the recent findings that emerged using this model organism concerning the role of reactive oxygen species (ROS) in the aging process. The accrual of oxidative stress and damage has been the predominant mechanistic explanation for the process of aging for many years, but reviewing the recent studies in *C. elegans* calls this theory into question. Thus, it becomes more and more evident that ROS are not merely toxic byproducts of the oxidative metabolism. Rather it seems more likely that tightly controlled concentrations of ROS and fluctuations in redox potential are important mediators of signaling processes. We therefore discuss some theories that explain how redox signaling may be involved in aging and provide some examples of ROS functions and signaling in *C. elegans* metabolism. To understand the role of ROS and the redox status in physiology, stress response, development, and aging, there is a rising need for accurate and reversible *in vivo* detection. Therefore, we comment on some methods of ROS and redox detection with emphasis on the implementation of genetically encoded biosensors in *C. elegans*.

1. Oxidative Stress Theory of Aging and Correlation of Oxidative Stress with Age

More than fifty years ago, Harman [1] postulated in his free-radical theory of aging that aging results from the accumulation of molecular damage caused by byproducts of the normal oxidative metabolism, called reactive oxygen species (ROS). The discovery of the superoxide dismutase enzyme, which detoxifies the superoxide anion [2], and the detection of the ROS hydrogen peroxide (H_2O_2) *in vivo* [3] further gave credibility to the free-radical theory of aging. Harman refined his theory to highlight the role of mitochondria in aging since mitochondria are considered as the main source of ROS [4]. Because there are many ROS that are not free-radicals and that can also cause oxidative damage, the free-radical theory of aging is now referred to as the oxidative stress theory of aging [5]. This new name for the theory also implies that oxidative stress can occur due to an imbalance between ROS production and removal. This imbalance leads to a progressive accumulation of oxidative damage with age, resulting in a decline of the functional efficiency of various cellular processes. Since its formulation, the oxidative stress theory of aging has been the most broadly tested theory of aging. Despite the tremendous effort to verify this theory, experimental studies do not support or remain inconclusive on whether oxidative damage is responsible for aging [6–10]. During the last years, many studies have yielded new insights on the role of ROS in aging. Many of these studies have been performed using the model organism *Caenorhabditis elegans* and will be discussed in this paper.

As organisms age, a number of behavioral, reproductive, morphological, and biochemical changes occur [11]. Previous studies have shown an age-related increase of oxidative damage in a variety of molecules (DNA, proteins, and lipids) in organisms ranging from invertebrates to humans [5, 12–14]. This finding is in accordance with the oxidative stress hypothesis of aging. In *C. elegans*, there is an increase in single-strand DNA breaks, 5-methylcytosine [15], and protein carbonyls accumulate with age [16, 17]. However, as in many other invertebrates [18, 19], in subsequent tests on extensively purified DNA of *C. elegans*, no cytosine methylation was detected during development and senescence [20].

Melov et al. [21] reported an increase in mitochondrial DNA deletions with age. However, these results could not be confirmed in our research group [22].

One way to investigate the role of ROS in aging is to examine oxidative stress resistance in long-lived strains. Two major interventions that greatly influence lifespan are the regulation of insulin/IGF-1 signaling (IIS) and dietary restriction. The IIS pathway is an evolutionary conserved pathway that regulates aging in organisms ranging from nematodes to mammals. In *C. elegans*, it comprises an insulin-like receptor *(daf-2)*, which negatively regulates a downstream FOXO transcription factor, DAF-16. A reduction of function of *daf-2* doubles the lifespan of *C. elegans*, and this lifespan extension is fully dependent on *daf-16*. Many of the transcriptional targets of DAF-16 are also activated in the dauer larvae, a special developmental stage of *C. elegans* that can survive harsh environmental conditions for several months, while the average lifespan of an adult *C. elegans* is about 2-3 weeks. The IIS pathway and pathways related to IIS and/or DAF-16 are induced by various environmental cues (nutrients, heat, UV, heavy metal, and oxidative stress) [23–26]. These pathways promote oxidative stress resistance and longevity and regulate the expression of ROS detoxification genes. Although oxidative stress resistance is increased in these long-lived strains, this does not mean that increased stress resistance is an essential prerequisite for longevity. To test this, the expression of *sod* genes was reduced in long-lived *daf-2* mutants. Although isolated mitochondria of *daf-2* mutants produce more superoxide compared to wild type [27], the loss of *sod* genes has little or no effect on *daf-2* longevity and in some cases, it even further increases lifespan of *daf-2* [28–31]. Thus, increased antioxidant defenses in *daf-2* do not significantly contribute to the extended lifespan of *daf-2* mutants.

A reduction of food intake by 40–60% without malnutrition, called dietary restriction (DR), has remarkable benefits for health and lifespan in many different species, including *C. elegans* [32]. It was long believed that DR would reduce metabolic rate and thereby decrease ROS production and ROS-induced damage, resulting in extended lifespan [33]. Measurement of the oxygen consumption and heat production rather shows an increase in metabolism in food-restricted worms, however [34, 35]. ROS production in worms imposed to at least one type of DR is not different from wild type [27]. Dietary restriction enhances SOD and catalase activity [36] and increases *sod* expression (*sod-1*, *sod-2*, *sod-4*, and *sod-5*), regulated by the FOXA transcription factor PHA-4 [37]. Although diet-restricted worms show increased antioxidant defense and oxidative stress resistance [36, 38], *sod* deletion does not reduce lifespan in two different types of DR [39, 40]. This suggests that enhanced antioxidant defense may not be essential for DR-induced longevity.

2. Evaluation of the Oxidative Stress Theory in *C. elegans*

Conform to the oxidative stress theory of aging, oxidatively damaged biomolecules accumulate with age in *C. elegans*. Moreover, it is often found that manipulations that slow down aging, such as dietary restriction, hormesis (discussed below), or reduced IIS, also increase oxidative stress resistance. Although such observations provide correlative evidence for the oxidative stress theory of aging, they do not allow concluding that this theory is correct. Long-lived strains are resistant to other types of stresses than oxidative stress, such as heat, UV, and pathogenic bacteria. Various studies show that enhanced oxidative stress resistance is not essential for the extended lifespan in long-lived strains [38]. The most conclusive approach to test the causal relation between ROS and aging is to change the antioxidant defense system and to examine the effect on aging. Antioxidant defenses can be altered by genetic (mutation or overexpression of antioxidant enzymes) or pharmacological interventions (SOD/catalase mimetics, vitamins).

2.1. Pharmacological Interventions. Numerous studies have examined the effect of exogenous antioxidant compounds on lifespan in *C. elegans* [41]. Various noncatalytic antioxidants, such as vitamin E and C, trolox, α-tocopherol, and N-acetylcysteine, affected lifespan differently in distinct studies [42–47]. In some cases, these compounds increased oxidative stress resistance without changing the lifespan [48]. The variability in the outcome of these reports may be explained by differences in dose, experimental conditions, and method of delivery. In *C. elegans*, the uptake of many drugs is rather poor as they are excluded by the thick cuticle [49]. The use of liposomes ameliorates the uptake of hydrophilic antioxidants, such as ascorbic acid, N-acetylcysteine, and reduced glutathione, and prolongs lifespan, while conventional delivery methods do not [50]. It should be noted that this lifespan extension was dose-independent and that liposomes can affect the fat metabolism of the worms, however.

The effect of the SOD/catalase mimetics EUK-8 and EUK-134 on lifespan in *C. elegans* has been tested by various research groups. Administration of these compounds increases SOD activity *in vivo*, primarily in the mitochondria, and it also enhances paraquat resistance [51–53]. EUK-8 and EUK-134 treatment extended lifespan in one study [54]. However, other groups detected no increase in lifespan for doses that protect the worm against paraquat [51, 53, 55]. In fact, they even found a dose-dependent decrease in lifespan at higher doses of EUK-8 and EUK-134. This toxic effect might be due to enhanced ROS production when EUK-8 is administered at high doses [56]. Thus, EUK-8 and EUK-134 clearly exhibit antioxidant activity *in vivo* and can enhance oxidative stress resistance, but without causing lifespan extension.

2.2. Genetic Interventions. *C. elegans* contains numerous antioxidant genes, and the effect on aging by deleting or overexpressing many of these genes has been examined recently. While most eukaryotes contain 2 or 3 different superoxide dismutase (*sod*) genes, *C. elegans* possesses at least six isoforms [64]. *sod-1* and *sod-5* encode for the cytosolic CuZnSODs [65, 66], while the *sod-4* gene expresses

Table 1: Overview of the effect *sod* knockdown/out or overexpression of *C. elegans* superoxide dismutases on ROS levels, oxidative damage, and lifespan under stressed and unstressed condition (paraquat or hyperoxia). OE: overexpression; ND: not determined; PQ: paraquat; J: juglone; DR: dietary restriction.

	sod gene	ROS levels	Oxidative damage	Effect on lifespan	Effect on stress survival	Effect on lifespan long-lived worms
			Knockdown/out			
MnSOD	*sod-2*	Increased [57] or no effect [27]	Increased [30, 40]	No effect [29–31, 39] or increased [27, 40, 57]	Decreased (PQ) [29–31, 40] (J) [40] or no effect (hyperoxia) [29]	No effect (*daf-2*) [29, 30, 40] (DR) [39] or decreased (*daf-2*) [31] or increased (*clk-1*) [30, 40]
	sod-3	Increased [57]	ND	No effect [29, 31, 39, 40] or increased [57]	Decreased (pathogenic bacteria) [58] (PQ-J) [40] or no effect (PQ-hyperoxia) [29, 31] or increased [39]	No effect (*daf-2*) [29] (DR) [39, 40] or increased (*daf-2*) [31] or decreased (*isp-1*) [40]
	sod-2; sod-3	Increased [59] or decreased [60]	No effect [29, 60]	No effect [29, 31] or increased [40]	Decreased (PQ-hyperoxia) [29, 31, 40] (J) [40]	Increased (*daf-2*) [31]
CuZnSOD	*sod-1*	Increased [61]	Increased [30] or no effect [29]	Decreased [29, 39, 61] or no effect [30, 39, 40]	Decreased (PQ) [29, 30, 39, 40, 61] (J) [40] (hyperoxia) [29]	Decreased (*daf-2*) [29] or no effect (DR) [39] or increased (*daf-2*) [30]
	sod-4	ND	ND	No effect [29, 31, 39, 40]	No effect [29] or increased [39]	Increased (*daf-2*) [29] or no effect (DR) [39]
	sod-5	ND	ND	No effect [29, 39, 40]	No effect [29, 39]	No effect (*daf-2*) [29] (DR) [39]
	sod-1; sod-4; sod-5	ND	ND	Decreased [29]	ND	ND
SOD	*sod-12345*	ND	No effect [62]	No effect [62]	Decreased [62]	ND
			OE			
MnSOD	*sod-2*	ND	No effect [63]	Increased [63]	ND	ND
CuZnSOD	*sod-1*	Increased [63]	Increased [63]	Increased [29, 63]	Decreased (PQ) [29]	ND

two extracellular CuZnSODs that are products of alternative splicing [67] and are either membrane bound or extracellular. *sod-2* and *sod-3* encode MnSOD enzymes that are localized in the mitochondrial matrix [68, 69]. SOD-1 and SOD-2 contribute to most of the SOD activity during normal development [29, 65], whereas the secondary SODs *sod-3* and *sod-5* are clearly upregulated in dauers [29, 70], in *daf-2* mutants [71] and under oxidative stress [72]. Very recently, a number of groups has examined the effect of *sod* deletion and overexpression on lifespan and oxidative stress resistance in *C. elegans* [27, 29–31, 39, 40, 57, 60, 61, 63]. An overview of these studies is represented in Table 1.

Loss of *sod-1* lowers the resistance to oxidative stress [29, 30, 39, 40, 61]. Although SOD-1 contributes to 80% of the total SOD activity in *C. elegans* [29], its deletion only slightly reduces lifespan [29, 39, 61] or even not at all in other studies [30, 39, 40]. Reduced *sod-1* expression increases ROS levels and oxidative damage in some studies [30, 61] but it did not in another one [29]. These contradicting results may reflect that the impact of *sod-1* reduction on oxidative stress is relatively subtle and difficult to detect. In contrast to *sod-1*, loss of *sod-4* or *sod-5* does not alter the lifespan under stressed or unstressed conditions [29, 31, 39, 40]. Unexpectedly, *sod-4* deletion prolongs lifespan in *daf-2* mutants [29]. Conform to the findings in mammalian studies [73],

it has been proposed that *sod-4* may stimulate IIS through the H_2O_2-dependent inactivation of the phosphatases [6]. This suggests that CuZnSOD is involved in the redox regulation of IIS. Consistent with this statement, mutation of *sod-4* or *sod-5* enhances dauer larva formation [29]. To resume, loss of CuZnSOD has little negative effect on oxidative stress and lifespan and it may regulate IIS via redox signaling.

While the loss of *sod-2*, *sod-3* or both, reduces oxidative stress resistance in most cases [29–31, 39, 40], it does not shorten lifespan under unstressed conditions [29–31, 39, 40], and in some studies it even increases lifespan [27, 40, 57]. Diminished MnSOD enhances ROS levels and oxidative damage to a minor extent in some reports [30, 40, 57, 59] but not in others [27, 29, 60]. MnSOD knockout/down experiments demonstrated that MnSOD is not required for lifespan extension in long-lived *daf-2* mutants and diet-restricted worms [29–31, 39, 40]. In contrast, abolished *sod-2* expression largely extends the lifespan of long-lived *clk-1* mutants [30, 40]. Consistent with this, *sod-2* mutation results in a Clk phenotype, that is, slow growth and defecation rate, and reduced and delayed fecundity [29, 40]. Thus, MnSOD is required for oxidative stress resistance, but it does not limit the lifespan of wild type or long-lived strains. The fact that loss of MnSOD enhances oxidative stress and concurrently prolongs lifespan in certain studies, contradicts a causal role for mitochondrial superoxide in the aging process.

Worms completely devoid of both Mn- and CuZnSOD have been recently obtained and are highly sensitive to multiple stresses (oxidative stress, heat, and osmotic stress), they develop slower and have a reduced brood size [62]. Remarkably, this total loss of SOD activity has no effect on lifespan at all. This lack of effect on lifespan can be explained by a counterbalance between superoxide toxicity on the one hand and an adaptive response to reduced superoxide detoxification on the other hand [62]. Most importantly, it shows that oxidative stress *per se* has no profound effect on aging in *C. elegans*.

If the oxidative stress hypothesis of aging is correct, an increase in SOD-1 should result in a decrease in oxidative damage and an extended lifespan. Overexpression of *sod-1* indeed prolongs lifespan [29, 63]. But instead of decreasing oxidative stress as expected by the oxidative stress hypothesis, *sod-1* overexpression does not reduce lipid oxidation and glycation and actually increases ROS levels and protein oxidation levels [63], and it increases paraquat sensitivity [29]. On the other hand, the lifespan extension of *sod-1* overexpressors is DAF-16- and partially HSF-1-dependent and *sod-1* overexpression enhances *hsp-4* and *hsp-6* expression, suggesting an unfolded protein response (UPR) [63]. This idea is further confirmed by the observation that knocking down genes involved in UPR, partly suppresses longevity of *sod-1* overexpressors. The exact mechanism by which *sod-1* overexpression increases lifespan through DAF-16 remains unclear, however. Like *sod-1*, *sod-2* overexpression also prolongs lifespan in a DAF-16-dependent manner, but it does not change the protein carbonylation levels [63]. These results imply that overexpression of *sod-1* or *sod-2* extends lifespan, not by reducing oxidative stress, but instead by activating longevity-related transcription factors, such as DAF-16. Therefore, lifespan extension by SOD overexpression does not support the oxidative stress hypothesis. The fact that *sod-1* overexpression increases oxidative damage and prolongs lifespan at the same time, even contradicts this hypothesis.

C. elegans contains three catalase enzymes with highly similar sequences in a tandem array: *ctl-3*, *ctl-1*, and *ctl-2* [74]. The peroxisomal catalase, CTL-2 [75], contributes to ~80% of the total catalase activity and a knockout of *ctl-2* can reduce mean lifespan by 16% while decreasing egg-laying capacity [74]. Remarkably, protein carbonyl levels increase more slowly with age in *ctl-2* mutants compared to wild type [74]. Moreover, overexpression of all three catalase genes reduces lifespan [29].

C. elegans contains three peroxiredoxins, two 2-Cys peroxiredoxins *prdx-2* (*CePrx2*), and *prdx-3* (*CePrx1*), and one 1-Cys peroxiredoxin *prdx-6* (*CePrx3*). *Prdx-3* is probably mitochondrial, and only deletion of *prdx-2* results in an altered phenotype, displaying a reduced size, fertility, and oxidative stress resistance [76]. At 25°C, the lifespan of *prdx-2* mutants is not reduced, but at 15°C and 20°C *prdx-2* knockout worms live shorter than wild-type worms [76–78]. *prdx-2* is expressed in pharyngeal neurons and in the reproductive system [76, 78]. Interestingly, overexpression of *prdx-2* in the intestine increases oxidative resistance but it does not prolong lifespan [78], indicating that the oxidative stress resistance obtained by tissue-specific *prdx-2* expression does not determine lifespan.

Analysis of the *C. elegans* genome reveals four glutaredoxins (GLRXs), at least eight thioredoxins (TRXs), and two thioredoxin reductases (TRXRs). Deletion of the cytosolic *trx-1*, expressed in specific pharyngeal neurons and the intestine, decreases lifespan with 19% and increases paraquat sensitivity [79, 80]. Furthermore, *trx-1* deletion partially suppresses lifespan extension in *daf-2* mutants and completely suppresses longevity induced by two forms of dietary restriction [81]. Overexpression of *trx-1* results in a moderate lifespan increase [80]. Deletion of mitochondrial *trx-2* and/or *trxr-2*, expressed in muscles, intestine, and neurons, does not affect lifespan in wild type and *daf-2* mutants and has no effect on heat shock and oxidative stress resistance [82]. Deletion of the methionine sulfoxide reductase, encoded by *msra-1*, reduces paraquat resistance and shortens lifespan of wild type and *daf-2* mutant worms [83].

C. elegans contains 57 genes that encode for glutathione-S-transferase (GST). *gst-4* is upregulated by oxidative stress, and although its overexpression enhances oxidative stress resistance, it does not extend lifespan [84]. GST-10 catalyzes the detoxification of the lipid peroxidation end-product HNE. *gst-10* overexpressors are more resistant to various forms of stress (heat, UV, and oxidative stress) and have an increased lifespan [85]. RNAi of 5 of the 26 tested *gst* genes causes an increase in HNE-mediated damage, while RNAi of only 2 of these *gst* genes (*gst-5* and *gst-10*) decreases lifespan [86]. This suggests that an increase in HNE-induced damage is not sufficient to reduce lifespan.

Iron-catalyzed ROS generation can increase protein damage and reduce oxidative stress resistance without affecting

lifespan [87]. Similarly, overexpression of the iron storage protein ferritin, *ftn-1*, reduces protein carbonylation and enhances oxidative stress resistance, but does not increase lifespan. These results suggest that high iron levels can increase oxidative stress, but iron levels under standard culture conditions do not limit lifespan. Moreover, deletion of *ftn-1* even increases lifespan and dauer formation of *daf-2* mutants, indicating a role for ferritin in IIS.

In general, deletion of some but not all antioxidant genes shortens lifespan to a minor extent. In some cases, loss of antioxidant enzymes can increase oxidative stress sensitivity without affecting lifespan and in some reports, it can even enhance longevity. Overexpression of a few antioxidant genes can moderately increase lifespan, but not necessarily increases oxidative stress resistance. In other instances, overexpression of antioxidant enzymes reduces lifespan. Therefore, genetic interventions in antioxidant defenses do not generally support the oxidative stress theory of aging in *C. elegans*.

Does this limited effect of antioxidants on aging in *C. elegans* support studies in higher model organisms, such as *Drosophila melanogaster* and mice (for review, see [7, 88])? Similar to worms, mice display a slightly reduced lifespan upon loss of CuZnSOD [89], while in the fruit fly, loss of CuZnSOD dramatically reduces lifespan [90, 91]. Heterozygous CuZnSOD$^{+/-}$ *Drosophila* has an unaltered lifespan, however [90]. Unlike *C. elegans*, loss of MnSOD is lethal to mice and flies [92–94], but heterozygotic MnSOD mutation has no effect on aging in mice. Interestingly, these heterozygous mice show a 100% increase in tumor incidence [95]. Thus, antioxidant genetic alteration can induce oxidative stress and pathology without affecting lifespan. Overexpression of CuZnSOD, MnSOD, catalase, and glutathione peroxidase does not prolong lifespan in mice, although oxidative stress resistance is increased [88, 96]. It was verified with various parameters, such as mean and maximum lifespan, body weight, and fecundity, that the mice were maintained under optimal health conditions, to avoid stress conditions which may affect lifespan of mice with altered antioxidant defense. In flies, lifespan is not changed or increased in SOD overexpressors, depending on the study [97–102]. This discrepancy between studies can be a result of artifacts, such as transformation method or genetic background [103, 104]. To conclude, like in *C. elegans*, antioxidant studies in other model organisms do not generally support the oxidative stress theory of aging.

2.3. Redox Signaling Theories on Aging in C. elegans. Since evidence against the oxidative stress theory of aging is accumulating, a few theories have been recently proposed to explain the correlation between ROS and aging. One alternative for the oxidative stress theory of aging is the redox stress hypothesis, which proposes to include a signaling role for ROS in the aging process. It states that functional loss during aging is caused by a progressive pro-oxidizing shift in the redox state of the cell. This leads to overoxidation of redox-sensitive proteins and consequently the disruption of redox-regulated signaling mechanisms [10]. Figure 1 represents an overview of signaling pathways regulated by ROS that we describe below in more detail.

While high concentrations of the $O_2^{\bullet -}$ generators juglone and paraquat are lethal, a life-long exposure to low concentrations of these compounds can extend lifespan [27, 105, 106]. Exposure to low doses of juglone enhances an antioxidant defense (increased GSH levels, SOD, catalase activity, and *sod-3* and *hsp-16.2* expression). In accordance with these findings, DAF-16, SIR-2.1, and 14-3-3, proteins involved in stress response, are required for the lifespan extension in worms exposed to low concentration of juglone [106]. In short, these results indicate that increased ROS production promotes a stress response, mediated by DAF-16, SIR-2.1 and the redox-dependent transcription factor SKN-1, and thereby extends lifespan. This phenomenon is called hormesis [107] and has also been observed in worms with decreased glucose metabolism, where it is described as mitohormesis more specifically [46]. Reduced glycolysis increases mitochondrial ROS production, which in turn increases oxidative stress resistance and catalase activity, prolonging lifespan [46]. However, pretreatment with antioxidants and vitamins prevents this lifespan extension, demonstrating that the increased ROS formation is an essential signal to activate lifespan extension. Consistent with these findings and the redox stress hypothesis, inhibition of glycolysis enhances stress resistance in *C. elegans* by activating the pentose phosphate pathway, which is crucial for maintaining the reducing cytosolic NADPH concentration [108]. More recently, it was found that impairment of *daf-2* reduces glucose uptake and induces a transient drop in ATP levels, thereby activating the energy sensor AAK-2. In its turn, AAK-2 mediates the generation of a transient ROS signal that ultimately promotes L-proline catabolism and partially extends lifespan. As a result, oxidative phosphorylation is enhanced and ROS levels are increased. This increase in ROS levels induces adaptive response that is partially mediated by the Nrf/SKN-1 transcription factor, resulting in enhanced stress resistance and extended lifespan [109].

Mitohormesis also plays a role in longevity of mutants with an impaired mitochondrial metabolism, or *mit* mutants. Some *mit* mutants (*clk-1*, *isp-1*, and *nuo-6*) show increased ROS production [27, 105, 110], an enhanced *sod* expression [30, 111, 112], and catalase activity [113]. While the increase in antioxidant defenses is dispensable for their long lifespan [30, 74], the increase in ROS levels is a prerequisite for *isp-1* and *nuo-6* [27] longevity, perhaps by provoking an adaptive response. The hypoxia-inducible transcription factor HIF-1 is activated by mild increases in ROS and HIF-1 is required for the extended lifespans of *clk-1* and *isp-1* mutants [105]. Thus, HIF-1 appears to link respiratory stress in the mitochondria to a nuclear transcriptional response that promotes longevity. Recently, it was shown that inhibition of mitochondrial respiration triggers the mitochondrial unfolded protein response (UPR^{mito}), increasing *hsp-6* expression [112]. This response is needed for the longevity in *mit* mutants and acts through unidentified signaling molecules, maybe ROS, between different tissues [114]. Like in *mit* mutants, UPR is essential for the longevity in *sod-1* overexpressors [63].

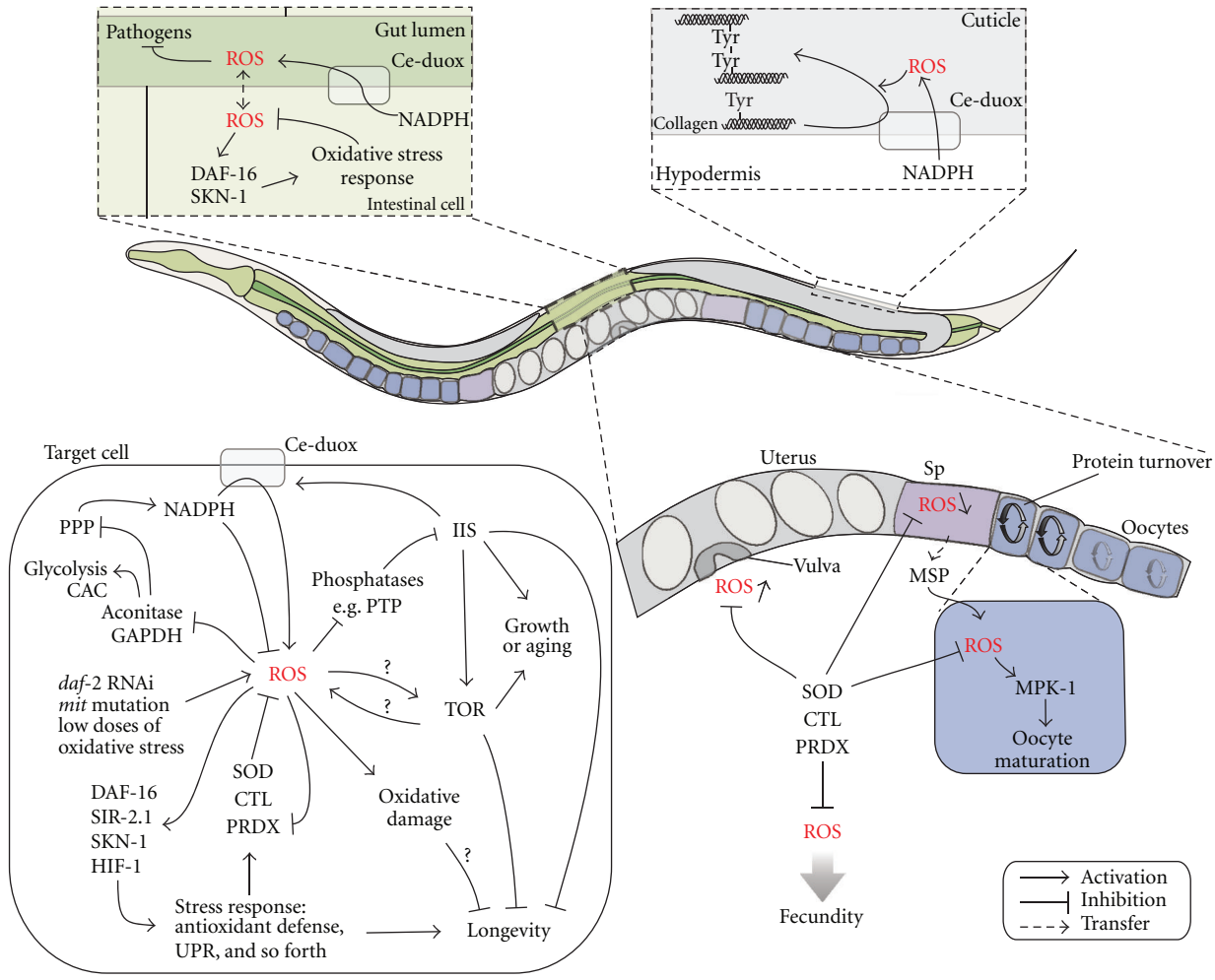


Figure 1: Schematic representation of signaling pathways regulated by ROS in *C. elegans*. PPP: pentose phosphate pathway; CAC: citric acid cycle; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; Sp: spermatheca.

To conclude, according to the (mito)hormesis theory, ROS can be regarded as a molecular signal that triggers a hormetic effect, inducing an overall increase in stress resistance and longevity. The specific antioxidant response may be important to maintain a reduced environment that remains sensitive to subsequent ROS signals.

Mammalian studies have reported that ROS (H_2O_2) activates the regulator of cell growth and proliferation, TOR, and its target S6 kinase [115, 116]. Moreover, TOR activation increases ROS production [117], whereas TOR inhibition reduces ROS production [118]. According to the TOR-centric model of aging, these multiple links between ROS and TOR indicate that ROS is a messenger molecule rather than a toxic byproduct, that accumulates life-limiting damage [119]. This TOR-centric model postulates that TOR is necessary during development, but that it is not switched off in adults. As a result, cells become hypertrophic and hyperactive. This hyperfunction causes cellular damage and age-related diseases [120]. According to this model, TOR limits lifespan by accelerating age-related diseases before oxidative damage accumulation can cause death.

2.4. Mechanisms of Redox Signaling in C. elegans. While the theories above can explain how ROS influence aging in another way than by causing oxidative damage, they do not provide details on how ROS can transfer a signal in a cellular environment. A few specific mechanisms of ROS or redox signaling has been described in *C. elegans* and are discussed below.

Protein-tyrosine phosphatases (PTP) contain a reduced cysteine residue that is a well-characterized target of ROS. PTP is oxidized by H_2O_2 to the sulfenic (–SOH) inactive form, which can be reversed by cellular thiols. The oxidized sulfenic acid rapidly reacts with amide nitrogen of its protein backbone generating inactive sulfenylamide, to prevent further irreversible oxidation to sulfonic ($–SO_2H$) and sulfinic species ($–SO_3H$). Inactivation of phosphatases can also occur by glutathionylation. Reactivation of the glutathiolated PTP may be catalyzed by glutaredoxin (GLRX) [73]. Other potential redox-sensitive phosphatases are the PTEN homolog DAF-18, the PP2A homolog PPTR-1, and the MKP-1 homolog VHP-1 [73].

In mammalian studies, insulin is found to stimulate H_2O_2 production by NADPH oxidase [121, 122]. H_2O_2 inactivates phosphatases which in turn negatively regulate insulin signaling. This leads to a positive feedback loop: ROS generated in response to insulin facilitate insulin signaling. In *C. elegans*, loss of *sod-4* enhances longevity and loss of either *sod-4* or *sod-5* enhances dauer formation in *daf-2* mutants [29], providing evidence for redox-sensitive regulation of IIS in *C. elegans*. Moreover, *sod-5* is specifically expressed in neurons involved in dauer formation in a *daf-16*-dependent manner [6, 96]. SOD-4 and SOD-5 generate extracellular and cytosolic H_2O_2, respectively, which can inactivate phosphatases, such as protein tyrosine phosphatase (PTP), thereby promoting IIS [73]. Therefore, SOD-4 and SOD-5 may facilitate a rapid exit from the dauer larvae stage to ensure further development and offspring during optimal conditions.

Another study that indicates a possible signaling function for ROS in *C. elegans*, describes redox changes in a limited set of proteins upon H_2O_2 treatment. A short-term H_2O_2 treatment causes immediate and reversible behavioral changes, such as reduced mobility, pharyngeal pumping, and reproduction, as well as decreased growth rate, and decreased ATP levels [77]. By using the redox proteomic technique OxICAT, 40 different proteins with redox-sensitive cysteines were identified which are involved in mobility and feeding (oxidative inactivation of MYO-2), protein translation (oxidative inactivation of EFT-1), protein homeostasis (oxidative activation of HSP-1), and ATP regeneration. Proteins involved in glycolysis are oxidatively inactivated, thus, redirecting glucose to the pentose phosphate pathway, which results in enhanced NADPH levels. These observations coincide with the finding that reduced glycolysis enhances oxidative stress resistance [46, 108]. Thus, oxidative stress induces oxidative modifications of specific redox-sensitive proteins to reduce energy consuming processes which are not essential for survival, such as protein synthesis and movement, thereby saving energy to restore redox homeostasis.

2.5. Functional Roles for ROS in C. elegans. Rather than being purely harmful, it has been established that ROS can also play an important role in metabolism. Here we provide a few examples of how ROS contribute to *C. elegans* metabolism, and therefore may be crucial for maintaining a normal lifespan.

Most organisms, including humans, *D. melanogaster*, and plants, produce ROS in phagocytic and nonphagocytic cells via NADPH oxidase in response to microbial infection [123–125]. *C. elegans* also produces extracellular ROS by a dual NADPH oxidase (CeDuox-1) in response to exposure to gut-infecting pathogenic bacteria *Enterococcus faecalis* or yeast [58, 126–128]. These studies suggest that *C. elegans* produces ROS in the intestine in response to pathogens while an oxidative stress response mediated by DAF-16 and the redox sensitive transcription factor SKN-1 is induced to protect neighbouring tissues. [58].

Another function for ROS in *C. elegans* metabolism, mediated by NADPH oxidase, is cuticle biogenesis. *C. elegans* encodes two dual oxidases, Ce-Duox1 (*bli-3*) and Ce-Duox2, which contain a NADPH oxidase domain and a peroxidase domain. The NADPH oxidase domain generates extracellular superoxide which dismutates spontaneously or by SOD-4 to H_2O_2. This peroxide is then used by the peroxidase domain for the cross-linking of tyrosine residues in collagen to create the cuticle of the worm [129].

The redox state of the cell has also been shown to regulate physiological processes during development, such as proliferation and differentiation [130]. Generally, a more reducing environment is associated with proliferation and an oxidizing environment initiates differentiation [131]. Consistent with mammalian studies, where GSH content is high during gametogenesis and fertilization [132], we find that in the spermatheca, where the oocytes are fertilized, GSSG/GSH levels are low [133]. Moreover, *in vivo* H_2O_2 levels are low and *sod-1* is highly expressed in the spermatheca (unpublished data). It was also reported that protein carbonylation levels are low in spermatheca as they are abruptly reduced by enhanced proteasome activity in the ovaries at the time of oocyte maturation [134]. Before oocytes can be fertilized in the spermatheca, they need to mature, a process that is characterized by their transition into the meiotic metaphase I [135]. The major sperm protein (MSP) secreted by the sperm in the spermatheca promotes ROS production in the most proximal oocytes, which augments MPK-1 activity, essential for this oocyte maturation. SOD-1 is found to inhibit MPK-1 activation [136]. Thus, ROS can act as a secondary messenger for oocyte maturation necessary for fertilization, while these oocytes are cleared from oxidative damage by enhancing proteasomal activity [134].

3. ROS and Redox Detection

A major challenge in establishing the exact function of ROS in metabolism and aging is their accurate detection. Because redox signaling acts through small, local, and transient changes, redox detection should ideally be selective, sensitive, instantaneous, reversible, compartment-specific, noninvasive, and applicable *in vivo*. Most conventional redox measurements are nonspecific, disruptive (which may create oxidation artifacts), irreversible (which precludes dynamic measurements), and some probes can generate ROS by themselves upon radiation (e.g., H_2DCF-DA) [137–139]. These exogenous chemical absorbent, fluorescent or luminescent probes need to be taken up by the biological model. This uptake is rather poor and variable in *C. elegans*, making comparison between samples complicated. In addition, the uptake of these chemical reporter molecules may not be identical for each subcellular compartment. Recently, small-molecule probes were designed to be targeted to the mitochondria to ensure the probe uptake into the mitochondria [140–142]. An extensive review on these chemical probes can be found elsewhere [137–139].

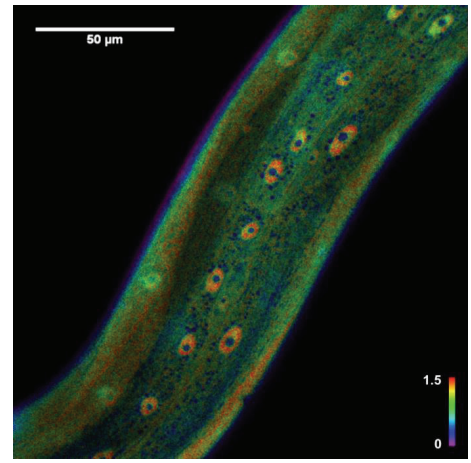
Many of the limitations of conventional redox probes were overcome by the development of genetically encoded

redox sensors based on the green fluorescent protein GFP. Generally, these chimeric proteins contain a regulatory domain that will specifically and reversibly bind the ROS or metabolite of interest, resulting in a conformational change altering the fluorescent properties of the biosensors. As a result, the fluorescent properties of the molecules are a direct measure of the levels of the ROS or metabolite *in vivo*. A major advantage of these genetically encoded sensors is that they can be targeted to specific tissues, cells or subcellular locations [143]. Hence, when choosing an accurate promoter for expression of the biosensor, real-time and *in vivo* analysis ranging from a specific single cell to a whole organism can be easily performed. Because of its genetic amenability and its transparency, *Caenorhabditis elegans* is ideally suited for such approaches, while its size allows analysis by both microplate fluorometry and confocal microscopy. A few examples of genetically encoded sensors that we successfully implemented to measure H_2O_2 levels and GSH in *C. elegans* will be further discussed.

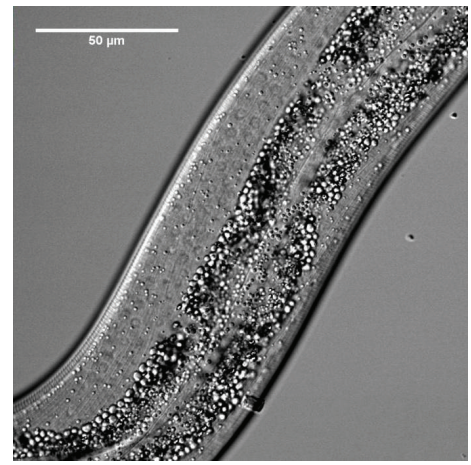
H_2O_2 has emerged as a widespread, physiologically relevant and selective signaling molecule [144–146], and it is relatively, abundant (1–100 nM) [145] and stable compared to other ROS. These features allow H_2O_2 to serve as an important second messenger. HyPer is a H_2O_2 (hydrogen peroxide)-specific intracellular biosensor. This sensor consists of a circularly permutated yellow fluorescent protein (cpYFP) inserted into the H_2O_2-sensitive regulatory domain of the *Escherichia coli* transcription factor OxyR (OxyR-RD) [147]. HyPer is a H_2O_2-sensitive, selective, and reversible biosensor, and its fluorescence ratio reflects the balance between H_2O_2-mediated oxidation and a GLRX/GSH-mediated reduction. Like most cpYFP's, HyPer fluorescence is influenced by pH (6–10): acidification decreases the 500 nm/420 nm excitation ratio [147]. HyPer has already been used in various cell culture [147–151] and zebra-fish [152] studies. Using HyPer, we identified local cells or tissues with distinct *in vivo* H_2O_2 levels in *C. elegans*. For example, we found that, consistent with the role of hypodermal cells in cuticle biogenesis, H_2O_2 levels are particularly high in these cells [133] (Figure 2).

The redox state of the cell is determined by the redox state of multiple redox pairs in the cell. Because of the high intracellular GSH concentration (1–11 mM) and the high GSH/GSSG ratio (≥100) [131], the redox state of the glutathione couple is a good proxy for the total redox state of the cell. The new generation of redox-sensitive sensors are the redox-sensitive GFP's (roGFP), such as roGFP2. These biosensors are ratiometric by excitation, thus minimizing measurement errors due to variable *in vivo* probe concentrations and photobleaching [153]. They are able to detect small changes in GSSG within a highly reduced glutathione pool (GSH/GSSG ≥ 100) [131]. The fusion of human glutaredoxin-1 (Grx1) to roGFP2 makes the redox response of the probe faster and more glutathione specific [154, 155]. Ubiquitous overexpression of this sensor in *C. elegans* shows a reduced state (low levels of GSSG/GSH) of the spermatheca (Figure 3).

Consistent with the redox hypothesis of aging, both Hyper and Grx-roGFP2 strains show an increase in H_2O_2 and GSSG/GSH with age. In dietary restricted populations, this increase is attenuated [133].



(a)

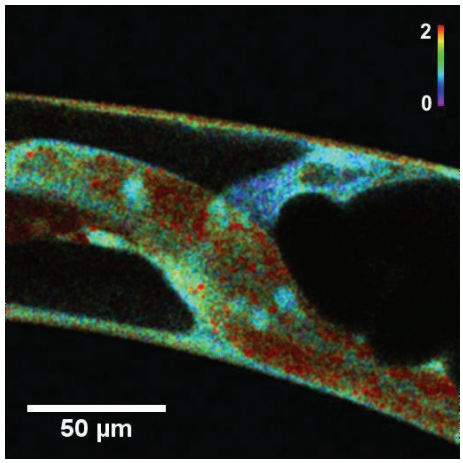


(b)

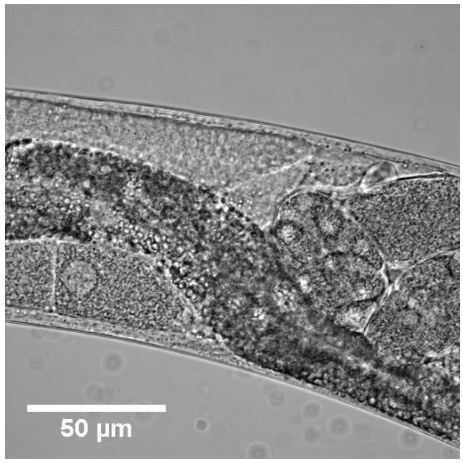
Figure 2: (a) Intensity normalized ratio image of a HyPer transgenic worm. Color represents oxidized/reduced HyPer ratio values, while color saturation represents fluorescence intensities. Hypodermal nuclei clearly show increased levels of H_2O_2. (b) Corresponding brightfield image. Construction of transgenic strains, confocal microscopy, and image analysis was performed as described in [133].

The fusion of the yeast peroxidase Orp1 (also known as Gpx3) to roGFP2 converts roGFP2 into a specific probe for H_2O_2 [156]. H_2O_2-specific oxidation of Orp1 induces the formation of a disulfide bridge in roGFP2. roGFP2-Orp1 can be selectively and reversibly oxidized by H_2O_2 [156] and reduced by thioredoxin (TRX) or glutaredoxin (GLRX) [155]. Like all other roGFP-based probes, this probe is independent of pH (5.5–8.0) [157]. roGFP2-Orp1 has been already applied in cell cultures [156] and *D. melanogaster* [158].

The last few years, many more *in vivo* ROS, redox, and metabolic sensors have been developed [147, 154, 156, 159–164], and their use and limitations have been reviewed recently [155]. It is very likely that this booming research



(a)



(b)

Figure 3: (a) Intensity normalized ratio image of a Grx1-roGFP2 transgenic worm. Color represents oxidized/reduced Grx1-roGFP2 ratio values, while color saturation represents fluorescence intensities. Spermatheca show clearly reduced GSSG/GSH ratios. (b) Corresponding brightfield image. Construction of transgenic strains, confocal microscopy, and image analysis was performed as described in [133].

field will yield more accurate, sensitive, and selective redox sensors in the near future.

4. General Conclusion

In recent years, there is mounting evidence against the oxidative stress theory of aging in *Caenorhabditis elegans*. Many intervention studies altering its antioxidant system have been performed, and most of them failed to support this theory. Gradually, it has become clear that a general increase in oxidative stress does not limit lifespan in this model organism. However, in the stressful conditions of its natural habitat oxidative stress resistance might be important to ensure a normal lifespan and reproduction. Although it cannot be ruled out that specific targets (proteins, lipids, or nucleic acids) accumulate some type of damage over age, and thereby contribute to the aging process, a global rise in oxidative damage is clearly not a major factor determining lifespan. In contrast, there is a growing body of evidence that ROS and redox signaling may be important in the aging process, and that ROS may exert essential functions in metabolism. However, to fully understand the mechanisms of ROS and redox signaling, it is crucial that the molecular details, the localization, and the regulation of the specific reactive oxygen species involved can be analyzed accurately. This will allow a critical validation of the latest aging theories that try to explain the correlation between ROS and aging. To this end, the recent development and implementation of genetically encoded biosensors is a promising tool that we believe will be highly valuable to further explore ROS and redox biology in aging *C. elegans*.

References

[1] D. Harman, "Aging: a theory based on free radical and radiation chemistry," *Journal of Gerontology*, vol. 11, no. 3, pp. 298–300, 1956.

[2] J. M. McCord and I. Fridovich, "Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein)," *Journal of Biological Chemistry*, vol. 244, no. 22, pp. 6049–6055, 1969.

[3] B. Chance, H. Sies, and A. Boveris, "Hydroperoxide metabolism in mammalian organs," *Physiological Reviews*, vol. 59, no. 3, pp. 527–605, 1979.

[4] D. Harman, "The biologic clock: the mitochondria?" *Journal of the American Geriatrics Society*, vol. 20, no. 4, pp. 145–147, 1972.

[5] R. S. Sohal and R. Weindruch, "Oxidative stress, caloric restriction, and aging," *Science*, vol. 273, no. 5271, pp. 59–63, 1996.

[6] D. Gems and R. Doonan, "Antioxidant defense and aging in *C. elegans*: is the oxidative damage theory of aging wrong?" *Cell Cycle*, vol. 8, no. 11, pp. 1681–1687, 2009.

[7] F. L. Muller, M. S. Lustgarten, Y. Jang, A. Richardson, and H. Van Remmen, "Trends in oxidative aging theories," *Free Radical Biology and Medicine*, vol. 43, no. 4, pp. 477–503, 2007.

[8] A. Bokov, A. Chaudhuri, and A. Richardson, "The role of oxidative damage and stress in aging," *Mechanisms of Ageing and Development*, vol. 125, no. 10-11, pp. 811–826, 2004.

[9] J. Lapointe and S. Hekimi, "When a theory of aging ages badly," *Cellular and Molecular Life Sciences*, vol. 67, no. 1, pp. 1–8, 2010.

[10] R. S. Sohal and W. C. Orr, "The redox stress hypothesis of aging," *Free Radical Biology and Medicine*, vol. 52, no. 3, pp. 539–555, 2012.

[11] J. J. Collins, C. Huang, S. Hughes, and K. Kornfeld, "The measurement and analysis of age-related changes in *Caenorhabditis elegans*," in *WormBook*, pp. 1–21, 2008.

[12] V. A. Bohr and R. M. Anson, "DNA damage, mutation and fine structure DNA repair in aging," *Mutation Research*, vol. 338, no. 1–6, pp. 25–34, 1995.

[13] H. R. Warner, "Superoxide dismutase, aging, and degenerative disease," *Free Radical Biology and Medicine*, vol. 17, no. 3, pp. 249–258, 1994.

[14] R. L. Levine and E. R. Stadtman, "Oxidative modification of proteins during aging," *Experimental Gerontology*, vol. 36, no. 9, pp. 1495–1502, 2001.

[15] M. Klass, P. N. Nguyen, and A. Dechavigny, "Age-correlated changes in the DNA template in the nematode *Caenorhabditis elegans*," *Mechanisms of Ageing and Development*, vol. 22, no. 3-4, pp. 253–263, 1983.

[16] H. Adachi, Y. Fujiwara, and N. Ishii, "Effects of oxygen on protein carbonyl and aging in *Caenorhabditis elegans* mutants with long (*age-1*) and short (*mev-1*) life spans," *Journals of Gerontology A*, vol. 53, no. 4, pp. B240–B244, 1998.

[17] K. Yasuda, H. Adachi, Y. Fujiwara, and N. Ishii, "Protein carbonyl accumulation in aging dauer formation-defective (*daf*) mutants of *Caenorhabditis elegans*," *Journals of Gerontology A*, vol. 54, no. 2, pp. B47–B51, 1999.

[18] J. H. Proffitt, J. R. Davie, D. Swinton, and S. Hattman, "5-methylcytosine is not detectable in *Saccharomyces cerevisiae* DNA," *Molecular and Cellular Biology*, vol. 4, no. 5, pp. 985–988, 1984.

[19] S. Urieli-Shoval, Y. Gruenbaum, J. Sedat, and A. Razin, "The absence of detectable methylated bases in *Drosophila melanogaster* DNA," *FEBS Letters*, vol. 146, no. 1, pp. 148–152, 1982.

[20] V. J. Simpson, T. E. Johnson, and R. F. Hammen, "*Caenorhabditis elegans* DNA does not contain 5-methylcytosine at any time during development or aging," *Nucleic Acids Research*, vol. 14, no. 16, pp. 6711–6719, 1986.

[21] S. Melov, G. J. Lithgow, D. R. Fischer, P. M. Tedesco, and T. E. Johnson, "Increased frequency of deletions in the mitochondrial genome with age of *Caenorhabditis elegans*," *Nucleic Acids Research*, vol. 23, no. 8, pp. 1419–1425, 1995.

[22] K. Brys, N. Castelein, F. Matthijssens, J. R. Vanfleteren, and B. P. Braeckman, "Disruption of insulin signalling preserves bioenergetic competence of mitochondria in ageing *Caenorhabditis elegans*," *BMC Biology*, vol. 8, article 91, 2010.

[23] S. T. Henderson and T. E. Johnson, "*daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*," *Current Biology*, vol. 11, no. 24, pp. 1975–1980, 2001.

[24] H. Inoue, N. Hisamoto, J. H. An et al., "The *C. elegans* p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response," *Genes & Development*, vol. 19, no. 19, pp. 2278–2283, 2005.

[25] S. W. Oh, A. Mukhopadhyay, N. Svrzikapa, F. Jiang, R. J. Davis, and H. A. Tissenbaum, "JNK regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 12, pp. 4494–4499, 2005.

[26] M. K. Lehtinen, Z. Yuan, P. R. Boag et al., "A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span," *Cell*, vol. 125, no. 5, pp. 987–1001, 2006.

[27] W. Yang and S. Hekimi, "A mitochondrial superoxide signal triggers increased longevity in *Caenorhabditis elegans*," *PLoS Biology*, vol. 8, no. 12, Article ID e1000556, 2010.

[28] C. T. Murphy, S. A. McCarroll, C. I. Bargmann et al., "Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*," *Nature*, vol. 424, no. 6946, pp. 277–283, 2003.

[29] R. Doonan, J. J. McElwee, F. Matthijssens et al., "Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in *Caenorhabditis elegans*," *Genes & Development*, vol. 22, no. 23, pp. 3236–3241, 2008.

[30] W. Yang, J. J. Li, and S. Hekimi, "A measurable increase in oxidative damage due to reduction in superoxide detoxification fails to shorten the life span of long-lived mitochondrial mutants of *Caenorhabditis elegans*," *Genetics*, vol. 177, no. 4, pp. 2063–2074, 2007.

[31] Y. Honda, M. Tanaka, and S. Honda, "Modulation of longevity and diapause by redox regulation mechanisms under the insulin-like signaling control in *Caenorhabditis elegans*," *Experimental Gerontology*, vol. 43, no. 6, pp. 520–529, 2008.

[32] K. Houthoofd, T. E. Johnson, and J. R. Vanfleteren, "Dietary restriction in the nematode *Caenorhabditis elegans*," *Journals of Gerontology A*, vol. 60, no. 9, pp. 1125–1131, 2005.

[33] B. Lakowski and S. Hekimi, "The genetics of caloric restriction in *Caenorhabditis elegans*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 22, pp. 13091–13096, 1998.

[34] K. Houthoofd, B. P. Braeckman, I. Lenaerts et al., "No reduction of metabolic rate in food restricted *Caenorhabditis elegans*," *Experimental Gerontology*, vol. 37, no. 12, pp. 1359–1369, 2002.

[35] N. A. Bishop and L. Guarente, "Two neurons mediate diet-restriction-induced longevity in *C. elegans*," *Nature*, vol. 447, no. 7144, pp. 545–549, 2007.

[36] K. Houthoofd, B. P. Braeckman, I. Lenaerts et al., "Axenic growth up-regulates mass-specific metabolic rate, stress resistance, and extends life span in *Caenorhabditis elegans*," *Experimental Gerontology*, vol. 37, no. 12, pp. 1371–1378, 2002.

[37] S. H. Panowski, S. Wolff, H. Aguilaniu, J. Durieux, and A. Dillin, "PHA-4/Foxa mediates diet-restriction-induced longevity of *C. elegans*," *Nature*, vol. 447, no. 7144, pp. 550–555, 2007.

[38] J. M. Van Raamsdonk and S. Hekimi, "Reactive oxygen species and aging in *Caenorhabditis elegans*: causal or casual relationship?" *Antioxidants & Redox Signaling*, vol. 13, no. 12, pp. 1911–1953, 2010.

[39] K. Yen, H. B. Patel, A. L. Lublin, and C. V. Mobbs, "SOD isoforms play no role in lifespan in ad lib or dietary restricted conditions, but mutational inactivation of SOD-1 reduces life extension by cold," *Mechanisms of Ageing and Development*, vol. 130, no. 3, pp. 173–178, 2009.

[40] J. M. Van Raamsdonk and S. Hekimi, "Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in *Caenorhabditis elegans*," *PLoS Genetics*, vol. 5, no. 2, Article ID e1000361, 2009.

[41] J. J. Collins, K. Evason, and K. Kornfeld, "Pharmacology of delayed aging and extended lifespan of *Caenorhabditis elegans*," *Experimental Gerontology*, vol. 41, no. 10, pp. 1032–1039, 2006.

[42] L. A. Harrington and C. B. Harley, "Effect of vitamin E on lifespan and reproduction in *Caenorhabditis elegans*," *Mechanisms of Ageing and Development*, vol. 43, no. 1, pp. 71–78, 1988.

[43] N. Ishii, N. Senoo-Matsuda, K. Miyake et al., "Coenzyme Q10 can prolong *C. elegans* lifespan by lowering oxidative stress," *Mechanisms of Ageing and Development*, vol. 125, no. 1, pp. 41–46, 2004.

[44] H. Adachi and N. Ishii, "Effects of tocotrienols on life span and protein carbonylation in *Caenorhabditis elegans*," *Journals of Gerontology A*, vol. 55, no. 6, pp. B280–B285, 2000.

[45] M. G. Benedetti, A. L. Foster, M. C. Vantipalli et al., "Compounds that confer thermal stress resistance and extended lifespan," *Experimental Gerontology*, vol. 43, no. 10, pp. 882–891, 2008.

[46] T. J. Schulz, K. Zarse, A. Voigt, N. Urban, M. Birringer, and M. Ristow, "Glucose restriction extends *Caenorhabditis elegans* life span by inducing mitochondrial respiration and increasing oxidative stress," *Cell Metabolism*, vol. 6, no. 4, pp. 280–293, 2007.

[47] M. K. Brown, J. L. Evans, and Y. Luo, "Beneficial effects of natural antioxidants EGCG and α-lipoic acid on life span and age-dependent behavioral declines in *Caenorhabditis elegans*," *Pharmacology Biochemistry and Behavior*, vol. 85, no. 3, pp. 620–628, 2006.

[48] P. B. Pun, J. Gruber, S. Y. Tang et al., "Ageing in nematodes: do antioxidants extend lifespan in *Caenorhabditis elegans*?" *Biogerontology*, vol. 11, no. 1, pp. 17–30, 2010.

[49] A. R. Burns, I. M. Wallace, J. Wildenhain et al., "A predictive model for drug bioaccumulation and bioactivity in *Caenorhabditis elegans*," *Nature Chemical Biology*, vol. 6, no. 7, pp. 549–557, 2010.

[50] A. Shibamura, T. Ikeda, and Y. Nishikawa, "A method for oral administration of hydrophilic substances to *Caenorhabditis elegans*: effects of oral supplementation with antioxidants on the nematode lifespan," *Mechanisms of Ageing and Development*, vol. 130, no. 9, pp. 652–655, 2009.

[51] M. Keaney, F. Matthijssens, M. Sharpe, J. Vanfleteren, and D. Gems, "Superoxide dismutase mimetics elevate superoxide dismutase activity *in vivo* but do not retard aging in the nematode *Caenorhabditis elegans*," *Free Radical Biology and Medicine*, vol. 37, no. 2, pp. 239–250, 2004.

[52] J. N. Sampayo, A. Olsen, and G. J. Lithgow, "Oxidative stress in *Caenorhabditis elegans*: protective effects of superoxide dismutase/catalase mimetics," *Aging Cell*, vol. 2, no. 6, pp. 319–326, 2003.

[53] J. Kim, M. Takahashi, T. Shimizu et al., "Effects of a potent antioxidant, platinum nanoparticle, on the lifespan of *Caenorhabditis elegans*," *Mechanisms of Ageing and Development*, vol. 129, no. 6, pp. 322–331, 2008.

[54] S. Melov, J. Ravenscroft, S. Malik et al., "Extension of lifespan with superoxide dismutase/catalase mimetics," *Science*, vol. 289, no. 5484, pp. 1567–1569, 2000.

[55] M. Keaney and D. Gems, "No increase in lifespan in *Caenorhabditis elegans* upon treatment with the superoxide dismutase mimetic EUK-8," *Free Radical Biology and Medicine*, vol. 34, no. 2, pp. 277–282, 2003.

[56] F. Matthijssens, P. Back, B. P. Braeckman, and J. R. Vanfleteren, "Prooxidant activity of the superoxide dismutase (SOD)-mimetic EUK-8 in proliferating and growth-arrested *Escherichia coli* cells," *Free Radical Biology and Medicine*, vol. 45, no. 5, pp. 708–715, 2008.

[57] S. Dingley, E. Polyak, R. Lightfoot et al., "Mitochondrial respiratory chain dysfunction variably increases oxidant stress in *Caenorhabditis elegans*," *Mitochondrion*, vol. 10, no. 2, pp. 125–136, 2010.

[58] V. Chávez, A. Mohri-Shiomi, A. Maadani, L. A. Vega, and D. A. Garsin, "Oxidative stress enzymes are required for DAF-16-mediated immunity due to generation of reactive oxygen species by *Caenorhabditis elegans*," *Genetics*, vol. 176, no. 3, pp. 1567–1577, 2007.

[59] P. Back, F. Matthijssens, J. R. Vanfleteren, and B. P. Braeckman, "A simplified hydroethidine method for fast and accurate detection of superoxide production in isolated mitochondria," *Analytical Biochemistry*, vol. 423, no. 1, pp. 147–151, 2012.

[60] J. Gruber, L. F. Ng, S. Fong et al., "Mitochondrial changes in ageing *Caenorhabditis elegans*—what do we learn from superoxide dismutase knockouts?" *PLoS ONE*, vol. 6, no. 5, Article ID e19444, 2011.

[61] S. Yanase, A. Onodera, P. Tedesco, T. E. Johnson, and N. Ishii, "SOD-1 deletions in *Caenorhabditis elegans* alter the localization of intracellular reactive oxygen species and show molecular compensation," *Journals of Gerontology A*, vol. 64, no. 5, pp. 530–539, 2009.

[62] J. M. Van Raamsdonk and S. Hekimi, "Superoxide dismutase is dispensable for normal animal lifespan," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 15, pp. 5785–5790, 2012.

[63] F. Cabreiro, D. Ackerman, R. Doonan et al., "Increased life span from overexpression of superoxide dismutase in *Caenorhabditis elegans* is not caused by decreased oxidative damage," *Free Radical Biology and Medicine*, vol. 51, no. 8, pp. 1575–1582, 2011.

[64] G. N. Landis and J. Tower, "Superoxide dismutase evolution and life span regulation," *Mechanisms of Ageing and Development*, vol. 126, no. 3, pp. 365–379, 2005.

[65] L. T. Jensen and V. C. Culotta, "Activation of CuZn superoxide dismutases from *Caenorhabditis elegans* does not require the copper chaperone CCS," *Journal of Biological Chemistry*, vol. 280, no. 50, pp. 41373–41379, 2005.

[66] P. L. Larsen, "Aging and resistance to oxidative damage in *Caenorhabditis elegans*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 19, pp. 8905–8909, 1993.

[67] M. Fujii, N. Ishii, A. Joguchi, K. Yasuda, and D. Ayusawa, "A novel superoxide dismutase gene encoding membrane-bound and extracellular isoforms by alternative splicing in *Caenorhabditis elegans*," *DNA Research*, vol. 5, no. 1, pp. 25–30, 1998.

[68] M. P. Giglio, T. Hunter, J. V. Bannister, W. H. Bannister, and G. J. Hunter, "The manganese superoxide dismutase gene of *Caenorhabditis elegans*," *Biochemistry and Molecular Biology International*, vol. 33, no. 1, pp. 37–40, 1994.

[69] T. Hunter, W. H. Bannister, and G. J. Hunter, "Cloning, expression, and characterization of two manganese superoxide dismutases from *Caenorhabditis elegans*," *Journal of Biological Chemistry*, vol. 272, no. 45, pp. 28652–28659, 1997.

[70] J. Wang and S. K. Kim, "Global analysis of dauer gene expression in *Caenorhabditis elegans*," *Development*, vol. 130, no. 8, pp. 1621–1634, 2003.

[71] Y. Honda and S. Honda, "The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in *Caenorhabditis elegans*," *The FASEB Journal*, vol. 13, no. 11, pp. 1385–1393, 1999.

[72] S. Yanase and N. Ishi, "Cloning of the oxidative stress-responsive genes in *Caenorhabditis elegans*," *Journal of Radiation Research*, vol. 40, no. 1, pp. 39–47, 1999.

[73] B. J. Goldstein, K. Mahadev, and X. Wu, "Redox paradox: insulin action is facilitated by insulin-stimulated reactive oxygen species with multiple potential signaling targets," *Diabetes*, vol. 54, no. 2, pp. 311–321, 2005.

[74] O. I. Petriv and R. A. Rachubinski, "Lack of peroxisomal catalase causes a progeric phenotype in *Caenorhabditis elegans*," *Journal of Biological Chemistry*, vol. 279, no. 19, pp. 19996–20001, 2004.

[75] J. Taub, J. F. Lau, C. Ma et al., "A cytosolic catalase is needed to extend adult lifespan in *C. elegans* daf-C and clk-1 mutants," *Nature*, vol. 399, no. 6732, pp. 162–166, 1999.

[76] K. Isermann, E. Liebau, T. Roeder, and I. Bruchhaus, "A peroxiredoxin specifically expressed in two types of pharyngeal neurons is required for normal growth and egg production

in *Caenorhabditis elegans*," *Journal of Molecular Biology*, vol. 338, no. 4, pp. 745–755, 2004.

[77] C. Kumsta, M. Thamsen, and U. Jakob, "Effects of oxidative stress on behavior, physiology, and the redox thiol proteome of *Caenorhabditis elegans*," *Antioxidants & Redox Signaling*, vol. 14, no. 6, pp. 1023–1037, 2011.

[78] M. Oláhová, S. R. Taylor, S. Khazaipoul et al., "A redox-sensitive peroxiredoxin that is important for longevity has tissue- and stress-specific roles in stress resistance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 50, pp. 19839–19844, 2008.

[79] C. Jee, L. Vanoaica, J. Lee, B. J. Park, and J. Ahnn, "Thioredoxin is related to life span regulation and oxidative stress response in *Caenorhabditis elegans*," *Genes to Cells*, vol. 10, no. 12, pp. 1203–1210, 2005.

[80] A. Miranda-Vizuete, J. C. F. González, G. Gahmon, J. Burghoorn, P. Navas, and P. Swoboda, "Lifespan decrease in a *Caenorhabditis elegans* mutant lacking TRX-1, a thioredoxin expressed in ASJ sensory neurons," *FEBS Letters*, vol. 580, no. 2, pp. 484–490, 2006.

[81] J. C. Fierro-González, M. González-Barrios, A. Miranda-Vizuete, and P. Swoboda, "The thioredoxin TRX-1 regulates adult lifespan extension induced by dietary restriction in *Caenorhabditis elegans*," *Biochemical and Biophysical Research Communications*, vol. 406, no. 3, pp. 478–482, 2011.

[82] B. Cacho-Valadez, F. Munoz-Lobato, J. R. Pedrajas et al., "The characterization of the *Caenorhabditis elegans* mitochondrial thioredoxin system uncovers an unexpected protective role of thioredoxin reductase 2 in beta-amyloid peptide toxicity," *Antioxidants & Redox Signaling*, vol. 16, no. 12, pp. 1384–1400, 2012.

[83] A. N. Minniti, R. Cataldo, C. Trigo et al., "Methionine sulfoxide reductase a expression is regulated by the DAF-16/FOXO pathway in *Caenorhabditis elegans*," *Aging Cell*, vol. 8, no. 6, pp. 690–705, 2009.

[84] B. Leiers, A. Kampkötter, C. G. Grevelding, C. D. Link, T. E. Johnson, and K. Henkle-Dührsen, "A stress-responsive glutathione S-transferase confers resistance to oxidative stress in *Caenorhabditis elegans*," *Free Radical Biology and Medicine*, vol. 34, no. 11, pp. 1405–1415, 2003.

[85] S. Ayyadevara, M. R. Engle, S. P. Singh et al., "Lifespan and stress resistance of *Caenorhabditis elegans* are increased by expression of glutathione transferases capable of metabolizing the lipid peroxidation product 4-hydroxynonenal," *Aging Cell*, vol. 4, no. 5, pp. 257–271, 2005.

[86] S. Ayyadevara, A. Dandapat, S. P. Singh et al., "Life span and stress resistance of *Caenorhabditis elegans* are differentially affected by glutathione transferases metabolizing 4-hydroxynon-2-enal," *Mechanisms of Ageing and Development*, vol. 128, no. 2, pp. 196–205, 2007.

[87] S. Valentini, F. Cabreiro, D. Ackerman et al., "Manipulation of *in vivo* iron levels can alter resistance to oxidative stress without affecting ageing in the nematode *C. elegans*," *Mechanisms of Ageing and Development*, vol. 133, no. 5, pp. 282–290, 2012.

[88] V. I. Pérez, A. Bokov, H. Van Remmen et al., "Is the oxidative stress theory of aging dead?" *Biochimica et Biophysica Acta*, vol. 1790, no. 10, pp. 1005–1014, 2009.

[89] S. Elchuri, T. D. Oberley, W. Qi et al., "CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life," *Oncogene*, vol. 24, no. 3, pp. 367–380, 2005.

[90] J. P. Phillips, S. D. Campbell, D. Michaud, M. Charbonneau, and A. J. Hilliker, "Null mutation of copper/zinc superoxide dismutase in *Drosophila* confers hypersensitivity to paraquat and reduced longevity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 8, pp. 2761–2765, 1989.

[91] I. Reveillaud, J. Phillips, B. Duyf, A. Hilliker, A. Kongpachith, and J. E. Fleming, "Phenotypic rescue by a bovine transgene in a Cu/Zn superoxide dismutase-null mutant of *Drosophila melanogaster*," *Molecular and Cellular Biology*, vol. 14, no. 2, pp. 1302–1307, 1994.

[92] K. Kirby, J. Hu, A. J. Hilliker, and J. P. Phillips, "RNA interference-mediated silencing of Sod2 in *Drosophila* leads to early adult-onset mortality and elevated endogenous oxidative stress," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 25, pp. 16162–16167, 2002.

[93] A. Duttaroy, A. Paul, M. Kundu, and A. Belton, "A Sod2 null mutation confers severely reduced adult life span in *Drosophila*," *Genetics*, vol. 165, no. 4, pp. 2295–2299, 2003.

[94] Y. Li, T. T. Huang, E. J. Carlson et al., "Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase," *Nature Genetics*, vol. 11, no. 4, pp. 376–381, 1995.

[95] H. Van Remmen, Y. Ikeno, M. Hamilton et al., "Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging," *Physiological Genomics*, vol. 16, no. 1, pp. 29–37, 2003.

[96] V. I. Pérez, H. Van Remmen, A. Bokov, C. J. Epstein, J. Vijg, and A. Richardson, "The overexpression of major antioxidant enzymes does not extend the lifespan of mice," *Aging Cell*, vol. 8, no. 1, pp. 73–75, 2009.

[97] N. O. Seto, S. Hayashi, and G. M. Tener, "Overexpression of Cu—Zn superoxide dismutase in *Drosophila* does not affect life-span," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 11, pp. 4270–4274, 1990.

[98] W. C. Orr and R. S. Sohal, "Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*," *Science*, vol. 263, no. 5150, pp. 1128–1130, 1994.

[99] T. L. Parkes, A. J. Elia, D. Dickinson, A. J. Hilliker, J. P. Phillips, and G. L. Boulianne, "Extension of *Drosophila* lifespan by overexpression of human SOD1 in motorneurons," *Nature Genetics*, vol. 19, no. 2, pp. 171–174, 1998.

[100] J. Sun and J. Tower, "FLP recombinase-mediated induction of Cu/Zn-superoxide dismutase transgene expression can extend the life span of adult *Drosophila melanogaster* flies," *Molecular and Cellular Biology*, vol. 19, no. 1, pp. 216–228, 1999.

[101] W. C. Orr, R. J. Mockett, J. J. Benes, and R. S. Sohal, "Effects of overexpression of copper-zinc and manganese superoxide dismutases, catalase, and thioredoxin reductase genes on longevity in *Drosophila melanogaster*," *Journal of Biological Chemistry*, vol. 278, no. 29, pp. 26418–26422, 2003.

[102] J. Sun, D. Folk, T. J. Bradley, and J. Tower, "Induced overexpression of mitochondrial Mn-superoxide dismutase extends the life span of adult *Drosophila melanogaster*," *Genetics*, vol. 161, no. 2, pp. 661–672, 2002.

[103] M. Kaiser, M. Gasser, R. Ackermann, and S. C. Stearns, "P-element inserts in transgenic flies: a cautionary tale," *Heredity*, vol. 78, no. 1, pp. 1–11, 1997.

[104] C. C. Spencer, C. E. Howell, A. R. Wright, and D. E. Promislow, "Testing an 'aging gene' in long-lived *Drosophila*

strains: increased longevity depends on sex and genetic background," *Aging Cell*, vol. 2, no. 2, pp. 123–130, 2003.

[105] S. J. Lee, A. B. Hwang, and C. Kenyon, "Inhibition of respiration extends *C. elegans* life span via reactive oxygen species that increase HIF-1 activity," *Current Biology*, vol. 20, no. 23, pp. 2131–2136, 2010.

[106] T. Heidler, K. Hartwig, H. Daniel, and U. Wenzel, "*Caenorhabditis elegans* lifespan extension caused by treatment with an orally active ROS-generator is dependent on DAF-16 and SIR-2.1," *Biogerontology*, vol. 11, no. 2, pp. 183–195, 2010.

[107] M. Ristow and K. Zarse, "How increased oxidative stress promotes longevity and metabolic health: the concept of mitochondrial hormesis (mitohormesis)," *Experimental Gerontology*, vol. 45, no. 6, pp. 410–418, 2010.

[108] M. Ralser, M. M. Wamelink, A. Kowald et al., "Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress," *Journal of Biology*, vol. 6, no. 4, article 10, 2007.

[109] K. Zarse, S. Schmeisser, M. Groth et al., "Impaired insulin/IGF1 signaling extends life span by promoting mitochondrial L-proline catabolism to induce a transient ROS signal," *Cell Metabolism*, vol. 15, no. 4, pp. 451–465, 2012.

[110] Y. Y. Yang, J. A. Gangoiti, M. M. Sedensky, and P. G. Morgan, "The effect of different ubiquinones on lifespan in *Caenorhabditis elegans*," *Mechanisms of Ageing and Development*, vol. 130, no. 6, pp. 370–376, 2009.

[111] J. Feng, F. Bussière, and S. Hekimi, "Mitochondrial electron transport is a key determinant of life span in *Caenorhabditis elegans*," *Developmental Cell*, vol. 1, no. 5, pp. 633–644, 2001.

[112] W. Yang and S. Hekimi, "Two modes of mitochondrial dysfunction lead independently to lifespan extension in *Caenorhabditis elegans*," *Aging Cell*, vol. 9, no. 3, pp. 433–447, 2010.

[113] B. P. Braeckman, K. Houthoofd, K. Brys et al., "No reduction of energy metabolism in Clk mutants," *Mechanisms of Ageing and Development*, vol. 123, no. 11, pp. 1447–1456, 2002.

[114] J. Durieux, S. Wolff, and A. Dillin, "The cell-non-autonomous nature of electron transport chain-mediated longevity," *Cell*, vol. 144, no. 1, pp. 79–91, 2011.

[115] G. U. Bae, D. W. Seo, H. K. Kwon et al., "Hydrogen peroxide activates p70(S6k) signaling pathway," *Journal of Biological Chemistry*, vol. 274, no. 46, pp. 32596–32602, 1999.

[116] C. Huang, J. Li, Q. Ke et al., "Ultraviolet-induced phosphorylation of p70S6K at Thr389 and Thr421/Ser424 involves hydrogen peroxide and mammalian target of rapamycin but not Akt and atypical protein kinase C," *Cancer Research*, vol. 62, no. 20, pp. 5689–5697, 2002.

[117] J. H. Kim, S. C. Chu, J. L. Gramlich et al., "Activation of the PI3K/mTOR pathway by BCR-ABL contributes to increased production of reactive oxygen species," *Blood*, vol. 105, no. 4, pp. 1717–1723, 2005.

[118] M. J. Tuñón, S. Sánchez-Campos, B. Gutiérrez, J. M. Culebras, and J. González-Gallego, "Effects of FK506 and rapamycin on generation of reactive oxygen species, nitric oxide production and nuclear factor kappa B activation in rat hepatocytes," *Biochemical Pharmacology*, vol. 66, no. 3, pp. 439–445, 2003.

[119] M. V. Blagosklonny, "Aging: ROS or TOR," *Cell Cycle*, vol. 7, no. 21, pp. 3344–3354, 2008.

[120] M. V. Blagosklonny, "Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition," *Cell Cycle*, vol. 5, no. 18, pp. 2087–2102, 2006.

[121] J. M. May and C. de Haen, "Insulin-stimulated intracellular hydrogen peroxide production in rat epididymal fat cells," *Journal of Biological Chemistry*, vol. 254, no. 7, pp. 2214–2220, 1979.

[122] B. J. Goldstein, K. Mahadev, X. Wu, L. Zhu, and H. Motoshima, "Role of insulin-induced reactive oxygen species in the insulin signaling pathway," *Antioxidants & Redox Signaling*, vol. 7, no. 7-8, pp. 1021–1031, 2005.

[123] E. M. Ha, C. T. Oh, Y. S. Bae, and W. J. Lee, "A direct role for dual oxidase in *Drosophila* gut immunity," *Science*, vol. 310, no. 5749, pp. 847–850, 2005.

[124] A. R. Cross and A. W. Segal, "The NADPH oxidase of professional phagocytes—prototype of the NOX electron transport chain systems," *Biochimica et Biophysica Acta*, vol. 1657, no. 1, pp. 1–22, 2004.

[125] K. Apel and H. Hirt, "Reactive oxygen species: metabolism, oxidative stress, and signal transduction," *Annual Review of Plant Biology*, vol. 55, pp. 373–399, 2004.

[126] C. Jain, M. Yun, S. M. Politz, and R. P. Rao, "A pathogenesis assay using *Saccharomyces cerevisiae* and *Caenorhabditis elegans* reveals novel roles for yeast AP-1, Yap1, and host dual oxidase BLI-3 in fungal pathogenesis," *Eukaryotic Cell*, vol. 8, no. 8, pp. 1218–1227, 2009.

[127] V. Chávez, A. Mohri-Shiomi, and D. A. Garsin, "Ce-Duox1/BLI-3 generates reactive oxygen species as a protective innate immune mechanism in *Caenorhabditis elegans*," *Infection and Immunity*, vol. 77, no. 11, pp. 4983–4989, 2009.

[128] R. van der Hoeven, K. C. McCallum, M. R. Cruz, and D. A. Garsin, "Ce-Duox1/BLI-3 generated reactive oxygen species trigger protective SKN-1 activity via p38 MAPK signaling during infection in *C. elegans*," *PLoS Pathogens*, vol. 7, no. 12, Article ID e1002453, 2011.

[129] W. A. Edens, L. Sharling, G. Cheng et al., "Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox," *Journal of Cell Biology*, vol. 154, no. 4, pp. 879–891, 2001.

[130] D. Hernández-García, C. D. Wood, S. Castro-Obregón, and L. Covarrubias, "Reactive oxygen species: a radical role in development?" *Free Radical Biology and Medicine*, vol. 49, no. 2, pp. 130–143, 2010.

[131] F. Q. Schafer and G. R. Buettner, "Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple," *Free Radical Biology and Medicine*, vol. 30, no. 11, pp. 1191–1212, 2001.

[132] M. J. Hitchler and F. E. Domann, "An epigenetic perspective on the free radical theory of development," *Free Radical Biology and Medicine*, vol. 43, no. 7, pp. 1023–1036, 2007.

[133] P. Back, W. H. De Vos, G. G. Depuydt, F. Matthijssens, J. R. Vanfleteren, and B. P. Braeckman, "Exploring real-time *in vivo* redox biology of developing and aging *Caenorhabditis elegans*," *Free Radical Biology and Medicine*, vol. 52, no. 5, pp. 850–859, 2012.

[134] J. Goudeau and H. Aguilaniu, "Carbonylated proteins are eliminated during reproduction in *C. elegans*," *Aging Cell*, vol. 9, no. 6, pp. 991–1003, 2010.

[135] D. Greenstein, "Control of oocyte meiotic maturation and fertilization," in *WormBook*, pp. 1–12, 2005.

[136] Y. Yang, S. M. Han, and M. A. Miller, "MSP hormonal control of the oocyte MAP kinase cascade and reactive oxygen species signaling," *Developmental Biology*, vol. 342, no. 1, pp. 96–107, 2010.

[137] A. Gomes, E. Fernandes, and J. L. Lima, "Fluorescence probes used for detection of reactive oxygen species," *Journal of Biochemical and Biophysical Methods*, vol. 65, no. 2-3, pp. 45–80, 2005.

[138] P. Wardman, "Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects," *Free Radical Biology and Medicine*, vol. 43, no. 7, pp. 995–1022, 2007.

[139] G. Bartosz, "Use of spectroscopic probes for detection of reactive oxygen species," *Clinica Chimica Acta*, vol. 368, no. 1-2, pp. 53–76, 2006.

[140] B. C. Dickinson, D. Srikun, and C. J. Chang, "Mitochondrial-targeted fluorescent probes for reactive oxygen species," *Current Opinion in Chemical Biology*, vol. 14, no. 1, pp. 50–56, 2010.

[141] H. M. Cochemé, C. Quin, S. J. McQuaker et al., "Measurement of H_2O_2 within living *Drosophila* during aging using a ratiometric mass spectrometry probe targeted to the mitochondrial matrix," *Cell Metabolism*, vol. 13, no. 3, pp. 340–350, 2011.

[142] D. Srikun, A. E. Albers, C. I. Nam, A. T. Iavarone, and C. J. Chang, "Organelle-targetable fluorescent probes for imaging hydrogen peroxide in living cells via SNAP-Tag protein labeling," *Journal of the American Chemical Society*, vol. 132, no. 12, pp. 4455–4465, 2010.

[143] E. A. Souslova and D. M. Chudakov, "Genetically encoded intracellular sensors based on fluorescent proteins," *Biochemistry*, vol. 72, no. 7, pp. 683–697, 2007.

[144] E. A. Veal, A. M. Day, and B. A. Morgan, "Hydrogen peroxide sensing and signaling," *Molecular Cell*, vol. 26, no. 1, pp. 1–14, 2007.

[145] J. R. Stone and S. Yang, "Hydrogen peroxide: a signaling messenger," *Antioxidants & Redox Signaling*, vol. 8, no. 3-4, pp. 243–270, 2006.

[146] M. Giorgio, M. Trinei, E. Migliaccio, and P. G. Pelicci, "Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals?" *Nature Reviews Molecular Cell Biology*, vol. 8, no. 9, pp. 722–728, 2007.

[147] V. V. Belousov, A. F. Fradkov, K. A. Lukyanov et al., "Genetically encoded fluorescent indicator for intracellular hydrogen peroxide," *Nature Methods*, vol. 3, no. 4, pp. 281–286, 2006.

[148] K. N. Markvicheva, D. S. Bilan, N. M. Mishina et al., "A genetically encoded sensor for H_2O_2 with expanded dynamic range," *Bioorganic and Medicinal Chemistry*, vol. 19, no. 3, pp. 1079–1084, 2011.

[149] A. Espinosa, A. García, S. Härtel, C. Hidalgo, and E. Jaimovich, "NADPH oxidase and hydrogen peroxide mediate insulin-induced calcium increase in skeletal muscle cells," *Journal of Biological Chemistry*, vol. 284, no. 4, pp. 2568–2575, 2009.

[150] Z. Ungvari, N. Labinskyy, P. Mukhopadhyay et al., "Resveratrol attenuates mitochondrial oxidative stress in coronary arterial endothelial cells," *American Journal of Physiology*, vol. 297, no. 5, pp. H1876–H1881, 2009.

[151] N. M. Mishina, P. A. Tyurin-Kuzmin, K. N. Markvicheva et al., "Does cellular hydrogen peroxide diffuse or act locally?" *Antioxidants & Redox Signaling*, vol. 14, no. 1, pp. 1–7, 2011.

[152] P. Niethammer, C. Grabher, A. T. Look, and T. J. Mitchison, "A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish," *Nature*, vol. 459, no. 7249, pp. 996–999, 2009.

[153] G. T. Hanson, R. Aggeler, D. Oglesbee et al., "Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators," *Journal of Biological Chemistry*, vol. 279, no. 13, pp. 13044–13053, 2004.

[154] M. Gutscher, A. L. Pauleau, L. Marty et al., "Real-time imaging of the intracellular glutathione redox potential," *Nature Methods*, vol. 5, no. 6, pp. 553–559, 2008.

[155] A. J. Meyer and T. P. Dick, "Fluorescent protein-based redox probes," *Antioxidants & Redox Signaling*, vol. 13, no. 5, pp. 621–650, 2010.

[156] M. Gutscher, M. C. Sobotta, G. H. Wabnitz et al., "Proximity-based protein thiol oxidation by H_2O_2-scavenging peroxidases," *Journal of Biological Chemistry*, vol. 284, no. 46, pp. 31532–31540, 2009.

[157] M. Schwarzländer, M. D. Fricker, C. Müller et al., "Confocal imaging of glutathione redox potential in living plant cells," *Journal of Microscopy*, vol. 231, no. 2, pp. 299–316, 2008.

[158] S. C. Albrecht, A. G. Barata, J. Grosshans, A. A. Teleman, and T. P. Dick, "*In vivo* mapping of hydrogen peroxide and oxidized glutathione reveals chemical and regional specificity of redox homeostasis," *Cell Metabolism*, vol. 14, no. 6, pp. 819–829, 2011.

[159] H. Ostergaard, A. Henriksen, F. G. Hansen, and J. R. Winther, "Shedding light on disulfide bond formation: engineering a redox switch in green fluorescent protein," *The EMBO Journal*, vol. 20, no. 21, pp. 5853–5862, 2001.

[160] W. Wang, H. Fang, L. Groom et al., "Superoxide flashes in single mitochondria," *Cell*, vol. 134, no. 2, pp. 279–290, 2008.

[161] J. Kishikawa, M. Fujikawa, H. Imamura et al., "MRT letter: expression of ATP sensor protein in *Caenorhabditis elegans*," *Microscopy Research and Technique*, vol. 75, no. 1, pp. 15–19, 2012.

[162] Y. P. Hung, J. G. Albeck, M. Tantama, and G. Yellen, "Imaging cytosolic NADH-NAD^+ redox state with a genetically encoded fluorescent biosensor," *Cell Metabolism*, vol. 14, no. 4, pp. 545–554, 2011.

[163] J. P. Klare and D. O. de Orue Lucana, "Conformational changes in the novel redox sensor protein HbpS studied by site-directed spin labeling and its turnover in dependence on the catalase-peroxidase CpeB," *Antioxidants & Redox Signaling*, vol. 16, no. 7, pp. 639–648, 2012.

[164] T. Yano, M. Oku, N. Akeyama et al., "A novel fluorescent sensor protein for visualization of redox states in the cytoplasm and in peroxisomes," *Molecular and Cellular Biology*, vol. 30, no. 15, pp. 3758–3766, 2010.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 741468, 20 pages
doi:10.1155/2012/741468

Review Article

Diet and Aging

Samo Ribarič

Institute of Pathophysiology, Medical Faculty, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia

Correspondence should be addressed to Samo Ribarič, samo.ribaric@mf.uni-lj.si

Received 1 June 2012; Revised 12 July 2012; Accepted 16 July 2012

Academic Editor: Paula Ludovico

Nutrition has important long-term consequences for health that are not only limited to the individual but can be passed on to the next generation. It can contribute to the development and progression of chronic diseases thus effecting life span. Caloric restriction (CR) can extend the average and maximum life span and delay the onset of age-associated changes in many organisms. CR elicits coordinated and adaptive stress responses at the cellular and whole-organism level by modulating epigenetic mechanisms (e.g., DNA methylation, posttranslational histone modifications), signaling pathways that regulate cell growth and aging (e.g., TOR, AMPK, p53, and FOXO), and cell-to-cell signaling molecules (e.g., adiponectin). The overall effect of these adaptive stress responses is an increased resistance to subsequent stress, thus delaying age-related changes and promoting longevity. In human, CR could delay many diseases associated with aging including cancer, diabetes, atherosclerosis, cardiovascular disease, and neurodegenerative diseases. As an alternative to CR, several CR mimetics have been tested on animals and humans. At present, the most promising alternatives to the use of CR in humans seem to be exercise, alone or in combination with reduced calorie intake, and the use of plant-derived polyphenol resveratrol as a food supplement.

1. Introduction

Nutrition has important long-term consequences for health. It is one of the lifestyle factors that contribute to the development and progression of chronic diseases including cardiovascular diseases, diabetes, and cancer [1]. The prevention or management of chronic diseases is a global priority since they account for more than half of the deaths worldwide [2]. The effects of nutrition on heath are not limited to the individual but can be passed on to the next generation. This observation has been confirmed by epidemiological studies and animal experiments. Epidemiologic observations linked smaller size or low weight at birth or during infancy to increased rates of coronary heart rate disease, type 2 diabetes mellitus, or adiposity in adult life [3–7]. In an animal model, for example, prenatal undernutrition reduced the offspring's life span [8] or lead to inadequate development of nephrons that increased the development of chronic kidney disease in later life [9].

2. Epigenetic Modifications by Dietary Factors

The effects of nutrition on the body are also mediated by epigenetic mechanisms [1]. The three known, closely interacting mechanisms are DNA methylation, histone modification, and noncoding microRNAs (miRNAs) as reviewed by McKay and Mathers [1]. Nutritional factors may induce epigenetic changes via three pathways: (a) a direct influence on gene expression, (b) activation of nuclear receptors by ligands, and (c) modification of membrane receptor signaling cascades [10]. Therefore, epigenetic mechanisms provide the organism with a robust, and time-responsive system for adapting gene expression that is (a) tissue-type specific, (b) appropriate for the developmental state of the organism, and (c) responsive to signals from the external and internal environment [1].

2.1. DNA Methylation by Diet. DNA methylation is tissue specific and is regulated by the enzyme DNA methyltransferase (DNMT) that modifies a cytosine base at the CpG dinucleotide residue with a methyl group to form 5-methylcytosine [11]. Examples of processes that are controlled by DNA methylation are X chromosome inactivation, imprinting, and silencing of germline-specific genes, carcinogenesis, and long-term memory formation [12]. Traditionally, DNA methylation was associated with suppression of gene expression. Thus, DNA methylation either physically impedes the binding of transcriptional proteins to the gene,

or the methylated DNA binds to proteins known as methyl-CpG-binding domain proteins that recruit additional proteins to the locus—such as histone deacetylases—that modify histones into compact, inactive chromatin as reviewed in [13, 14]. However, in some patients with cancer, both global DNA-hypomethylation and localized DNA-hypermethylation are present [15, 16].

Dietary constituents that are known to modulate DNA methylation are, for example, folate, vitamin B12, selenium, green tee polyphenols (e.g., epigallocatechin-3-gallate (EGCG), epicatechin, ganistein), and bioflavonoids (quercetin, fisetin and myricetin). Folate and vitamin B12 promote global DNA-methylation, whereas selenium, green tee polyphenols, and bioflavonoids reduce global DNA-methylation as reviewed in Davis et al. [17]. However, the local effect of these constituents on DNA methylation can differ from their global one. For example, long-term selenium consumption increases exon-specific DNA methylation of the p53 gene in rat liver and colon mucosa [18].

2.2. Histone Modification by Diet. Eukaryotic cell nuclei contain alkaline proteins (due to highly positively charged N-terminus with many lysine and arginine residues) called histones, that package and order the DNA into structural units called nucleosomes. Histones act as spools around which DNA winds and play a role in gene regulation, since genes that are active are less bound to histones; inactive genes are highly associated with histones [19]. The histones N-terminus (i.e., the histone tail) or the side chains at the globular histone core are the sites of epigenetic modifications [20].

Posttranslational modification of histones is significantly more diverse then DNA methylation. Some of the best understood histone modifications are methylation, acetylation, phosphorylation, ribosylation, ubiquitination, sumoylation, or biotinylation [20]. Examples of enzymes involved in posttranslational modification of histones are histone acetyltransferases (HATs), methyltransferases (HMTs), deacetylases (HDACs), and demethylases (HDMs). The effects of diet on histone posttranslational modification were recently reviewed by Link et al. [21]. For example, polyphenols from garlic or cinnamon inhibit HDAC; green tea polyphenols and copper inhibit HAT; EGCG inhibits HMT.

Histone methylation can modulate DNA methylation patterns, and DNA methylation might serve as a template for some histone modifications after DNA replication [20, 22]. It has been suggested that these interactions could be accomplished via direct interactions between histone and DNA methyltransferases [20, 22]. Such DNA-histone interactions could also be initiated or modulated by diet.

2.3. miRNA Modification by Diet. miRNAs are a family of approximately 22-nucleotides long noncoding RNAs in eukaryotic cells. miRNAs are posttranscriptional regulators and bind to complementary sequences on target messenger RNA transcripts (mRNAs) leading to posttranscriptional gene silencing due to mRNA translational repression or increased RNA degradation. However, miRNAs can also cause histone modification and DNA methylation of promoter sites thus regulating the expression of target genes by an alternative pathway. [23, 24]. The human genome encodes over 1000 miRNAs, which target more than 50% of mammalian genes in many human cell types [25–30]. Thus, miRNAs influence the expression of many transcription factors, receptors and transporters [31]. Recent evidence from experiments in human and in animal models suggests that nutrition (e.g., the consumption of fat, protein, alcohol or vitamin E) effects the expression of many miRNA [32].

Polyphenols (e.g., anthocyanin, curcumin and quercetin,) at nutritional doses modulate the expression of liver miRNA in mice *in vivo* [33]. Dietary modulation of miRNA expression could contribute to the cancer protective effects of genistein, curcumin, retinoic acid, or fish oil. Genistein (an isoflavone) inhibits uveal melanoma cell growth in a time and dose-related manner by inhibiting the of miRNA-27a expression [34]. Curcumin treatment upregulates miRNA-22 and downregulated miRNA-199a in a pancreatic cancer cell line [35] and also upregulates the expression of miRNA-15a and miRNA-16 in breast cancer cells [36]. Patients with acute promyelocytic leukemia that were successfully treated with chemotherapy and all-trans-retinoic acid had a downregulation of miRNA-181b and an upregulation of several miRNAs [37]. miRNA-10a downregulation, induced by treatment with retinoic acid, prevented pancreatic cancer metastasis in xenotransplantation experiments in zebrafish embryos [38]. Fish oil reduced the number of differentially expressed miRNAs in experimental animals and may be useful in prevention of colon carcinoma [39]. Indol-3-carbinol downregulated the expression of several miRNAs (i.e., miRNAs -21, -31, -130a, -146b and -377) in mice with induced mouse lung tumors [40]. Inadequate nutrition can also modulate miRNA expression. For example, dietary deficiency of folate was associated with significant overexpression of miRNA -222 in human with low folate intake [41]. Also, rats on a folate-methionine-choline deficient diet developed hepatocellular carcinoma with concurrent overexpression of miRNAs -17 to -92, -21, -23, -130, and -190 [42].

2.4. TOR Signaling Pathway and Nutrition. TOR (target of Rapamycin) is a protein kinase that functions as a central controller of cell growth and aging as reviewed elsewhere [43, 44]. Inactivation of the TOR signaling pathway promotes autophagy and prolongs life span [45]. Its function was first characterized in yeast but was also identified in other eukaryotes including mammals (hence mammalian TOR or mTOR). *In vivo*, mTOR exists in two multiprotein complexes, the mTORC1 and mTORC2. The mTORC1 functions as a nutrient-energy-redox sensor and modulates protein synthesis. Therefore, the upstream factors that stimulate the activity of this complex are insulin and other growth factors, amino acids (e.g., leucine), and stress (temperature change, caffeine, oxidative stress). Caffeine, hypoxia, and DNA damage inhibit mTORC1 activity. The upstream regulators of TORC1 activity are the AGC family of kinases (e.g., PKA; PKG and PKC) that are activated by phosphorylation [46]. In mammals, mTORC1 targets are S6 K1 and the eukaryotic initiation factor (4E-BP1) [47–52]. mTORC1-mediated phosphorylation of S6 K1 promotes protein synthesis and

4E-BP1 phosphorylation promotes localization of ribosomes to the cap structure of mRNAs. The phosphorylating activity of mTORC1 is regulated through its association with the RAPTOR (regulatory-associated protein of mTOR) protein [53, 54]. High nutrient or ATP levels activate mTORC1 by phosphorylating and thus inhibiting the TSC1-TSC2 complex as reviewed by Loewith and Hall [43]. This complex is a GTPase activating protein that modifies a second GTPase RHEB into a GTP bound state. RHEB, in the GTP bound state, directly binds and activates mTORC1 thus allowing mTORC1 to phosphorylate downstream targets [55]. Low cellular energy (high AMP levels) or low nutrient levels, in association with the tumor suppressor kinase LBK1, activate AMPK and an activated AMPK phosphorylates both TSC2 and RAPTOR thus inhibiting mTORC1 activity by two pathways [56]. In yeast, TORC1 promotes protein synthesis, ribosome biogenesis, regulates the relationship between cell cycle and cell size, promotes cell growth by inhibiting stress responses, stimulates autophagy, and regulates the signaling of mitochondrial dysfunction to the nucleus via the negative regulator of RTG1-dependent transcription [43, 44]. At the organ and whole body levels, the TORC1/S6 K1 signaling pathway regulates glucose homeostasis, insulin sensitivity, adipocyte metabolism, body mass and energy balance, tissue and organ size, learning, memory formation, and aging [57]. For example, the S6 K1 modulates the differentiation of mesenchymal stem cells into adipocytes. Overstimulation of the mTORC1/S6 K1 signaling pathway by excessive quantities of leucine in infant milk formulas could be the cause of increased adipogenesis and early childhood obesity [58].

The best understood functions of mTORC2 are the control of cell cycle-dependent polarization of actin cytoskeleton, endocytosis, and sphingolipid biosynthesis [43, 59, 60]. The upstream regulators of mTORC2 are insulin and IGF1 [44, 61]. The ribosome maturation factor Nip7 is required for mTORC2 kinase activity in yeast and mammalian cells [44, 61] and the substrates of mTORC2 are the AGC family of kinases including AKT, SGK1, and PKC [44, 62]. For example, mTORC2 promotes cell survival via AKT [63, 64] and also regulates hepatic glucose and lipid metabolism via insulin induced AKT signaling [62].

Although the signaling pathways of TORC1 and TORC2 are to some extent distinct they have a cooperative function to coordinate growth, mitosis, and cell size control. For example, TORC2 activates TORC1 via the AKT signaling pathway. TORC1 activation stimulates anabolic cellular pathways and TORC1 inhibition stimulates catabolic cellular pathways [65]. As a general rule, the sensitivity of the TORC1 and TORC2 signaling pathways could be not only cell-tissue specific but also TORC isoform dependent. For example, the activity of mTORC2 depends on the type of mammalian stress-activated protein kinase interacting protein (mSin1) isoform that constitutes this multiprotein-complex [66].

3. Nutrition and Aging

The possibility that mammalian life span could be significantly extended by diet modification was demonstrated in a rodent study published by McCay and coworkers. in 1935 [67]. Rats, as opposed to primates, have the ability to grow their entire life. One of the objects of this study was to determine the effect of retarding growth on the total length of life of rats of both sexes. Growth was retarded by limiting the intake of diet to the quantity necessary for maintaining the rats at fixed levels of body weights at the time of weaning or 2 weeks after weaning. At the same time, care was taken to provide adequate levels of all other diet constituents. Animals of both sexes, subject to diet restriction, had a prolonged total lenght of life. However, the effect of diet restriction on life span was more pronounced in male than in female rats [67]. In summary, this seminal experiment suggests that life span can be extended by diet restriction without malnutrition as opposed to diet restriction with malnutrition that can have an opposite effect as discussed elsewhere [1].

The standard protocol for studying the positive effects of a limited food intake is the use of caloric restriction, or calorie restriction (CR) diet that does not lead to malnutrition (due to lack of vitamins, minerals or essential biomolecules). CR means limiting calorie intake by 10–30% compared to the base line unrestricted intake for the studied life form and has been shown to improve health at all ages and also to slow the aging process in many eukaryotes [68]. The relevance of CR life span prolonging effects for primates was explored in a 20-year longitudinal adult-onset CR study in rhesus monkeys. The animals' baseline intake of calories was reduced progressively by 10% per month to a final 30% reduction that was maintained for the duration of the experiment. The effect of CR, compared to control, was evaluated by comparing the delay in mortality and the onset of some age-associated conditions most prevalent in humans (e.g., diabetes, cancer, cardiovascular disease, and brain atrophy). The conclusions of the study were that CR lowered the incidence of aging-related deaths (50% in control fed animals versus 20% in CR-fed animals) and also lowered the incidence of diabetes, cancer, cardiovascular disease, and brain atrophy [68].

4. Caloric Restriction Effects in Humans

The fundamental assumption, that caloric restriction can extend the average and maximum life span and delay the onset of age-associated changes, has been proven in many organisms from yeast, worms, and flies to mammals [69–71]. In higher mammals, CR delays many diseases associated with aging including cancer, diabetes, atherosclerosis, cardiovascular disease, and neurodegenerative diseases [68, 72–74]. The incidence of these diseases increases with age and they contribute significantly to mortality. Therefore, CR could increase life span by increasing the body's general state of health and providing a nonspecific, resistance to chronic diseases and metabolic derangements [68, 72–74]. However, the ultimate question, how does CR effect the human body, was studied in a limited number of experiments [73–93]. The study of CR effects on human longevity faces ethical and logistical challenges since the average life span is close to 80 years for the population in developed countries. Therefore, human studies are focused on measuring the CR-related changes that could slow the aging process and

the progression of chronic diseases thus increasing life span. The most convincing evidence that CR could have a positive effect in humans was provided by experiments by Fontana and coworkers, by the Comprehensive Assessment of Long-Term Effects of Reducing Calorie Intake (CALERIE Phase 1), and by data obtained on the members of the Caloric Restriction Society (as discussed below).

Fontana and coworkers evaluated the effect of a 6-year long CR diet on risk factors for atherosclerosis in adult male and female adults (age range 35–82 years) and compared them to age-matched healthy individuals on typical American diets (control group). The total serum cholesterol level and low-density lipoprotein (LDL) cholesterol levels, the ratio of total cholesterol to high-density lipoprotein cholesterol (HDL), triglycerides, fasting glucose, fasting insulin, C-reactive protein (CRP), platelet-derived growth factor AB, and systolic and diastolic blood pressures were all markedly lower in the CR group. The HDL cholesterol was higher after CR. Medical records of individuals in the CR group indicated that, before they began CR, they had serum lipid-lipoprotein and blood pressure levels in the expected range for individuals on typical American diets, and similar to those of the comparison group. The conclusion of the study was that long-term CR can reduce the risk factors for atherosclerosis [74].

The effect of fat loss induced by either (a) a long-term 20% CR or (b) a 20% increased energy expenditure (IEE) by exercise on coronary heart disease (CHD) risk factors was evaluated in a one-year randomized, controlled trial on 48 nonobese male and female subjects. The CR or exercise induced reductions in body fat were quantitatively similar and were accompanied by similar reductions in most of the major CHD risk factors, including plasma LDL-cholesterol, total cholesterol/HDL ratio, and CRP concentrations. The authors concluded that long-term CR or IEE of the same magnitude lead to substantial and similar improvements in the major risk factors for CHD in normal-weight and overweight middle-aged adults [83].

The effects of a 1-year, 20% CR regime or 20% IEE by exercise, on the oxidative damage of DNA and RNA, was evaluated by white blood cell and urine analyses in normal-to-overweight adults. Both interventions significantly reduced oxidative damage to both DNA and RNA in white blood cells compared to baseline. However, urinary levels of DNA and RNA oxidation products did not differ from baseline values following either 1-year intervention program. The conclusion of the study was that either CR or IEE by exercise reduce systemic oxidative stress which is reflected in a decreased DNA or RNA oxidative damage [85].

CALERIE is a research program initiated by the National Institute on aging that involves three research centers. The Phase 1 of CALERIE included three pilot studies to determine whether long-term (6–12 months) effects of 20–25% CR in free-living, nonobese humans could be investigated and to evaluate the adaptive responses to CR. The conclusions of this randomized, controlled, clinical trial were that CR subjects had a lower body weight, a decreased whole body and visceral fat, a reduced activity energy expenditure, improved fasting insulin levels, improvements in cardiovascular disease markers (LDL, total cholesterol to HDL ratio, and CRP), and no change in bone density compared to controls [76, 77, 83, 86, 92]. The objective of the ongoing CALERIE Phase 2 is to test if 2 years sustained 25% CR of *ad libitum* energy intake, results in beneficial effects that would be similar to those observed in animal studies [91].

Members of the Caloric Restriction Society (CRS) restrict food intake with the expectation that this would delay the disease processes responsible for secondary aging and to slow the primary aging process. Compared to age-matched individuals eating typical American diets, CRS members (average age 50 ± 10 yr) had a lower body mass index, a reduced body fat, significantly lower values for total serum cholesterol, LDL cholesterol, total cholesterol/LDL, and higher HDL cholesterol. Also fasting plasma insulin and glucose values were significantly lower than in the age-matched control group. Left ventricular diastolic function in CRS members was similar to that of about 16 years younger individuals. Chronic inflammation was reduced by CR and this was reflected in significantly lower levels of plasma CRP and tumor necrosis factor alpha (TNFα) [74, 78, 84].

Aging is associated with a progressive reduction in heart-rate-variability (HRV)—a measure of declining autonomic function—and also a worse health outcome. The effect of a 30% CR on heart autonomic function was assessed by 24-hour monitoring of HRV in adults on self-imposed CR for 3 to 15 years and compared with an age-matched control eating a Western diet. The CR group had a significantly lower heart rate and significantly higher values for HRV. Also, HRV in the CR individuals was comparable to published norms for healthy individuals 20 years younger. The authors suggest that CR reset the balance between the sympathetic/parasympathetic modulation of heart frequency in favor of the parasympathetic drive thus increasing the circadian variability of heart rate [93].

5. Cellular Mechanisms of Caloric Restriction

Most age-related changes in gene expression are less then two folds and are tissue specific [94]. Yet despite tissue-specific differences in the effect of age on gene transcription, the rate of aging across tissues appears to be coordinated, suggesting a role for systemic factors in coordinating the aging process at a whole body level [95]. The most common age-related changes include increased expression of genes involved in inflammation and immune responses, and reduced expression of genes involved in mitochondrial (MTH) energy metabolism and CR prevents the majority of these age-associated changes in gene expression [96, 97]. CR is suggested to counteract the age-associated changes by modulating the mTOR signaling pathway, IGF1/insulin signaling, adiponectin expression, DNA methylation, and histone acetylation and deacetylation.

5.1. Adiponectin Secretion in Caloric Restriction. A consistent change during CR is a reduction in body fat (i.e., a reduction

in white adipose tissue). White adipose tissue is not only a storage site for lipids but has an important role in blood glucose homeostasis, immune, and inflammatory responses that are mediated by adipocyte-derived, cell-to-cell, signaling molecules adipokines (e.g., adiponectin) [98, 99]. Therefore, adipose tissue could be an important factor for aging and CR-related metabolic changes.

The secretion of adiponectin is increased by reduced calorie intake (e.g., CR) and reduced by both insulin and IGF1 that decrease its synthesis. Cross-sectional studies demonstrate a consistent inverse relationship between plasma insulin and adiponectin concentrations. An increase in adipocyte size will also reduce the secretion of adiponectin [100]. Adiponectin promotes fatty acid oxidation in adipose tissue and reduces lipid accumulation in other peripheral tissues [101]; CR is associated with increased levels of adiponectin [102]. In humans, this hormone suppresses metabolic derangements that may lead to *type 2 diabetes, obesity, atherosclerosis*, or *metabolic syndrome* [103–105]. Adiponectin regulates mitochondrial energy production via AMPK. AMPK has many functions, it up-regulates cellular uptake of glucose, β-oxidation of fatty acids, expression of glucose transporter 4 (GLUT4), and mitochondrial energy production. The enzyme has an "energy-sensing capability" and responds to fluctuations in the intracellular AMP/ATP ratio. For example, adiponectin treatment of human myotubes leads to an AMPK-dependent increase in MTH biogenesis and reduces reactive oxygen species (ROS) production [106]. AMPK regulates MTH energy production by activating peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) directly, or through the endothelial nitric oxide synthase (eNOS) and NAD-dependent-deacetylase sirtuin1 (i.e., SIRT1 or silent mating type information regulation 2 homolog 1) signaling pathway. Increased AMPK activity during CR has also a cardioprotective effect, which is abolished in transgenic adiponectin antisense mice or in mice treated with an AMPK inhibitor [102]. Increased AMPK activity also stimulates eNOS activity thus reducing the chances of cerebral ischemic injury [107]. Additional cardioprotective effects that are mediated by increased secretion of adiponectin during CR are (a) inhibition of TNFα release and (b) inhibition of synthesis of adhesion molecules in endothelial cells. The latter suppresses the attachment of monocytes to the endothelial cells and delays the progression of atherosclerosis. Adiponectin modulated inflammatory responses are due to inhibiting the secretion of TNFα (a cytokine involved in systemic inflammation) from monocyte/macrophages and foam cells [108–110]; this may explain the reduced plasma concentration of inflammatory protein CRP in humans subjected to CR.

5.2. Insulin/IGF1 Signaling in Caloric Restriction. Insulin resistance is a well-known age-related metabolic change in mammals that can be reversed by CR [94]. CR has been reported to reduce IGF1 blood levels in mice but not in humans [111, 112]. Insulin and IGF1 inhibit FOXOs (an O subclass of the forkhead family of transcription factors) by a signaling pathway that includes insulin receptor substrate proteins (IRS), 3-phosphoinositide-dependent protein kinase-1 (PDPK1), and phosphatidylinositol 3-kinase (PTDINS-3 K), thus translocating FOXOs from the nucleus. FOXOs regulate the rate of aging in response to dietary cues and the disregulation of this pathway in mammals is associated with obesity and insulin resistance [113]. In a cell-type-specific manner, mammalian FOXO factors control various cellular functions including apoptosis, cell cycle, differentiation, and the expression of genes involved in DNA repair and oxidative stress resistance. These functions are assumed to be the basis for FOXO's ability to control life span [114]. The actions of insulin/IGF1 signaling pathway on FOXO1a are mimicked by black tea polyphenols [113] and polymorphisms in the FOXO3a gene were associated with longevity in humans [115]. CR stimulates SIRT1-mediated deacetylation of the FOXO3a, preventing nuclear FOXO3a activity and inhibiting Rho-associated protein kinase-1 expression thus activating nonamyloidogenic α-secretase processing of APP and lowering Aβ generation. This reduced Aβ generation is associated with the prevention of Alzheimer's disease type amyloid neuropathology and spatial memory deterioration in a mouse model [114]. The positive effect of CR on the insulin/IGF1 signaling pathway was also associated with a reduction in ROS production in MTH [116].

5.3. mTOR Signaling Pathway in Caloric Restriction. The regulation of life span by the mTOR signaling pathway is not completely understood. However, recent experimental work implies that it plays an important role in the cell's aging process [44]. Inhibition of mTOR with rapamycin expands maximal and median life span in mice. This effect was observed even when the treatment was initiated late in life, corresponding roughly to an age of 60 years in humans [44, 117]. The above mentioned, rapamycin-mediated life extension was not associated with change in disease patterns or causes of death suggesting that rapamycin increases life span by slowing-down age-related tissue and organ degeneration [44, 117]. mTORC1 inhibition could prevent tissue degeneration and extend life span by improving stem cell function. For example, reducing mTORC1 signaling with rapamycin restores hematopoietic stem cells self-renewal and hematopoietic function, improves immunity, and increases life span in mice [118]. S6 K1 and 4E-BP1 were suggested as effectors of the mTORC1 signaling pathway that regulates the aging process. As reviewed in Kapahi et al., reduced S6 K1 activity increases life span in various species including in mice [119] and overexpression of 4E-BP1 extends life span under rich nutrient conditions by enhancing mitochondrial activity in flies [120].

mTORC1 could also influence life span through mechanisms that are not associated with modulation of protein synthesis; for example, stimulation of autophagy, as a consequence of mTORC1 inhibition, could promote longevity by stimulating degradation of aberrant proteins and damaged organelles that are accumulating over time and impairing cellular homeostasis [44]. An example how disregulation of mTORC1 activity can affect life span is seen in the liver

of old mice with impaired fasting-induced ketogenesis and increased mTORC1 activity [121]. This impaired ketogenesis limits the supply of available energy substrates to the peripheral tissues thus reducing the organism's chances of survival during food deprivation.

The age-related decline in MTH function is counteracted by CR that increases the transcription of nuclear-encoded genes involved in the electron transport system [69]. The effects of CR on MTH could also be mediated by the mTOR signaling pathway since mTOR is necessary for the maintenance of mitochondrial oxidative function [122]. Two, S6 K1 and 4E-BP1 independent, mTOR/MTH signaling pathways have been suggested: the TORC1-YY1-PGC-1α complex [122] demonstrated in a mouse model or the TORC1-regulated complex of BCL-XL and VDAC1 located at the mitochondrial outer membrane in a T-cell leukemic cell model [123].

5.4. DNA Methylation in Caloric Restriction. The aging process is associated with a progressively reduced cell homeostasis and altered gene expression [124]. Aging causes a significant change in the distribution of 5-methylcytosine (the product of DNA methylation) across the genome and a decrease in global genome DNA methylation [124–130]. However, the promoter regions of some specific genes tend to switch from unmethylated to methylated status, leading to gene silencing (e.g., promoters of tumor or aging-related genes, such as *RUNX3* and *TIG1* [129, 131]). In summary, the aging process is associated with globally decreased but locally increased DNA methylation [132]. CR is assumed to delay the aging process by reversing aging-related DNA methylation changes thus increasing genomic stability [133, 134]. For example, CR increased the methylation level of proto-oncogene *RAS* in a rat model when compared to *ad libitum* fed animals [135]. A hypermethylated gene promoter is often recognized by transcriptional repressor complexes, thus leading to silencing the expression of these oncogenes, which contributes to the cancer prevention effects of CR [132]. In an *in vitro* human cell model of CR, the E2F-1 binding site in the promoter of the $p16^{INK4\alpha}$ gene (a tumor suppressor and aging-associated gene) was hypermethylated. This DNA hypermethylation blocked access of E2F-1 (an active transcription factor of $p16^{INK4\alpha}$) to the $p16^{INK4\alpha}$ promoter, resulting in $p16^{INK4\alpha}$ downregulation, thus contributing to the CR induced life span extension [136].

Obesity is an important metabolic disorder in humans that is closely associated with recognized causes of accelerated aging and increased mortality such as diabetes, hypertension or cancer [137]. Therefore, the antiageing effects of CR should have an impact on the progression of obesity and are used in clinical weight control interventions [138]. The practice of CR by obese humans revealed that hypocaloric diets cause DNA methylation changes in specific loci *ATP10A*, *WT1*, and *TNF-α*, which could be used as early indicators of a response to CR [139–141]. Further CR studies in humans are necessary to characterize the pool of DNA methylation-controlled candidate genes that could be closely correlated with metabolic pathways [132].

5.5. Posttranslational Modification of Histones in Caloric Restriction

5.5.1. Histone Acetylation/Deacetylation. Histone modifications are associated with gene activation or gene repression. The combination of modifications within histone tails directly changes nucleosomes configuration switching chromatin to either a compacted (tight-close) or a relaxed configuration (loose-open) [142]. Therefore, histone modifications determine the (tight-close: loose-open) ratio of chromatin and thus the degree of gene activity within a certain DNA region. For example, a deacetylated histone lysine residue has the positive charge, which attracts the negatively charged DNA strands producing a compact chromatin state that is associated with transcriptional repression. Alternatively, histone acetylation removes the positive charge and results in an open chromatin structure, which promotes gene transcription [132]. HDAC activity is increased during CR, therefore, global deacetylation may be a protective mechanism against nutrition stress and may influence the aging processes [136]. For example, enhanced activity of HDAC1 on the promoter regions of the $p16^{INK4\alpha}$ and human telomerase reverse transcriptase (*hTERT*) genes, the former a tumor suppressor in many human cancers and the latter a key regulator of telomerase activity modified by aging regulation, leads to beneficial expression changes of these two genes and contributes to longevity under CR conditions [136, 143, 144].

Several HDAC families have been identified, including class III NAD^+-dependent HDACs like Sirtuin1. Sirtuin1 (SIRT1 in mammals), and its orthologs in other species (e.g., Sirtuin2 in yeast) are important for aging regulation and CR-related lifespan extension [145–149]. The enzymatic activity of SIRT1 depends on NAD^+/NADH ratio, a key indicator for oxygen consumption, thus suggesting that this protein is responsive to the metabolic state of cells. The role of SIRT1 in mediating CR and lifespan extension is supported by several animal models, human subjects, and *in vitro* CR cellular systems [136, 145, 146, 148–154]. CR induces SIRT1 expression in several tissues of mice or rats [146]. SIRT1 is assumed to mediate CR-induced metabolic alterations and subsequent aging retardation by (a) increasing stress resistance by negative regulation of p53 and FOXO [155–159] and (b) by initiating a series of endocrine responses, including inhibition of adipogenesis and insulin secretion in pancreatic β cells by regulation of key metabolism-associated genes such as peroxisome proliferator-activated receptor G coactivator 1α (PGC-1α) [160, 161]. Although SIRT1 is classified as an HDAC, it also deacetylates nonhistone substrates [146, 152] including key transcription factors (e.g., FOXO), regulatory proteins (e.g., p53, $p16^{INK4\alpha}$), and DNA repair proteins (e.g., Ku70) that are involved in lifespan extension by CR. For example, downregulation of p53 by SIRT1 deacetylation may affect lifespan by inhibiting cellular apoptosis and replicative senescence processes [155–157, 162–164]. FOXO protein can be directly deacetylated by SIRT1 at lysine residues and its expression is reduced, thereby repressing FOXO-mediated apoptosis [158, 159]. The DNA repair protein, Ku70, can become deacetylated by SIRT1, allowing it to inactivate

the proapoptotic factor BAX, thus inhibiting apoptosis [165, 166]. $p16^{INK4\alpha}$ is a cyclin-dependent kinase inhibitor, an important tumor suppressor protein and a potential aging biomarker since it is significantly accumulated during the aging processes [167–171]. CR-activated SIRT1 can directly bind to the $p16^{INK4\alpha}$ promoter and decrease its expression through a deacetylation effect, which contributes to delaying the aging process and to life span extension in human cells *in vitro* [153]. As stated previously, SIRT1 also regulates the expression of genes that are involved in metabolic pathways. PGC-1α is a key regulator of gluconeogenesis and fatty acid oxidation [160, 161] and is upregulated during CR by SIRT1-mediated deacetylation, which increases its ability to coactivate HNF4α, a transcription factor that promotes the expression of gluconeogenic genes and represses genes involved in glycolysis [147, 152]. In summary, SIRT1 plays an important role in the cross-talk between epigenetic and genetic pathways [132].

5.5.2. Histone Methylation. In contrast to histone acetylation, associated with open chromatin status and subsequent gene activation, differentially methylated forms of histones show unique association patterns with specific proteins that recognize these markers and thus lead to gene silencing or activation [132]. Histone lysine residues can be mono-, di-or, tri-methylated, leading to either activation or repression depending upon the particular lysine residue that is modified [172, 173]. For example, CR elicited histone methylation modifications such as di- or tri-methylated histone H3 at lysine residue 3 or 4 regulate expression of key aging-related genes, $p16^{INK4\alpha}$ and *hTERT*, thereby contributing to CR-induced lifespan extension of human cells [136, 153].

5.6. miRNA Expression in Caloric Restriction. miRNA expression patterns change with age; some miRNAs are downregulated and some are upregulated. Expression profile analysis of 800 miRNAs in human peripheral blood mononuclear cells revealed that the majority of miRNAs decreased in quantity including miRNAs involved in cancer development [174]. Since human tumors are often associated with a general downregulation of miRNAs, the reported age-related global decrease in miRNA could increase the frequency of cellular transformation and tumor genesis thus reducing life span. The decrease of these latter miRNAs with advanced age was also associated with an increased expression of target proteins phosphatidylinositol 3-kinase, stem cell factor receptor (c-KIT) and histone H2A [174]. Animal studies also support the role of miRNAs in aging. For example compared to wild-type controls, *C. elegans mutants* with deletion of miRNA-239 have a significantly prolonged lifespan and *C. elegans* mutants with deletions of miRNA-71, miRNA-238, and miRNA-246 have a significantly reduced lifespan [175]. The longevity of Ames dwarf mouse—attributed to their increased insulin sensitivity, increased stress resistance and reduced tumor frequency as a result of reduced IGF-1 activity—was associated with liver miRNA-27a suppression of regulatory proteins ornithine decarboxylase and spermidine synthase that occurred sooner in postnatal life that in wild-type mice [176].

CR changes miRNA expression profile. In mouse breast tissue, of animals restricted to 70% of normal diet for 6 months, CR increased the expression of miR-203 that targets caveolin-1 and p63 important factors affecting growth and invasive potential of cancer cells [177]. The authors concluded that CR could reduce the incidence, progression and metastasis of breast cancer thus contributing to an increased life span [177]. The brain of CR mice—after an 8-month reduction of calories to 60% of normal *ad libitum* intake—shows a decreased expression of miRNA-181a, miRNA-30e, and miRNA-34a with a concomitant increase in BCL2 expression and a concomitant decrease in BAX expression with reduced activities of Caspases 9 and -3. Decreased activities of Caspases 9 and 3 are associated with a reduced rate of apoptosis [178]. BAX and Caspase 3 activity is increased in Alzheimer's and Parkinson's disease; therefore, the progress of these common neurodegenerative diseases could be delayed by CR thus prolonging life span [179–183].

6. Mimetics of Caloric Restriction

Since long-term CR is necessary to produce beneficial effects on health and longevity observed in experimental conditions, alternatives have been investigated that could produce the positive effects of CR without food restriction. An ideal calorie restriction mimetic (CRM) should (a) elicit metabolic, hormonal and physiological effects similar to CR, (b) not require a significant reduction in long-term food intake, (c) activate stress response pathways similar to CR and (d) extend life span and reduce or delay the onset of age-related diseases [184]. To speedup the search for a candidate CRM the National Institute on Aging established the Interventions Testing Program as a multi-institutional program to test substances predicted to "extend lifespan and delay disease and dysfunction" [185–189].

6.1. Caloric Restriction Combined with Exercise. Male rats are the favorite animal model to study whether exercise in combination with CR (i.e., CE) potentiates the health-promoting benefits caused by CR alone, because these animals do not increase their caloric intake to compensate for their exercise-induced caloric expenditure [180]. Some studies concluded that CE does not have health-promoting benefits beyond those elicited by CR [111, 190–192]; there was no significant change in oxidative stress levels or pro-inflammatory protein levels in exercised animals fed an 80% CR diet [191, 192] and no effect on the animal's maximal life span [190]. On the other hand, CE reduced CRP levels to a greater extent than CR by itself [193] and reduced the chances of developing both myocardial necrosis and myocardial ischemia [194, 195].

Several human CE studies investigated the effect of a 25% total caloric reduction with 12.5% coming from exercise induced expenditure and another 12.5% coming from CR. The majority of them found no significant difference between CR and CE in respect to fasting insulin levels, DNA damage, muscle mitochondrial gene expression, triglyceride levels, and liver lipid content [76, 196–198]. The exceptions are two studies that reported a further reduction in both

diastolic blood pressure and LDL cholesterol when CR with exercise was compared to CR alone [198, 199]. Also, CE has been shown to increase bone mineral density at the femoral neck and reduce sTNFR1, an inflammatory biomarker, in overweight postmenopausal women [200]. The main advantage of combining CR with exercise over CR alone is that it may be easier for an individual to comply with a CE regimen where the total energy (i.e., caloric reduction) is divided between exercise-induced expenditure and calorie restriction [201].

6.2. Dietary Restriction. Dietary restriction (DR) refers to the modification of the quantity ratio between protein, fat, and carbohydrates with or without reducing the total intake of calories. Neither carbohydrate restriction nor lipid restriction are effective alternatives to CR and both failed to decrease reactive oxygen species production or oxidative DNA damage [202–208]. In an animal model, protein DR seems to be an alternative to CR. Protein DR was reported to increase the maximum lifespan in rodents by 20% [206]. The life-extending benefits of protein DR were attributed to a methionine restriction in diet [209–215]. For example, a 40% methionine restriction has been reported to decrease both mitochondrial reactive oxygen species generation and oxidative damage in mitochondrial DNA [216, 217]. Evidence that supports the link between methionine restriction and increased life span includes (a) inverse relationship between methionine content and maximum life span in mammals [218], (b) methionine supplementation increases LDL cholesterol oxidation [219] and (c) increased methionine intake increases plasma homocysteine concentrations, thus increasing the risk of cardiovascular disease and mortality [219]. Also, it has been demonstrated that a 40% restriction of all dietary amino acids except methionine failed to reduce both mitochondrial reactive oxygen species generation and oxidative damage in mitochondrial DNA [220]. In summary, animal experiments suggest that about half of the life extension effect of CR can be attributed to methionine restriction [206]. Therefore, further work in humans is justifiable since methionine DR is feasible and tolerable [221].

6.3. Alternate Day Fasting. Alternate day fasting (ADF) alternates 24-hour periods of *ad libitum* intake with partial or complete restriction of calorie intake. Therefore, ADF does not necessarily reduce overall caloric consumption or bodyweight, since subjects may compensate for the reduced caloric intake during fasting periods by overeating in the *ad libitum* intake period [222, 223]. ADF extended lifespan in animal trials [223–225]. Some authors attributed the increased life span during ADF to the concomitant increase in brain-derived neurotrophic factor [215]. ADF also attenuated or prevented the development of age-related disease processes, including cardiovascular disease, kidney disease, cancers, and diabetes [222, 223, 225–230]. Human trials have established the feasibility of ADF in humans [231]. The preliminary results of ADF-human trials [231–233] cannot be compared to CR-human trials since the ADF-trial periods ware relatively short (from a few days to 20 weeks) compared to the CR-trial periods (6 months to 6 years) [74, 83, 85]. However, even during such brief trial periods, some potentially beneficial effects were noted: a decrease in fasting insulin with no difference in fasting glucose [231] and an improved bronchial responsiveness to medication [233]. It has been reported that peripheral blood mononuclear cells of normal weight middle-age male and female subjects on a 2-month long ADF responded with a reduced capability to produce cytokines upon stimulation [234]. To date, there are no reports in regard to changes in biomarkers specific to blood lipids and oxidative stress in ADF subjects.

6.4. Resveratrol. Resveratrol (RSV), a plant-derived polyphenol in the skins of red grapes, is the most studied caloric restriction mimetic. RSV is reported to activate Sir2 (SIRT1 homolog) [235], thus mimicking the benefits of CR—without restricting calorie intake—such as increasing lifespan in yeast, worms, flies, and fish [235–238]. Recently, the assumption that activation of Sir2 by direct binding with RSV is responsible for extended lifespan has been challenged in experiments in multiple organisms [239–248]. For example, RSV is known to produce a wide array of effects in mammalian cells, including activation of AMP-activated protein kinase (AMPK) that is involved in some of the same pathways as SIRT1 and directly phosphorylates PGC-1α. [249, 250]. SIRT1 can activate the kinase upstream of AMPK, but this pathway does not appear to be necessary for AMPK stimulation by RSV [251]. Recently, it was reported that SIRT1 is essential for moderate doses of resveratrol to stimulate AMPK and improve mitochondrial function *in vitro* and *in vivo* [252].

Although the mechanism of RSV-mediated CR-like effects are not fully understood, it appears that RSV treatment produces a transcriptional response similar to CR [253], and in the presence of a high-fat diet, both health and longevity benefits have been reported with RSV use in a mouse model [249]. The beneficial effects of RSV use in obese mice were increased insulin sensitivity, improved motor coordination, and decreased incidence of cataracts [253, 254]. There was no significant life span increase in adult mice when RSV was added to a normal diet [254, 255]. This finding implies that RSV is not a true CRM [256]. A one-year treatment with RSV increased resting metabolic rate and total daily energy expenditure in nonhuman primate with any adverse health effects, implying that long-term use of RSV is effective and safe [257, 258]. CR, in the same animal model and experimental protocol, reduced total daily energy expenditure but did not change resting metabolic rate [258].

There have been only a few studies on RSV effects in humans, however the results are encouraging. The use of 0.1 mM RVS in cultures of human mesenchymal stem cells promotes cell regeneration by inhibiting cellular senescence; at higher concentrations (5 mM or more) RSV inhibits cell regeneration by increasing senescence rate, cell doubling time, and S-phase cell cycle arrest [259]. In human peritoneal mesothelial cells RSV delays replicative senescence by mobilization of antioxidative and DNA repair mechanisms

as measured by increased expression of proliferating cell nuclear antigen, augmented fraction of cells in the S phase of the cell cycle, increased number of divisions, diminished expression and activity of senescence-associated β-galactosidase, upregulated biogenesis of mitochondria, increased activity of superoxide dismutase and reduced DNA damage [260]. RSV and other polyphenols have low bioavailability in humans. However, RSV and its metabolites do accumulate within human cells *in vivo* in a tissue-specific and dose-dependent manner [261]. A six-week supplementation regime with RSV suppressed the binding of nuclear factor kappa B (NF-κB), decreased ROS generation, and decreased the levels of TNFα and interleukin-6 (IL-6) in mononuclear cells. The plasma levels of TNFα and CRP were significantly reduced as well. There were no significant changes in fasting plasma concentrations of cholesterol (total, LDL and HDL), triglycerides, or leptin in RSV-treated group compared to the control group of healthy individuals receiving placebo [262]. A high-fat, high-carbohydrate diet induces inflammation and oxidative stress [263]. Healthy humans on a high-fat, high-carbohydrate meal, taking a single-dose supplement of RSV and other grape polyphenols, had a significantly increased messenger RNA (mRNA) expression of the *NAD(P)H dehydrogenase [quinone] 1* and *glutathione S-transferase-p1* genes—implying a strong anti-oxidant effect. The single-dose RSV supplement also attenuated the meal-induced increase of plasma endotoxin and lipoprotein binding protein concentrations and attenuated the expression of $p47^{phox}$, TLR-4, CD14, SOCS-3, IL-1β, and KEAP-1 [264]. Therefore, RSV reduces the oxidative and inflammatory responses of a high-fat, high-carbohydrate meal, and it may reduce the risk of atherosclerosis and diabetes [261]. Preliminary results suggest that RSV also improves the glucose tolerance and insulin sensitivity [265]. The improved insulin sensitivity was attributed to decreased oxidative stress [265].

The causal association between red wine and grape juice consumption and the reduction of risk factors for cardiovascular disease (reduced blood flow, increased oxidative stress and inflammation) is well known [266–269]. RSV upregulates eNOS, thus promoting nitric oxide mediated vasodilatation and increased blood flow [270–272]. Also, RSV attenuates hemostasis-related activation of human platelets [273]. Increased arterial blood flow, after a single bolus of RSV, was measured in the brain and arm [274, 275]. However, increased brain blood flow after RSV treatment was not associated with an enhanced cognitive function [274].

Improved insulin resistance, arterial blood flow, and decreased oxidative stress and inflammation are associated with short-term use of RSV but there are no human data on the long-term health benefits [261]. In summary, further research is needed to clarify the biochemical pathways of RSV mediated effects and to establish its long-term effects in humans [276].

6.5. Rapamycin. Rapamycin (RAP) is an antibiotic and inhibitor of TOR (target of rapamycin) signaling in cells, with known immunosuppressive and antiproliferative effects [277]. TOR is a mediator of nutrient signaling in cells and is proposed to play a role in aging and the CR response (see Section 6.3). When RAP was administered to mice at about 20 months of age there was a significant, about 10% increase in mean life span extension in male and female mice. Since there were no significant changes in the organ pathology in the RAP feed mice, compared to control, the authors suggested that the longevity benefits of RAP could be at least partially mediated by biochemical pathways independent of the CR response [117]. The existence of multiple, RAP activated life-extension biochemical pathways were also suggested in flies. RAP feed adult drosophila had an increased life span. The suggested mechanism for this RAP increased longevity was by the TORC1 branch of the TOR pathway, with alterations to autophagy and translation. However, RAP could increase life span of weak insulin/IGF1 signaling pathway mutants and of flies with life span maximized by CR, suggesting additional mechanisms for life span extension [278]. Lifelong administration of rapamycin, administrated intermittently 2 weeks per month, extended lifespan in normal inbred female mice. Significantly, rapamycin inhibited age-related weight gain, decreased aging rate, increased life span and delayed spontaneous cancer [279]. Adult mice treated with rapamycin, starting at 2 months of age, perform significantly better on a task measuring spatial learning and memory compared to age-matched mice on the control diet. However, rapamycin did not improve cognition in adult mice with pre-existing, age-dependent learning and memory deficits. The rapamycin-mediated improvement in learning and memory was associated with a decrease in IL-1β levels and an increase in NMDA signaling. [280]. Since rapamycin is used as an immunosuppressive, its relevance for longevity in humans has yet to be established [117].

7. Diet and the Aging Population

An important demographic tendency in the developed world is a progressive increase in the percentage of the population over 65 years of age and a simultaneous decline in the percentage of the working age population. The health implications of these trends are a shift from acute to chronic and age related illnesses (e.g., Alzheimer's disease, osteoporosis, cardiovascular diseases, and cancer), increasing heath costs and an increasing economic burden to the society and to the individual [281–283]. Therefore, any dietary intervention that has the potential of delaying the progression of chronic and age-related illnesses could have a significant impact not only on the individual's quality of life but also on the society's ability to deal with the health and economic implications of an aging population. There is a body of data suggesting that CR significantly reduces the rate of age-related changes in humans [73–93]. However, there is no data that CR promotes longevity in humans. Studies of people with exceptional longevity suggest that a family history of longevity and of a low prevalence of age-related diseases enables a significantly prolonged life span even when the subjects were obese, smoked or did not exercise regularly. Therefore, exceptional longevity in humans could be more dependent on genetics than lifestyle [284–286].

8. Conclusions

Caloric restriction or calorie restriction mimetics elicit coordinated adaptive stress responses at the cellular and whole-organism level by modulating the signaling pathways of adiponectin, insulin/IGF1, AMPK, mTOR, FOXO, p53, and sirtuins [287]. Sirtuins could play an important role in the cross-talk between epigenetic and genetic pathways [132]. The activation of these adaptive stress responses may prevent the initiation of apoptosis by the intrinsic pathway [288]. Furthermore, it may stimulate autophagy to provide substrates for energy production and for the anabolic processes involved in cellular regeneration and synthesis of antioxidants and heat-shock proteins [287]. A large body of experimental evidence proves that the overall effect of these adaptive stress responses is an increased resistance to subsequent stress, thus delaying age related changes and promoting longevity. Therefore, CR, alone or in combination with caloric restriction mimetics, could improve the quality of life of the aging population.

Abbreviations

4E-BP1: Eukaryotic translation initiation factor 4E binding protein 1
ADF: Alternate day fasting
AGC: Acronym of the protein kinase A, G, and C families
AKT: Serine-threonine-specific proteinkinase also known as protein kinase B (PKB)
AMP: Adenosine monophosphate
AMPK: 5′ adenosine monophosphate-activated protein kinase
ATP: Adenosine-5′-triphosphate
ATP10A: Probable phospholipid-transporting ATPase VA also known as ATPase class V type 10A or aminophospholipid translocase VA gene
Aβ: Amyloid beta
B12 vitamin: Cobalamin
BAX: Bcl-2 associated X protein
BCL-XL: B-cell lymphoma-extra large, a transmembrane mitochondrial protein
CALERIE: Comprehensive Assessment of Long-Term Effects of Reducing Calorie Intake
CD14: Cluster of differentiation 14 protein also known as CD14 protein
CE: Exercise in combination with CR
CHD: Coronary heart disease
CpG dinucleotide: Cytosine-phosphate-guanine dinucleotide
CR: Caloric restriction or calorie restriction diet
CRM: Calorie restriction mimetic
CRP: C-reactive protein
CRS: Caloric Restriction Society
DNA: Deoxyribonucleic acid
DNMT: DNA methyltransferase
DR: Dietary restriction
E2F-1: Transcription factor E2F1 protein
EGCG: Epigallocatechin-3-gallate
eNOS: Endothelial nitric oxide synthase
FOXO: O subclass of the forkhead family of transcription factors; known FOXO family members are FOXO1, FOXO3, FOXO4 and FOXO6
GLUT4: Glucose transporter 4
GTP: Guanosine-5′-triphosphate
GTPase: Enzyme that hydrolyses GTP
HAT(s): Histone acetlytransferase(s)
HDAC(s): Histone deacetylase(s)
HDAC(s)s: Histone deacetylase(s)
HDL: High-density lipoprotein
HDM(s): Histone demethylase(s)
hmdC: 5-hyd0072oxymethyl-2′-deoxycytidine
HNF4α: Hepatocyte nuclear factor 4 α also known as nuclear receptor subfamily 2, group A, member 1
HMT(s): Histone methyltransferase(s)
HNF4α: Hepatocyte nuclear factor 4α
HRV: Heart-rate-variability
hTERT: Gene encoding human telomerase reverse transcriptase a catalytic subunit of the enzymetelomerase
IEE: Increased energy expenditure
IGF1: Insulin-like growth factor 1 also known as somatomedin C
IL-1β: Human interleukin 1β
c-KIT: Proto-oncogene c-Kit also known as mast/stem cell growth factor receptor, also known as tyrosine-protein kinase Kit or CD117
IRS: Insulin receptor substrate
KEAP-1: Kelch-like ECH-associated protein 1
Ku70: Protein encoded in humans by the gene *XRCC*6
LBK1: Tumor suppressor kinase enzyme that activates AMPK
LDL: Low-density lipoprotein
miRNA(s): microRNA(s)
mRNA: Messenger RNA
mSin1: Mammalian stress-activated protein kinase-interacting protein
MTH: Mitochondrion, mitochondrial
mTOR: Mammalian target of rapamycin
mTORC1: Mammalian target of rapamycin complex 1
mTORC2: Mammalian target of rapamycin complex 2
NAD^+: Nicotinamide adenine dinucleotide
NADH: NADH dehydrogenase
NF-κB: nuclear factor kappa B
NIP7: 60S ribosome subunit biogenesis protein NIP7 homolog
NMDA: N-Methyl-D-aspartic acid or N-Methyl-D-aspartate

$p16^{INK4a}$: Gene encoding the tumor suppressor protein cyclin-dependent kinase inhibitor 2A or CDKN2A or multiple tumor suppressor 1 (MTS-1)
PDPK1: 3-phosphoinositide-dependent protein kinase-1
PGC1-α: Peroxisome proliferator-activated receptor G co-activator 1α
p53: Tumor suppressor protein p53 also known as tumor protein 53
p47phox: Subunit of NADPH oxidase, that has to be phosphorilated for the activation of NADPH oxidase
PKA: Protein kinase A
PKC: Protein kinase C
PKG: Protein kinase G, or cGMP-dependent protein kinase
PtdIns-3K: Phosphatidylinositol 3-kinase
RAP: Rapamycin
RAPTOR: Regulatory-associated protein of mTOR
RHEB: RAS homolog enriched in brain protein, binds GTP
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RSV: Resveratrol
RAS: Protein superfamily of small GTPases
RTG1: Retrograde regulation protein 1
RUNX3: Gene encoding runt-related transcription factor 3
S6 K1: Ribosomal protein S6 kinase β-1
SGK1: Serum-and glucocorticoid-regulated kinase; a serine/threonine protein kinase
SIRT1: NAD-dependent-deacetylase sirtuin1 also known as silent mating type information regulation 2 homolog 1
SOCS-3: Suppressor of cytokine signaling 3
sTNRF1: Soluble tumor necrosis factor receptor 1
TLR-4: Toll-like receptor 4
TNFα: Tumor necrosis factor α
TOR: Target of rapamycin
TSC1: Tuberous sclerosis protein 1 also known as hamartin
TSC2: Tuberous sclerosis protein 2 also known as tuberin
VDAC1: Voltage-dependent anion-selective channel protein 1
TIG1: Tazarotene-induced gene-1
WT1: Gene encoding Wilms tumor protein
YY1: Transcriptional repressor protein YY1.

References

[1] J. A. Mckay and J. C. Mathers, "Diet induced epigenetic changes and their implications for health," *Acta Physiologica*, vol. 202, no. 2, pp. 103–118, 2011.

[2] "Diet, nutrition and the prevention of chronic diseases," *World Health Organization Technical Report Series*, vol. 916, no. 1–8, pp. 1–149, 2003.

[3] D. J. P. Barker and C. Osmond, "Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales," *The Lancet*, vol. 1, no. 8489, pp. 1077–1081, 1986.

[4] O. A. Kensara, S. A. Wootton, D. I. Phillips, M. Patel, A. A. Jackson, and M. Elia, "Fetal programming of body composition: relation between birth weight and body composition measured with dual-energy X-ray absorptiometry and anthropometric methods in older Englishmen," *The American Journal of Clinical Nutrition*, vol. 82, no. 5, pp. 980–987, 2005.

[5] C. Osmond, D. J. P. Barker, P. D. Winter, C. H. D. Fall, and S. J. Simmonds, "Early growth and death from cardiovascular disease in women," *British Medical Journal*, vol. 307, no. 6918, pp. 1519–1524, 1993.

[6] C. N. Hales and D. J. P. Barker, "Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis," *Diabetologia*, vol. 35, no. 7, pp. 595–601, 1992.

[7] C. Cooper, C. Fall, P. Egger, R. Hobbs, R. Eastell, and D. Barker, "Growth in infancy and bone mass in later life," *Annals of the Rheumatic Diseases*, vol. 56, no. 1, pp. 17–21, 1997.

[8] S. E. Ozanne and C. N. Hales, "Lifespan: catch-up growth and obesity in male mice," *Nature*, vol. 427, no. 6973, pp. 411–412, 2004.

[9] V. M. Vehaskari, "Prenatal programming of kidney disease," *Current Opinion in Pediatrics*, vol. 22, no. 2, pp. 176–182, 2010.

[10] A. Gabory, L. Attig, and C. Junien, "Sexual dimorphism in environmental epigenetic programming," *Molecular and Cellular Endocrinology*, vol. 304, no. 1-2, pp. 8–18, 2009.

[11] A. Bird, "DNA methylation patterns and epigenetic memory," *Genes & Development*, vol. 16, no. 1, pp. 6–21, 2002.

[12] H. Wu, J. Tao, and Y. E. Sun, "Regulation and function of mammalian DNA methylation patterns: a genomic perspective," *Briefings in Functional Genomics*, vol. 11, no. 3, pp. 240–250, 2012.

[13] X. Zou, W. Ma, I. A. Solov'yov, C. Chipot, and K. Schulten, "Recognition of methylated DNA through methyl-CpG binding domain proteins," *Nucleic Acids Research*, vol. 40, no. 6, pp. 2747–2758, 2012.

[14] K. S. Crider, T. P. Yang, R. J. Berry, and L. B. Bailey, "Folate and DNA methylation: a review of molecular mechanisms and the evidence for folate's role," *Advances in Nutrition*, vol. 3, no. 1, pp. 21–38, 2012.

[15] T. A. Rauch, X. Zhong, X. Wu et al., "High-resolution mapping of DNA hypermethylation and hypomethylation in lung cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 1, pp. 252–257, 2008.

[16] M. Ehrlich, "DNA hypomethylation in cancer cells," *Epigenomics*, vol. 1, pp. 239–259, 2009.

[17] C. D. Davis, E. O. Uthus, and J. W. Finley, "Dietary selenium and arsenic affect DNA methylation in vitro in Caco-2 cells and in vivo in rat liver and colon," *The Journal of Nutrition*, vol. 130, no. 12, pp. 2903–2909, 2000.

[18] H. Zeng, L. Yan, W. H. Cheng, and E. O. Uthus, "Dietary selenomethionine increases exon-specific DNA methylation of the p53 gene in rat liver and colon mucosa," *The Journal of Nutrition*, vol. 141, no. 8, pp. 1464–1468, 2011.

[19] E. M. E. Van Straten, V. W. Bloks, N. C. A. Huijkman et al., "The liver X-receptor gene promoter is hypermethylated in a mouse model of prenatal protein restriction," *American Journal of Physiology*, vol. 298, no. 2, pp. R275–R282, 2010.

[20] A. J. Bannister and T. Kouzarides, "Regulation of chromatin by histone modifications," *Cell Research*, vol. 21, no. 3, pp. 381–395, 2011.

[21] A. Link, F. Balaguer, and A. Goel, "Cancer chemoprevention by dietary polyphenols: promising role for epigenetics," *Biochemical Pharmacology*, vol. 80, no. 12, pp. 1771–1792, 2010.

[22] X. Cheng and R. M. Blumenthal, "Coordinated chromatin control: structural and functional linkage of DNA and histone methylation," *Biochemistry*, vol. 49, no. 14, pp. 2999–3008, 2010.

[23] Y. Tan, B. Zhang, T. Wu et al., "Transcriptional inhibiton of Hoxd4 expression by miRNA-10a in human breast cancer cells," *BMC Molecular Biology*, vol. 10, article 12, 2009.

[24] P. G. Hawkins and K. V. Morris, "RNA and transcriptional modulation of gene expression," *Cell Cycle*, vol. 7, no. 5, pp. 602–607, 2008.

[25] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell*, vol. 136, no. 2, pp. 215–233, 2009.

[26] B. Kusenda, M. Mraz, J. Mayer, and S. Pospisilova, "MicroRNA biogenesis, functionality and cancer relevance," *Biomedical papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia*, vol. 150, no. 2, pp. 205–215, 2006.

[27] I. Bentwich, A. Avniel, Y. Karov et al., "Identification of hundreds of conserved and nonconserved human microRNAs," *Nature Genetics*, vol. 37, no. 7, pp. 766–770, 2005.

[28] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.

[29] R. C. Friedman, K. K. H. Farh, C. B. Burge, and D. P. Bartel, "Most mammalian mRNAs are conserved targets of microRNAs," *Genome Research*, vol. 19, no. 1, pp. 92–105, 2009.

[30] L. P. Lim, N. C. Lau, E. G. Weinstein et al., "The microRNAs of *Caenorhabditis elegans*," *Genes & Development*, vol. 17, no. 8, pp. 991–1008, 2003.

[31] A. Esquela-Kerscher and F. J. Slack, "Oncomirs—microRNAs with a role in cancer," *Nature Reviews Cancer*, vol. 6, no. 4, pp. 259–269, 2006.

[32] J. C. Mathers, G. Strathdee, and C. L. Relton, "Induction of epigenetic alterations by dietary and other environmental factors," *Advances in Genetics*, vol. 71, pp. 4–39, 2010.

[33] D. Milenkovic, C. Deval, E. Gouranton et al., "Modulation of miRNA expression by dietary polyphenols in apoE deficient mice: a new mechanism of the action of polyphenols," *PLoS One*, vol. 7, no. 1, Article ID e29837, 2012.

[34] Q. Sun, R. Cong, H. Yan et al., "Genistein inhibits growth of human uveal melanoma cells and affects microRNA-27a and target gene expression," *Oncology Reports*, vol. 22, no. 3, pp. 563–567, 2009.

[35] M. Sun, Z. Estrov, Y. Ji, K. R. Coombes, D. H. Harris, and R. Kurzrock, "Curcumin (diferuloylmethane) alters the expression profiles of microRNAs in human pancreatic cancer cells," *Molecular Cancer Therapeutics*, vol. 7, no. 3, pp. 464–473, 2008.

[36] J. Yang, Y. Cao, J. Sun, and Y. Zhang, "Curcumin reduces the expression of Bcl-2 by upregulating miR-15a and miR-16 in MCF-7 cells," *Medical Oncology*, vol. 27, no. 4, pp. 1114–1118, 2010.

[37] S. Careccia, S. Mainardi, A. Pelosi et al., "A restricted signature of miRNAs distinguishes APL blasts from normal promyelocytes," *Oncogene*, vol. 28, no. 45, pp. 4034–4040, 2009.

[38] F. U. Weiss, I. J. Marques, J. M. Woltering et al., "Retinoic acid receptor antagonists inhibit miR-10a expression and block metastatic behavior of pancreatic cancer," *Gastroenterology*, vol. 137, no. 6, pp. 2136–2145, 2009.

[39] L. A. Davidson, N. Wang, M. S. Shah, J. R. Lupton, I. Ivanov, and R. S. Chapkin, "n-3 Polyunsaturated fatty acids modulate carcinogen-directed non-coding microRNA signatures in rat colon," *Carcinogenesis*, vol. 30, no. 12, pp. 2077–2084, 2009.

[40] T. Melkamu, X. Zhang, J. Tan, Y. Zeng, and F. Kassie, "Alteration of microRNA expression in vinyl carbamate-induced mouse lung tumors and modulation by the chemopreventive agent indole-3-carbinol," *Carcinogenesis*, vol. 31, no. 2, Article ID bgp208, pp. 252–258, 2010.

[41] C. J. Marsit, K. Eddy, and K. T. Kelsey, "MicroRNA responses to cellular stress," *Cancer Research*, vol. 66, no. 22, pp. 10843–10848, 2006.

[42] H. Kutay, S. Bai, J. Datta et al., "Downregulation of miR-122 in the rodent and human hepatocellular carcinomas," *Journal of Cellular Biochemistry*, vol. 99, no. 3, pp. 671–678, 2006.

[43] R. Loewith and M. N. Hall, "Target of rapamycin (TOR) in nutrient signaling and growth control," *Genetics*, vol. 189, no. 4, pp. 1177–1201, 2011.

[44] M. Laplante and D. M. Sabatini, "mTOR signaling in growth control and disease," *Cell*, vol. 149, no. 2, pp. 274–293, 2012.

[45] W. L. Yen and D. J. Klionsky, "How to live long and prosper: autophagy, mitochondria, and aging," *Physiology*, vol. 23, no. 5, pp. 248–262, 2008.

[46] L. R. Pearce, D. Komander, and D. R. Alessi, "The nuts and bolts of AGC protein kinases," *Nature Reviews*, vol. 11, no. 1, pp. 9–22, 2010.

[47] Y. Sancak, C. C. Thoreen, T. R. Peterson et al., "PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase," *Molecular Cell*, vol. 25, no. 6, pp. 903–915, 2007.

[48] C. A. Easley IV, A. Ben-Yehudah, C. J. Redinger et al., "MTOR-mediated activation of p70 S6K induces differentiation of pluripotent human embryonic stem cells," *Cellular Reprogramming*, vol. 12, no. 3, pp. 263–273, 2010.

[49] L. A. Julien, A. Carriere, J. Moreau, and P. P. Roux, "mTORC1-activated S6K1 phosphorylates rictor on threonine 1135 and regulates mTORC2 signaling," *Molecular and Cellular Biology*, vol. 30, no. 4, pp. 908–921, 2010.

[50] A. Y. Choo, S. O. Yoon, S. G. Kim, P. P. Roux, and J. Blenis, "Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 45, pp. 17414–17419, 2008.

[51] D. Zhang, R. Contu, M. V. G. Latronico et al., "MTORC1 regulates cardiac function and myocyte survival through 4E-BP1 inhibition in mice," *The Journal of Clinical Investigation*, vol. 120, no. 8, pp. 2805–2816, 2010.

[52] R. J. O. Dowling, I. Topisirovic, T. Alain et al., "mTORCI-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs," *Science*, vol. 328, no. 5982, pp. 1172–1176, 2010.

[53] K. G. Foster, H. A. Acosta-Jaquez, Y. Romeo et al., "Regulation of mTOR complex 1 (mTORC1) by raptor Ser863 and multisite phosphorylation," *The Journal of Biological Chemistry*, vol. 285, no. 1, pp. 80–94, 2010.

[54] D. Kwak, S. Choi, H. Jeong et al., "Osmotic stress regulates mammalian target of rapamycin(mTOR) complex 1 via c-Jun N-terminal Kinase (JNK)-mediated Raptor protein phosphorylation," *The Journal of Biological Chemistry*, vol. 287, no. 22, pp. 18398–18407, 2012.

[55] T. Sato, A. Nakashima, L. Guo, and F. Tamanoi, "Specific activation of mTORC1 by Rheb G-protein in vitro involves

enhanced recruitment of its substrate protein," *The Journal of Biological Chemistry*, vol. 284, no. 19, pp. 12783–12791, 2009.

[56] D. M. Gwinn, D. B. Shackelford, D. F. Egan et al., "AMPK phosphorylation of raptor mediates a metabolic checkpoint," *Molecular Cell*, vol. 30, no. 2, pp. 214–226, 2008.

[57] B. Magnuson, B. Ekim, and D. C. Fingar, "Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks," *The Biochemical Journal*, vol. 441, no. 1, pp. 1–21, 2012.

[58] B. C. Melnik, "Excessive Leucine-mTORC1-signalling of cow milk-based infant formula: the missing link to understand early childhood obesity," *Journal of Obesity*, vol. 2012, Article ID 197653, 2012.

[59] A. K. A. DeHart, J. D. Schnell, D. A. Allen, J. Y. Tsai, and L. Hicke, "Receptor internalization in yeast requires the Tor2-Rho1 signaling pathway," *Molecular Biology of the Cell*, vol. 14, no. 11, pp. 4676–4684, 2003.

[60] T. Powers, S. Aronova, and B. Niles, "TORC2 and sphingolipid biosynthesis and signaling. lessons from budding yeast," *The Enzymes*, vol. 27, pp. 177–197, 2010.

[61] V. Zinzalla, D. Stracka, W. Oppliger, and M. N. Hall, "Activation of mTORC2 by association with the ribosome," *Cell*, vol. 144, no. 5, pp. 757–768, 2011.

[62] A. Hagiwara, M. Cornu, N. Cybulski et al., "Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c," *Cell Metabolism*, vol. 15, no. 5, pp. 725–738, 2012.

[63] N. Cybulski and M. N. Hall, "TOR complex 2: a signaling pathway of its own," *Trends in Biochemical Sciences*, vol. 34, no. 12, pp. 620–627, 2009.

[64] C. A. Sparks and D. A. Guertin, "Targeting mTOR: prospects for mTOR complex 2 inhibitors in cancer therapy," *Oncogene*, vol. 29, no. 26, pp. 3733–3744, 2010.

[65] N. Ikai, N. Nakazawa, T. Hayashi, and M. Yanagida, "The reverse, but coordinated, roles of Tor2 (TORC1) and Tor1 (TORC2) kinases for growth, cell cycle and separase-mediated mitosis in *Schizosaccharomyces pombe*," *Open Biology*, vol. 1, 2011.

[66] M. A. Frias, C. C. Thoreen, J. D. Jaffe et al., "mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s," *Current Biology*, vol. 16, no. 18, pp. 1865–1870, 2006.

[67] C. M. McCay, M. F. Crowell, and L. A. Maynard, "The effect of retarded growth upon the Length of life span and upon the ultimate body size," *The Journal of Nutrition*, vol. 10, no. 1, pp. 63–79, 1935.

[68] R. J. Colman, R. M. Anderson, S. C. Johnson et al., "Caloric restriction delays disease onset and mortality in rhesus monkeys," *Science*, vol. 325, no. 5937, pp. 201–204, 2009.

[69] R. Anderson and R. Weindruch, "Metabolic reprogramming in dietary restriction," *Interdisciplinary Topics in Gerontology*, vol. 35, pp. 18–38, 2007.

[70] B. K. Kennedy, K. K. Steffen, and M. Kaeberlein, "Ruminations on dietary restriction and aging," *Cellular and Molecular Life Sciences*, vol. 64, no. 11, pp. 1323–1328, 2007.

[71] M. D. W. Piper and A. Bartke, "Diet and aging," *Cell Metabolism*, vol. 8, no. 2, pp. 99–104, 2008.

[72] G. S. Roth, D. K. Ingram, and M. A. Lane, "Caloric restriction in primates and relevance to humans," *Annals of the New York Academy of Sciences*, vol. 928, pp. 305–315, 2001.

[73] R. L. Walford, D. Mock, R. Verdery, and T. MacCallum, "Calorie restriction in biosphere 2: alterations in physiologic, hematologic, hormonal, and biochemical parameters in humans restricted for a 2-year period," *The Journals of Gerontology A*, vol. 57, no. 6, pp. B211–B224, 2002.

[74] L. Fontana, T. E. Meyer, S. Klein, and J. O. Holloszy, "Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 17, pp. 6659–6663, 2004.

[75] V. Tsagareli, M. Noakes, and R. J. Norman, "Effect of a very-low-calorie diet on in vitro fertilization outcomes," *Fertility and Sterility*, vol. 86, no. 1, pp. 227–229, 2006.

[76] L. K. Heilbronn, L. De Jonge, M. I. Frisard et al., "Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial," *Journal of the American Medical Association*, vol. 295, no. 13, pp. 1539–1548, 2006.

[77] S. B. Racette, E. P. Weiss, D. T. Villareal et al., "One year of caloric restriction in humans: feasibility and effects on body composition and abdominal adipose tissue," *The Journals of Gerontology A*, vol. 61, no. 9, pp. 943–950, 2006.

[78] T. E. Meyer, S. J. Kovács, A. A. Ehsani, S. Klein, J. O. Holloszy, and L. Fontana, "Long-term caloric restriction ameliorates the decline in diastolic function in humans," *Journal of the American College of Cardiology*, vol. 47, no. 2, pp. 398–402, 2006.

[79] L. Fontana, S. Klein, J. O. Holloszy, and B. N. Premachandra, "Effect of long-term calorie restriction with adequate protein and micronutrients on thyroid hormones," *The Journal of Clinical Endocrinology & Metabolism*, vol. 91, no. 8, pp. 3232–3235, 2006.

[80] L. Fontana and S. Klein, "Aging, adiposity, and calorie restriction," *Journal of the American Medical Association*, vol. 297, no. 9, pp. 986–994, 2007.

[81] J. O. Holloszy and L. Fontana, "Caloric restriction in humans," *Experimental Gerontology*, vol. 42, no. 8, pp. 709–712, 2007.

[82] B. J. Willcox, D. C. Willcox, H. Todoriki et al., "Caloric restriction, the traditional okinawan diet, and healthy aging: the diet of the world's longest-lived people and its potential impact on morbidity and life span," *Annals of the New York Academy of Sciences*, vol. 1114, pp. 434–455, 2007.

[83] L. Fontana, D. T. Villareal, E. P. Weiss et al., "Calorie restriction or exercise: effects on coronary heart disease risk factors. A randomized, controlled trial," *American Journal of Physiology*, vol. 293, no. 1, pp. E197–E202, 2007.

[84] J. O. Holloszy and L. Fontana, "Caloric restriction in humans," *Experimental Gerontology*, vol. 42, no. 8, pp. 709–712, 2007.

[85] T. Hofer, L. Fontana, S. D. Anton et al., "Long-term effects of caloric restriction or exercise on DNA and RNA oxidation levels in white blood cells and urine in humans," *Rejuvenation Research*, vol. 11, no. 4, pp. 793–799, 2008.

[86] L. M. Redman, J. Rood, S. D. Anton, C. Champagne, S. R. Smith, and E. Ravussin, "Calorie restriction and bone health in young, overweight individuals," *Archives of Internal Medicine*, vol. 168, no. 17, pp. 1859–1866, 2008.

[87] C. Cruzen and R. J. Colman, "Effects of caloric restriction on cardiovascular aging in non-human primates and humans," *Clinics in Geriatric Medicine*, vol. 25, no. 4, pp. 733–743, 2009.

[88] R. Cangemi, A. J. Friedmann, J. O. Holloszy, and L. Fontana, "Long-term effects of calorie restriction on serum sex-hormone concentrations in men," *Aging Cell*, vol. 9, no. 2, pp. 236–242, 2010.

[89] J. F. Trepanowski and R. J. Bloomer, "The impact of religious fasting on human health," *Nutrition Journal*, vol. 9, no. 1, article 57, 2010.

[90] A. Soare, R. Cangemi, D. Omodei, J. O. Holloszy, and L. Fontana, "Long-term calorie restriction, but not endurance exercise, lowers core body temperature in humans," *Aging*, vol. 3, no. 4, pp. 374–379, 2011.

[91] J. Rochon, C. W. Bales, E. Ravussin et al., "Design and conduct of the CALERIE study: comprehensive assessment of the long-term effects of reducing intake of energy," *The Journals of Gerontology A*, vol. 66, no. 1, pp. 97–108, 2011.

[92] C. K. Martin, S. K. Das, L. Lindblad et al., "Effect of calorie restriction on the free-living physical activity levels of nonobese humans: results of three randomized trials," *Journal of Applied Physiology*, vol. 110, no. 4, pp. 956–963, 2011.

[93] K. Stein, A. Soare, T. E. Meyer, R. Cangemi, J. O. Holloszy, and L. Fontana, "Caloric restriction may reverse age-related autonomic decline in humans," *Aging Cell*, vol. 11, no. 4, pp. 644–650, 2012.

[94] R. M. Anderson and R. Weindruch, "Metabolic reprogramming, caloric restriction and aging," *Trends in Endocrinology and Metabolism*, vol. 21, no. 3, pp. 134–141, 2010.

[95] J. M. Zahn, S. Poosala, A. B. Owen et al., "AGEMAP: a gene expression database for aging in mice," *PLoS Genetics*, vol. 3, no. 11, p. e201, 2007.

[96] J. P. de Magalhães, J. Curado, and G. M. Church, "Meta-analysis of age-related gene expression profiles identifies common signatures of aging," *Bioinformatics*, vol. 25, no. 7, pp. 875–881, 2009.

[97] S. K. Park and T. A. Prolla, "Lessons learned from gene expression profile studies of aging and caloric restriction," *Ageing Research Reviews*, vol. 4, no. 1, pp. 55–65, 2005.

[98] G. S. Hotamisligil, "Inflammation and metabolic disorders," *Nature*, vol. 444, no. 7121, pp. 860–867, 2006.

[99] F. Lago, C. Dieguez, J. Gómez-Reino, and O. Gualillo, "The emerging role of adipokines as mediators of inflammation and immune responses," *Cytokine & Growth Factor Reviews*, vol. 18, no. 3-4, pp. 313–325, 2007.

[100] U. Meier and A. M. Gressner, "Endocrine regulation of energy metabolism: review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin," *Clinical Chemistry*, vol. 50, no. 9, pp. 1511–1525, 2004.

[101] M. Zhu, G. D. Lee, L. Ding et al., "Adipogenic signaling in rat white adipose tissue: modulation by aging and calorie restriction," *Experimental Gerontology*, vol. 42, no. 8, pp. 733–744, 2007.

[102] K. Shinmura, K. Tamaki, K. Saito, Y. Nakano, T. Tobe, and R. Bolli, "Cardioprotective effects of short-term caloric restriction are mediated by adiponectin via activation of AMP-activated protein kinase," *Circulation*, vol. 116, no. 24, pp. 2809–2817, 2007.

[103] J. J. Díez and P. Iglesias, "The role of the novel adipocyte-derived hormone adiponectin in human disease," *European Journal of Endocrinology*, vol. 148, no. 3, pp. 293–300, 2003.

[104] O. Ukkola and M. Santaniemi, "Adiponectin: a link between excess adiposity and associated comorbidities?" *Journal of Molecular Medicine*, vol. 80, no. 11, pp. 696–702, 2002.

[105] O. Renaldi, B. Pramono, H. Sinorita, L. B. Purnomo, R. H. Asdie, and A. H. Asdie, "Hypoadiponectinemia: a risk factor for metabolic syndrome," *Acta medica Indonesiana*, vol. 41, no. 1, pp. 20–24, 2009.

[106] A. E. Civitarese, B. Ukropcova, S. Carling et al., "Role of adiponectin in human skeletal muscle bioenergetics," *Cell Metabolism*, vol. 4, no. 1, pp. 75–87, 2006.

[107] M. Nishimura, Y. Izumiya, A. Higuchi et al., "Adiponectin prevents cerebral ischemic injury through endothelial nitric oxide synthase-dependent mechanisms," *Circulation*, vol. 117, no. 2, pp. 216–223, 2008.

[108] J. M. Fernández-Real, A. López-Bermejo, R. Casamitjana, and W. Ricart, "Novel interactions of adiponectin with the endocrine system and inflammatory parameters," *The Journal of Clinical Endocrinology & Metabolism*, vol. 88, no. 6, pp. 2714–2718, 2003.

[109] W. Aldhahi and O. Hamdy, "Adipokines, inflammation, and the endothelium in diabetes," *Current Diabetes Reports*, vol. 3, no. 4, pp. 293–298, 2003.

[110] N. Ouchi, S. Kihara, T. Funahashi, Y. Matsuzawa, and K. Walsh, "Obesity, adiponectin and vascular inflammatory disease," *Current Opinion in Lipidology*, vol. 14, no. 6, pp. 561–566, 2003.

[111] D. M. Huffman, D. R. Moellering, W. E. Grizzle, C. R. Stockard, M. S. Johnson, and T. R. Nagy, "Effect of exercise and calorie restriction on biomarkers of aging in mice," *American Journal of Physiology*, vol. 294, no. 5, pp. R1618–R1627, 2008.

[112] L. Fontana, E. P. Weiss, D. T. Villareal, S. Klein, and J. O. Holloszy, "Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans," *Aging Cell*, vol. 7, no. 5, pp. 681–687, 2008.

[113] A. R. Cameron, S. Anton, L. Melville et al., "Black tea polyphenols mimic insulin/insulin-like growth factor-1 signalling to the longevity factor FOXO1a," *Aging Cell*, vol. 7, no. 1, pp. 69–77, 2008.

[114] W. Qin, W. Zhao, L. Ho et al., "Regulation of forkhead transcription factor FOXO3a contributes to calorie restriction-induced prevention of Alzheimer's disease-type amyloid neuropathology and spatial memory deterioration," *Annals of the New York Academy of Sciences*, vol. 1147, pp. 335–347, 2008.

[115] F. Flachsbart, A. Caliebe, R. Kleindorp et al., "Association of FOX03A variation with human longevity confirmed in German centenarians," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 8, pp. 2700–2705, 2009.

[116] E. J. Anderson, M. E. Lustig, K. E. Boyle et al., "Mitochondrial H_2O_2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans," *The Journal of Clinical Investigation*, vol. 119, no. 3, pp. 573–581, 2009.

[117] D. E. Harrison, R. Strong, Z. D. Sharp et al., "Rapamycin fed late in life extends lifespan in genetically heterogeneous mice," *Nature*, vol. 460, no. 7253, pp. 392–395, 2009.

[118] C. Chen, Y. Liu, Y. Liu, and P. Zheng, "mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells," *Science Signaling*, vol. 2, no. 98, p. ra75, 2009.

[119] P. Kapahi, D. Chen, A. N. Rogers et al., "With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging," *Cell Metabolism*, vol. 11, no. 6, pp. 453–465, 2010.

[120] B. M. Zid, A. N. Rogers, S. D. Katewa et al., "4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in *Drosophila*," *Cell*, vol. 139, no. 1, pp. 149–160, 2009.

[121] S. Sengupta, T. R. Peterson, M. Laplante, S. Oh, and D. M. Sabatini, "mTORC1 controls fasting-induced ketogenesis and its modulation by ageing," *Nature*, vol. 468, no. 7327, pp. 1100–1106, 2010.

[122] J. T. Cunningham, J. T. Rodgers, D. H. Arlow, F. Vazquez, V. K. Mootha, and P. Puigserver, "mTOR controls mitochondrial oxidative function through a YY1-PGC-1α transcriptional complex," *Nature*, vol. 450, no. 7170, pp. 736–740, 2007.

[123] A. Ramanathan and S. L. Schreiber, "Direct control of mitochondrial function by mTOR," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 52, pp. 22229–22232, 2009.

[124] J. Knapowski, K. Wieczorowska-Tobis, and J. Witowski, "Pathophysiology of ageing," *Journal of Physiology and Pharmacology*, vol. 53, no. 2, pp. 135–146, 2002.

[125] J. P. J. Issa, N. Ahuja, M. Toyota, M. P. Bronner, and T. A. Brentnall, "Accelerated age-related CpG island methylation in ulcerative colitis," *Cancer Research*, vol. 61, no. 9, pp. 3573–3577, 2001.

[126] J. P. J. Issa, Y. L. Ottaviano, P. Celano, S. R. Hamilton, N. E. Davidson, and S. B. Baylin, "Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon," *Nature Genetics*, vol. 7, no. 4, pp. 536–540, 1994.

[127] J. P. J. Issa, P. M. Vertino, C. D. Boehm, I. F. Newsham, and S. B. Baylin, "Switch from monoallelic to biallelic human IGF2 promoter methylation during aging and carcinogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 21, pp. 11757–11762, 1996.

[128] R. P. Singhal, L. L. Mays-Hoopes, and G. L. Eichhorn, "DNA methylation in aging of mice," *Mechanisms of Ageing and Development*, vol. 41, no. 3, pp. 199–210, 1987.

[129] T. Waki, G. Tamura, M. Sato, and T. Motoyama, "Age-related methylation of tumor suppressor and tumor-related genes: an analysis of autopsy samples," *Oncogene*, vol. 22, no. 26, pp. 4128–4133, 2003.

[130] V. L. Wilson, R. A. Smith, S. Ma, and R. G. Cutler, "Genomic 5-methyldeoxycytidine decreases with age," *The Journal of Biological Chemistry*, vol. 262, no. 21, pp. 9948–9951, 1987.

[131] T. Y. Kim, H. J. Lee, K. S. Hwang et al., "Methylation of RUNX3 in various types of human cancers and premalignant stages of gastric carcinoma," *Laboratory Investigation*, vol. 84, no. 4, pp. 479–484, 2004.

[132] Y. Li, M. Daniel, and T. O. Tollefsbol, "Epigenetic regulation of caloric restriction in aging," *BMC Medicine*, vol. 9, article 98, 2011.

[133] A. Vaquero and D. Reinberg, "Calorie restriction and the exercise of chromatin," *Genes & Development*, vol. 23, no. 16, pp. 1849–1869, 2009.

[134] U. Muñoz-Najar and J. M. Sedivy, "Epigenetic control of aging," *Antioxidants & Redox Signaling*, vol. 14, no. 2, pp. 241–259, 2011.

[135] B. S. Hass, R. W. Hart, M. H. Lu, and B. D. Lyn-Cook, "Effects of caloric restriction in animals on cellular function, oncogene expression, and DNA methylation in vitro," *Mutation Research*, vol. 295, no. 4–6, pp. 281–289, 1993.

[136] Y. Li, L. Liu, and T. O. Tollefsbol, "Glucose restriction can extend normal cell lifespan and impair precancerous cell growth through epigenetic control of hTERT and p16 expression," *The FASEB Journal*, vol. 24, no. 5, pp. 1442–1453, 2010.

[137] R. S. Ahima, "Connecting obesity, aging and diabetes," *Nature Medicine*, vol. 15, no. 9, pp. 996–997, 2009.

[138] T. M. Larsen, S. Dalskov, M. Van Baak et al., "The diet, obesity and genes (diogenes) dietary study in eight European countries—a comprehensive design for long-term intervention," *Obesity Reviews*, vol. 11, no. 1, pp. 76–91, 2010.

[139] F. I. Milagro, J. Campión, P. Cordero et al., "A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss," *The FASEB Journal*, vol. 25, no. 4, pp. 1378–1389, 2011.

[140] L. Bouchard, R. Rabasa-Lhoret, M. Faraj et al., "Differential epigenomic and transcriptomic responses in subcutaneous adipose tissue between low and high responders to caloric restriction," *The American Journal of Clinical Nutrition*, vol. 91, no. 2, pp. 309–320, 2010.

[141] J. Campión, F. I. Milagro, E. Goyenechea, and J. A. Martínez, "TNF-α promoter methylation as a predictive biomarker for weight-loss response," *Obesity*, vol. 17, no. 6, pp. 1293–1297, 2009.

[142] A. L. Clayton, C. A. Hazzalin, and L. C. Mahadevan, "Enhanced histone acetylation and transcription: a dynamic perspective," *Molecular Cell*, vol. 23, no. 3, pp. 289–296, 2006.

[143] M. Meyerson, C. M. Counter, E. N. Eaton et al., "hEST2, the putative human telomerase catalytic subunit gene, is upregulated in tumor cells and during immortalization," *Cell*, vol. 90, no. 4, pp. 785–795, 1997.

[144] T. Kanaya, S. Kyo, M. Takakura, H. Ito, M. Namiki, and M. Inoue, "hTERT is a critical determinant of telomerase activity in renal-cell carcinoma," *International Journal of Cancer*, vol. 78, no. 5, pp. 539–543, 1998.

[145] S. J. Lin, P. A. Defossez, and L. Guarente, "Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*," *Science*, vol. 289, no. 5487, pp. 2126–2128, 2000.

[146] L. Guarente and F. Picard, "Calorie restriction—the SIR2 connection," *Cell*, vol. 120, no. 4, pp. 473–482, 2005.

[147] I. B. Leibiger and P. O. Berggren, "Sirt1: a metabolic master switch that modulates lifespan," *Nature Medicine*, vol. 12, no. 1, pp. 34–36, 2006.

[148] L. Bordone, D. Cohen, A. Robinson et al., "SIRT1 transgenic mice show phenotypes resembling calorie restriction," *Aging Cell*, vol. 6, no. 6, pp. 759–767, 2007.

[149] H. Y. Cohen, C. Miller, K. J. Bitterman et al., "Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase," *Science*, vol. 305, no. 5682, pp. 390–392, 2004.

[150] Y. Kanfi, V. Peshti, Y. M. Gozlan, M. Rathaus, R. Gil, and H. Y. Cohen, "Regulation of SIRT1 protein levels by nutrient availability," *FEBS Letters*, vol. 582, no. 16, pp. 2417–2423, 2008.

[151] A. B. Crujeiras, D. Parra, E. Goyenechea, and J. A. Martínez, "Sirtuin gene expression in human mononuclear cells is modulated by caloric restriction," *European Journal of Clinical Investigation*, vol. 38, no. 9, pp. 672–678, 2008.

[152] L. A. Wakeling, L. J. Ions, and D. Ford, "Could Sirt1-mediated epigenetic effects contribute to the longevity response to dietary restriction and be mimicked by other dietary interventions?" *Age*, vol. 31, no. 4, pp. 327–341, 2009.

[153] Y. Li and T. O. Tollefsbol, "$P16^{INK4a}$ suppression by glucose restriction contributes to human cellular lifespan extension through SIRT1-mediated epigenetic and genetic mechanisms," *PLoS ONE*, vol. 6, no. 2, Article ID e17421, 2011.

[154] M. C. Haigis and L. P. Guarente, "Mammalian sirtuins—emerging roles in physiology, aging, and calorie restriction," *Genes & Development*, vol. 20, no. 21, pp. 2913–2921, 2006.

[155] J. Luo, A. Y. Nikolaev, S. I. Imai et al., "Negative control of p53 by Sir2α promotes cell survival under stress," *Cell*, vol. 107, no. 2, pp. 137–148, 2001.

[156] E. Langley, M. Pearson, M. Faretta et al., "Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence," *The EMBO Journal*, vol. 21, no. 10, pp. 2383–2396, 2002.

[157] H. Vaziri, S. K. Dessain, E. N. Eaton et al., "*hSIR2*^{SIRT1} functions as an NAD-dependent p53 deacetylase," *Cell*, vol. 107, no. 2, pp. 149–159, 2001.

[158] A. Brunet, L. B. Sweeney, J. F. Sturgill et al., "Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase," *Science*, vol. 303, no. 5666, pp. 2011–2015, 2004.

[159] M. C. Motta, N. Divecha, M. Lemieux et al., "Mammalian SIRT1 represses forkhead transcription factors," *Cell*, vol. 116, no. 4, pp. 551–563, 2004.

[160] M. M. Schilling, J. K. Oeser, J. N. Boustead, B. P. Flemming, and R. M. O'Brien, "Gluconeogenesis: re-evaluating the FOXO1-PGC-1α connection," *Nature*, vol. 443, no. 7111, pp. E10–E11, 2006.

[161] R. B. Vega, J. M. Huss, and D. P. Kelly, "The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor α in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes," *Molecular and Cellular Biology*, vol. 20, no. 5, pp. 1868–1876, 2000.

[162] J. Koubova and L. Guarente, "How does calorie restriction work?" *Genes & Development*, vol. 17, no. 3, pp. 313–321, 2003.

[163] R. S. Sohal and R. Weindruch, "Oxidative stress, caloric restriction, and aging," *Science*, vol. 273, no. 5271, pp. 59–63, 1996.

[164] B. J. Merry, "Molecular mechanisms linking calorie restriction and longevity," *The International Journal of Biochemistry & Cell Biology*, vol. 34, no. 11, pp. 1340–1354, 2002.

[165] J. Jeong, K. Juhn, H. Lee et al., "SIRT1 promotes DNA repair activity and deacetylation of Ku70," *Experimental & Molecular Medicine*, vol. 39, no. 1, pp. 8–13, 2007.

[166] H. Y. Cohen, S. Lavu, K. J. Bitterman et al., "Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis," *Molecular Cell*, vol. 13, no. 5, pp. 627–638, 2004.

[167] H. Wong and K. Riabowol, "Differential CDK-inhibitor gene expression in aging human diploid fibroblasts," *Experimental Gerontology*, vol. 31, no. 1-2, pp. 311–325, 1996.

[168] J. Gil and G. Peters, "Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all," *Nature Reviews*, vol. 7, no. 9, pp. 667–677, 2006.

[169] J. Krishnamurthy, C. Torrice, M. R. Ramsey et al., "Ink4a/Arf expression is a biomarker of aging," *The Journal of Clinical Investigation*, vol. 114, no. 9, pp. 1299–1307, 2004.

[170] D. A. Alcorta, Y. Xiong, D. Phelps, G. Hannon, D. Beach, and J. C. Barrett, "Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 24, pp. 13742–13747, 1996.

[171] A. Melk, B. M. W. Schmidt, O. Takeuchi, B. Sawitzki, D. C. Rayner, and P. F. Halloran, "Expression of p16^{INK4a} and other cell cycle regulator and senescence associated genes in aging human kidney," *Kidney International*, vol. 65, no. 2, pp. 510–520, 2004.

[172] W. Fischle, Y. Wang, and C. D. Allis, "Histone and chromatin cross-talk," *Current Opinion in Cell Biology*, vol. 15, no. 2, pp. 172–183, 2003.

[173] T. Kouzarides, "Histone methylation in transcriptional control," *Current Opinion in Genetics & Development*, vol. 12, no. 2, pp. 198–209, 2002.

[174] N. Noren Hooten, K. Abdelmohsen, M. Gorospe, N. Ejiogu, A. B. Zonderman, and M. K. Evans, "microRNA expression patterns reveal differential expression of target genes with age," *PloS One*, vol. 5, no. 5, Article ID e10724, 2010.

[175] A. De Lencastre, Z. Pincus, K. Zhou, M. Kato, S. S. Lee, and F. J. Slack, "MicroRNAs both promote and antagonize longevity in *C. elegans*," *Current Biology*, vol. 20, no. 24, pp. 2159–2168, 2010.

[176] D. J. Bates, N. Li, R. Liang et al., "MicroRNA regulation in Ames dwarf mouse liver may contribute to delayed aging," *Aging Cell*, vol. 9, no. 1, pp. 1–18, 2010.

[177] U. A. Ørom, M. K. Lim, J. E. Savage et al., "MicroRNA-203 regulates caveolin-1 in breast tissue during caloric restriction," *Cell Cycle*, vol. 11, no. 7, pp. 1291–1295, 2012.

[178] A. Khanna, S. Muthusamy, R. Liang, H. Sarojini, and E. Wang, "Gain of survival signaling by down-regulation of three key miRNAs in brain of calorie-restricted mice," *Aging*, vol. 3, no. 3, pp. 223–236, 2011.

[179] E. Paradis, H. Douillard, M. Koutroumanis, C. Goodyer, and A. LeBlanc, "Amyloid β peptide of Alzheimer's disease down-regulates bcl-2 and upregulates bax expression in human neurons," *Journal of Neuroscience*, vol. 16, no. 23, pp. 7533–7539, 1996.

[180] C. Perier, J. Bové, D. C. Wu et al., "Two molecular pathways initiate mitochondria-dependent dopaminergic neurodegeneration in experimental Parkinson's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 19, pp. 8161–8166, 2007.

[181] N. Louneva, J. W. Cohen, L. Y. Han et al., "Caspase-3 is enriched in postsynaptic densities and increased in Alzheimer's disease," *The American Journal of Pathology*, vol. 173, no. 5, pp. 1488–1495, 2008.

[182] M. Yamada, K. Kida, W. Amutuhaire, F. Ichinose, and M. Kaneki, "Gene disruption of caspase-3 prevents MPTP-induced Parkinson's disease in mice," *Biochemical and Biophysical Research Communications*, vol. 402, no. 2, pp. 312–318, 2010.

[183] W. Kudo, H. P. Lee, M. A. Smith, X. Zhu, S. Matsuyama, and H. G. Lee, "Inhibition of Bax protects neuronal cells from oligomeric Aβ neurotoxicity," *Cell Death & Disease*, vol. 3, Article ID e309, 2012.

[184] D. K. Ingram, M. Zhu, J. Mamczarz et al., "Calorie restriction mimetics: an emerging research field," *Aging Cell*, vol. 5, no. 2, pp. 97–108, 2006.

[185] H. R. Warner, D. Ingram, R. A. Miller, N. L. Nadon, and A. G. Richardson, "Program for testing biological interventions to promote healthy aging," *Mechanisms of Ageing and Development*, vol. 115, no. 3, pp. 199–207, 2000.

[186] N. L. Nadon, R. Strong, R. A. Miller et al., "Design of aging intervention studies: the NIA interventions testing program," *Age*, vol. 30, no. 4, pp. 187–199, 2008.

[187] R. A. Miller, D. E. Harrison, C. M. Astle et al., "An aging interventions testing program: study design and interim report," *Aging Cell*, vol. 6, no. 4, pp. 565–575, 2007.

[188] R. Strong, R. A. Miller, C. M. Astle et al., "Nordihydroguaiaretic acid and aspirin increase lifespan of genetically heterogeneous male mice," *Aging Cell*, vol. 7, no. 5, pp. 641–650, 2008.

[189] N. L. Nadon, "Exploiting the rodent model for studies on the pharmacology of lifespan extension," *Aging Cell*, vol. 5, no. 1, pp. 9–15, 2006.

[190] J. O. Holloszy, "Mortality rate and longevity of food-restricted exercising male rats: a reevaluation," *Journal of Applied Physiology*, vol. 82, no. 2, pp. 399–403, 1997.

[191] K. C. Deruisseau, A. N. Kavazis, S. Judge et al., "Moderate caloric restriction increases diaphragmatic antioxidant enzyme mRNA, but not when combined with lifelong

exercise," *Antioxidants & Redox Signaling*, vol. 8, no. 3-4, pp. 539–547, 2006.

[192] A. Y. Seo, T. Hofer, B. Sung, S. Judge, H. Y. Chung, and C. Leeuwenburgh, "Hepatic oxidative stress during aging: effects of 8% long-term calorie restriction and lifelong exercise," *Antioxidants & Redox Signaling*, vol. 8, no. 3-4, pp. 529–538, 2006.

[193] R. Kalani, S. Judge, C. Carter, M. Pahor, and C. Leeuwenburgh, "Effects of caloric restriction and exercise on age-related, chronic inflammation assessed by C-reactive protein and interleukin-6," *The Journals of Gerontology A*, vol. 61, no. 3, pp. 211–217, 2006.

[194] P. Abete, G. Testa, G. Galizia et al., "Tandem action of exercise training and food restriction completely preserves ischemic preconditioning in the aging heart," *Experimental Gerontology*, vol. 40, no. 1-2, pp. 43–50, 2005.

[195] D. L. Crandall, R. P. Feirer, D. R. Griffith, and D. C. Beitz, "Relative role of caloric restriction and exercise training upon susceptibility to isoproterenol-induced myocardial infarction in male rats," *The American Journal of Clinical Nutrition*, vol. 34, no. 5, pp. 841–847, 1981.

[196] A. E. Civitarese, S. Carling, L. K. Heilbronn et al., "Calorie restriction increases muscle mitochondrial biogenesis in healthy humans," *PLoS Medicine*, vol. 4, no. 3, article e76, 2007.

[197] D. E. Larson-Meyer, B. R. Newcomer, L. K. Heilbronn et al., "Effect of 6-month calorie restriction and exercise on serum and liver lipids and markers of liver function," *Obesity*, vol. 16, no. 6, pp. 1355–1362, 2008.

[198] M. Lefevre, L. M. Redman, L. K. Heilbronn et al., "Caloric restriction alone and with exercise improves CVD risk in healthy non-obese individuals," *Atherosclerosis*, vol. 203, no. 1, pp. 206–213, 2009.

[199] D. E. Larson-Meyer, L. Redman, L. K. Heilbronn, C. K. Martin, and E. Ravussin, "Caloric restriction with or without exercise: the fitness versus fatness debate," *Medicine and Science in Sports and Exercise*, vol. 42, no. 1, pp. 152–159, 2010.

[200] N. E. Silverman, B. J. Nicklas, and A. S. Ryan, "Addition of aerobic exercise to a weight loss program increases BMD, with an associated reduction in inflammation in overweight postmenopausal women," *Calcified Tissue International*, vol. 84, no. 4, pp. 257–265, 2009.

[201] J. F. Trepanowski, R. E. Canale, K. E. Marshall, M. M. Kabir, and R. J. Bloomer, "Impact of caloric and dietary restriction regimens on markers of health and longevity in humans and animals: a summary of available findings," *Nutrition Journal*, vol. 10, article 107, 2011.

[202] K. Iwasaki, C. A. Gleiser, E. J. Masoro, C. A. McMahan, E. Seo, and B. P. Yu, "The influence of dietary protein source on longevity and age-related disease processes of Fischer rats," *Journals of Gerontology*, vol. 43, no. 1, pp. B5–B12, 1988.

[203] I. Shimokawa, Y. Higami, B. P. Yu, E. J. Masoro, and T. Ikeda, "Influence of dietary components on occurrence of and mortality due to neoplasms in male F344 rats," *Aging*, vol. 8, no. 4, pp. 254–262, 1996.

[204] M. Khorakova, Z. Deil, D. Khausman, and K. Matsek, "Effect of carbohydrate-enriched diet and subsequent food restriction on life prolongation in Fischer 344 male rats," *Fiziologicheskii Zhurnal*, vol. 36, no. 5, pp. 16–21, 1990.

[205] C. Kubo, B. C. Johnson, A. Gajjar, and R. A. Good, "Crucial dietary factors in maximizing life span and longevity in autoimmune-prone mice," *The Journal of Nutrition*, vol. 117, no. 6, pp. 1129–1135, 1987.

[206] R. Pamplona and G. Barja, "Mitochondrial oxidative stress, aging and caloric restriction: the protein and methionine connection," *Biochimica et Biophysica Acta*, vol. 1757, no. 5-6, pp. 496–508, 2006.

[207] A. Sanz, P. Caro, J. G. Sanchez, and G. Barja, "Effect of lipid restriction on mitochondrial free radical production and oxidative DNA damage," *Annals of the New York Academy of Sciences*, vol. 1067, no. 1, pp. 200–209, 2006.

[208] A. Sanz, J. Gómez, P. Caro, and G. Barja, "Carbohydrate restriction does not change mitochondrial free radical generation and oxidative DNA damage," *Journal of Bioenergetics and Biomembranes*, vol. 38, no. 5-6, pp. 327–333, 2006.

[209] P. E. Segall and P. S. Timiras, "Pathophysiologic findings after chronic tryptophan deficiency in rats: a model for delayed growth and aging," *Mechanisms of Ageing and Development*, vol. 5, no. 2, pp. 109–124, 1976.

[210] H. Ooka, P. E. Segall, and P. S. Timiras, "Histology and survival in age-delayed low-tryptophan-fed rats," *Mechanisms of Ageing and Development*, vol. 43, no. 1, pp. 79–98, 1988.

[211] R. A. Miller, G. Buehner, Y. Chang, J. M. Harper, R. Sigler, and M. Smith-Wheelock, "Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance," *Aging Cell*, vol. 4, no. 3, pp. 119–125, 2005.

[212] N. Orentreich, J. R. Matias, A. DeFelice, and J. A. Zimmerman, "Low methionine ingestion by rats extends life span," *The Journal of Nutrition*, vol. 123, no. 2, pp. 269–274, 1993.

[213] J. P. Richie Jr., Y. Leutzinger, S. Parthasarathy, V. Malloy, N. Orentreich, and J. A. Zimmerman, "Methionine restriction increases blood glutathione and longevity in F344 rats," *The FASEB Journal*, vol. 8, no. 15, pp. 1302–1307, 1994.

[214] J. P. Richie Jr., D. Komninou, Y. Leutzinger et al., "Tissue glutathione and cysteine levels in methionine-restricted rats," *Nutrition*, vol. 20, no. 9, pp. 800–805, 2004.

[215] J. A. Zimmerman, V. Malloy, R. Krajcik, and N. Orentreich, "Nutritional control of aging," *Experimental Gerontology*, vol. 38, no. 1-2, pp. 47–52, 2003.

[216] A. Sanz, P. Caro, V. Ayala, M. Portero-Otin, R. Pamplona, and G. Barja, "Methionine restriction decreases mitochondrial oxygen radical generation and leak as well as oxidative damage to mitochondrial DNA and proteins," *The FASEB Journal*, vol. 20, no. 8, pp. 1064–1073, 2006.

[217] P. Caro, J. Gómez, M. López-Torres et al., "Forty percent and eighty percent methionine restriction decrease mitochondrial ROS generation and oxidative stress in rat liver," *Biogerontology*, vol. 9, no. 3, pp. 183–196, 2008.

[218] M. C. Ruiz, V. Ayala, M. Portero-Otín, J. R. Requena, G. Barja, and R. Pamplona, "Protein methionine content and MDA-lysine adducts are inversely related to maximum life span in the heart of mammals," *Mechanisms of Ageing and Development*, vol. 126, no. 10, pp. 1106–1114, 2005.

[219] N. Hidiroglou, G. S. Gilani, L. Long et al., "The influence of dietary vitamin E, fat, and methionine on blood cholesterol profile, homocysteine levels, and oxidizability of low density lipoprotein in the gerbil," *The Journal of Nutritional Biochemistry*, vol. 15, no. 12, pp. 730–740, 2004.

[220] P. Caro, J. Gomez, I. Sanchez et al., "Effect of 40% restriction of dietary amino acids (except methionine) on mitochondrial oxidative stress and biogenesis, AIF and SIRT1 in rat liver," *Biogerontology*, vol. 10, no. 5, pp. 579–592, 2009.

[221] M. F. McCarty, J. Barroso-Aranda, and F. Contreras, "The low-methionine content of vegan diets may make

methionine restriction feasible as a life extension strategy," *Medical Hypotheses*, vol. 72, no. 2, pp. 125–128, 2009.

[222] K. A. Varady and M. K. Hellerstein, "Alternate-day fasting and chronic disease prevention: a review of human and animal trials," *The American Journal of Clinical Nutrition*, vol. 86, no. 1, pp. 7–13, 2007.

[223] R. M. Anson, Z. Guo, R. de Cabo et al., "Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 6216–6220, 2003.

[224] O. Descamps, J. Riondel, V. Ducros, and A. M. Roussel, "Mitochondrial production of reactive oxygen species and incidence of age-associated lymphoma in OF1 mice: effect of alternate-day fasting," *Mechanisms of Ageing and Development*, vol. 126, no. 11, pp. 1185–1191, 2005.

[225] W. Duan, Z. Guo, H. Jiang, M. Ware, and M. P. Mattson, "Reversal of behavioral and metabolic abnormalities, and insulin resistance syndrome, by dietary restriction in mice deficient in brain-derived neurotrophic factor," *Endocrinology*, vol. 144, no. 6, pp. 2446–2453, 2003.

[226] I. Ahmet, R. Wan, M. P. Mattson, E. G. Lakatta, and M. Talan, "Cardioprotection by intermittent fasting in rats," *Circulation*, vol. 112, no. 20, pp. 3115–3121, 2005.

[227] D. E. Mager, R. Wan, M. Brown et al., "Caloric restriction and intermittent fasting alter spectral measures of heart rate and blood pressure variability in rats," *The FASEB Journal*, vol. 20, no. 6, pp. 631–637, 2006.

[228] C. R. Pedersen, I. Hagemann, T. Bock, and K. Buschard, "Intermittent feeding and fasting reduces diabetes incidence in BB rats," *Autoimmunity*, vol. 30, no. 4, pp. 243–250, 1999.

[229] K. Tikoo, D. N. Tripathi, D. G. Kabra, V. Sharma, and A. B. Gaikwad, "Intermittent fasting prevents the progression of type I diabetic nephropathy in rats and changes the expression of Sir2 and p53," *FEBS Letters*, vol. 581, no. 5, pp. 1071–1078, 2007.

[230] R. Wan, S. Camandola, and M. P. Mattson, "Intermittent fasting and dietary supplementation with 2-deoxy-D-glucose improve functional and metabolic cardiovascular risk factors in rats," *The FASEB Journal*, vol. 17, no. 9, pp. 1133–1134, 2003.

[231] L. K. Heilbronn, S. R. Smith, C. K. Martin, S. D. Anton, and E. Ravussin, "Alternate-day fasting in nonobese subjects: effects on body weight, body composition, and energy metabolism," *The American Journal of Clinical Nutrition*, vol. 81, no. 1, pp. 69–73, 2005.

[232] N. Halberg, M. Henriksen, N. Söderhamn et al., "Effect of intermittent fasting and refeeding on insulin action in healthy men," *Journal of Applied Physiology*, vol. 99, no. 6, pp. 2128–2136, 2005.

[233] J. B. Johnson, W. Summer, R. G. Cutler et al., "Alternate day calorie restriction improves clinical findings and reduces markers of oxidative stress and inflammation in overweight adults with moderate asthma," *Free Radical Biology and Medicine*, vol. 42, no. 5, pp. 665–674, 2007.

[234] V. D. Dixit, H. Yang, K. S. Sayeed et al., "Controlled meal frequency without caloric restriction alters peripheral blood mononuclear cell cytokine production," *Journal of Inflammation*, vol. 8, article 6, 2011.

[235] K. T. Howitz, K. J. Bitterman, H. Y. Cohen et al., "Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan," *Nature*, vol. 425, no. 6954, pp. 191–196, 2003.

[236] J. G. Wood, B. Rogina, S. Lavu et al., "Sirtuin activators mimic caloric restriction and delay ageing in metazoans," *Nature*, vol. 430, no. 7000, pp. 686–689, 2004.

[237] H. Yang, J. A. Baur, A. Chen, C. Miller, and D. A. Sinclair, "Design and synthesis of compounds that extend yeast replicative lifespan," *Aging Cell*, vol. 6, no. 1, pp. 35–43, 2007.

[238] D. R. Valenzano, E. Terzibasi, T. Genade, A. Cattaneo, L. Domenici, and A. Cellerino, "Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate," *Current Biology*, vol. 16, no. 3, pp. 296–300, 2006.

[239] M. Kaeberlein, T. McDonagh, B. Heltweg et al., "Substrate-specific activation of sirtuins by resveratrol," *The Journal of Biological Chemistry*, vol. 280, no. 17, pp. 17038–17045, 2005.

[240] M. Kaeberlein and R. W. Powers III, "Sir2 and calorie restriction in yeast: a skeptical perspective," *Ageing Research Reviews*, vol. 6, no. 2, pp. 128–140, 2007.

[241] M. Kaeberlein and B. K. Kennedy, "Does resveratrol activate yeast Sir2 in vivo?" *Aging Cell*, vol. 6, no. 4, pp. 415–416, 2007.

[242] D. L. Smith Jr., C. Li, M. Matecic, N. Maqani, M. Bryk, and J. S. Smith, "Calorie restriction effects on silencing and recombination at the yeast rDNA," *Aging Cell*, vol. 8, no. 6, pp. 633–642, 2009.

[243] T. M. Bass, D. Weinkove, K. Houthoofd, D. Gems, and L. Partridge, "Effects of resveratrol on lifespan in *Drosophila melanogaster* and *Caenorhabditis elegans*," *Mechanisms of Ageing and Development*, vol. 128, no. 10, pp. 546–552, 2007.

[244] E. L. Greer and A. Brunet, "Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*," *Aging Cell*, vol. 8, no. 2, pp. 113–127, 2009.

[245] T. L. Kaeberlein, E. D. Smith, M. Tsuchiya et al., "Lifespan extension in *Caenorhabditis elegans* by complete removal of food," *Aging Cell*, vol. 5, no. 6, pp. 487–494, 2006.

[246] S. Zou, J. R. Carey, P. Liedo et al., "The prolongevity effect of resveratrol depends on dietary composition and calorie intake in a tephritid fruit fly," *Experimental Gerontology*, vol. 44, no. 6-7, pp. 472–476, 2009.

[247] M. Riesen and A. Morgan, "Calorie restriction reduces rDNA recombination independently of rDNA silencing," *Aging Cell*, vol. 8, no. 6, pp. 624–632, 2009.

[248] M. Pacholec, J. E. Bleasdale, B. Chrunyk et al., "SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1," *The Journal of Biological Chemistry*, vol. 285, no. 11, pp. 8340–8351, 2010.

[249] J. A. Baur, K. J. Pearson, N. L. Price et al., "Resveratrol improves health and survival of mice on a high-calorie diet," *Nature*, vol. 444, no. 7117, pp. 337–342, 2006.

[250] M. Zang, S. Xu, K. A. Maitland-Toolan et al., "Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice," *Diabetes*, vol. 55, no. 8, pp. 2180–2191, 2006.

[251] B. Dasgupta and J. Milbrandt, "Resveratrol stimulates AMP kinase activity in neurons," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 17, pp. 7217–7222, 2007.

[252] N. L. Price, A. P. Gomes, A. J. Ling et al., "SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function," *Cell Metabolism*, vol. 15, no. 5, pp. 675–690, 2012.

[253] J. L. Barger, T. Kayo, J. M. Vann et al., "A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice," *PLoS ONE*, vol. 3, no. 6, Article ID e2264, 2008.

[254] K. J. Pearson, J. A. Baur, K. N. Lewis et al., "Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span," *Cell Metabolism*, vol. 8, no. 2, pp. 157–168, 2008.

[255] R. A. Miller, D. E. Harrison, C. M. Astle et al., "Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice," *The Journals of Gerontology A*, vol. 66, no. 2, pp. 191–201, 2011.

[256] D. L. Smith Jr., T. R. Nagy, and D. B. Allison, "Calorie restriction: what recent results suggest for the future of ageing research," *European Journal of Clinical Investigation*, vol. 40, no. 5, pp. 440–450, 2010.

[257] A. Dal-Pan, S. Blanc, and F. Aujard, "Resveratrol suppresses body mass gain in a seasonal non-human primate model of obesity," *BMC Physiology*, vol. 10, no. 1, article 11, 2010.

[258] A. Dal-Pan, J. Terrien, F. Pifferi et al., "Caloric restriction or resveratrol supplementation and ageing in a non-human primate: first-year outcome of the RESTRIKAL study in *Microcebus murinus*," *Age*, vol. 33, no. 1, pp. 15–31, 2011.

[259] L. Peltz, J. Gomez, M. Marquez et al., "Resveratrol exerts dosage and duration dependent effect on human mesenchymal stem cell development," *PLoS One*, vol. 7, no. 5, Article ID e37162, 2012.

[260] J. Mikuła-Pietrasik, A. Kuczmarska, B. Rubiś et al., "Resveratrol delays replicative senescence of human mesothelial cells via mobilization of antioxidative and DNA repair mechanisms," *Free Radical Biology & Medicine*, vol. 52, pp. 2234–2245, 2012.

[261] J. M. Smoliga, J. A. Baur, and H. A. Hausenblas, "Resveratrol and health—a comprehensive review of human clinical trials," *Molecular Nutrition & Food Research*, vol. 55, no. 8, pp. 1129–1141, 2011.

[262] H. Ghanim, C. L. Sia, S. Abuaysheh et al., "An antiinflammatory and reactive oxygen species suppressive effects of an extract of *Polygonum cuspidatum* containing resveratrol," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 9, pp. E1–E8, 2010.

[263] H. Ghanim, S. Abuaysheh, C. L. Sia et al., "Increase in plasma endotoxin concentrations and the expression of toll-like receptors and suppressor of cytokine signaling-3 in mononuclear cells after a high-fat, high-carbohydrate meal: implications for insulin resistance," *Diabetes Care*, vol. 32, no. 12, pp. 2281–2287, 2009.

[264] H. Ghanim, C. L. Sia, K. Korzeniewski et al., "A resveratrol and polyphenol preparation suppresses oxidative and inflammatory stress response to a high-fat, high-carbohydrate meal," *The Journal of Clinical Endocrinology & Metabolism*, vol. 96, no. 5, pp. 1409–1414, 2011.

[265] P. Brasnyó, G. A. Molnár, M. Mohás et al., "Resveratrol improves insulin sensitivity, reduces oxidative stress and activates the Akt pathway in type 2 diabetic patients," *The British Journal of Nutrition*, vol. 106, no. 3, pp. 383–389, 2011.

[266] L. M. Vislocky and M. L. Fernandez, "Biomedical effects of grape products," *Nutrition Reviews*, vol. 68, no. 11, pp. 656–670, 2010.

[267] A. A. A. Bertelli and D. K. Das, "Grapes, wines, resveratrol, and heart health," *Journal of Cardiovascular Pharmacology*, vol. 54, no. 6, pp. 468–476, 2009.

[268] M. M. Dohadwala and J. A. Vita, "Grapes and cardiovascular disease," *The Journal of Nutrition*, vol. 139, no. 9, pp. 17885–17935, 2009.

[269] W. R. Leifert and M. Y. Abeywardena, "Grape seed and red wine polyphenol extracts inhibit cellular cholesterol uptake, cell proliferation, and 5-lipoxygenase activity," *Nutrition Research*, vol. 28, no. 11, pp. 729–737, 2008.

[270] T. Wallerath, G. Deckert, T. Ternes et al., "Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase," *Circulation*, vol. 106, no. 13, pp. 1652–1658, 2002.

[271] T. Wallerath, H. Li, U. Gödtel-Ambrust, P. M. Schwarz, and U. Förstermann, "A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase," *Nitric Oxide*, vol. 12, no. 2, pp. 97–104, 2005.

[272] J. F. Leikert, T. R. Räthel, P. Wohlfart, V. Cheynier, A. M. Vollmar, and V. M. Dirsch, "Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells," *Circulation*, vol. 106, no. 13, pp. 1614–1617, 2002.

[273] P. Gresele, P. Pignatelli, G. Guglielmini et al., "Resveratrol, at concentrations attainable with moderate wine consumption, stimulates human platelet nitric oxide production," *The Journal of Nutrition*, vol. 138, no. 9, pp. 1602–1608, 2008.

[274] D. O. Kennedy, E. L. Wightman, J. L. Reay et al., "Effects of resveratrol on cerebral blood flow variables and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation," *The American Journal of Clinical Nutrition*, vol. 91, no. 6, pp. 1590–1597, 2010.

[275] R. H. X. Wong, P. R. C. Howe, J. D. Buckley, A. M. Coates, I. Kunz, and N. M. Berry, "Acute resveratrol supplementation improves flow-mediated dilatation in overweight/obese individuals with mildly elevated blood pressure," *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 21, no. 11, pp. 851–856, 2011.

[276] B. Agarwal and J. A. Baur, "Resveratrol and life extension," *Annals of the New York Academy of Sciences*, vol. 1215, no. 1, pp. 138–143, 2011.

[277] J. L. Crespo and M. N. Hall, "Elucidating TOR signaling and rapamycin action: lessons from *Saccharomyces cerevisiae*," *Microbiology and Molecular Biology Reviews*, vol. 66, no. 4, pp. 579–591, 2002.

[278] I. Bjedov, J. M. Toivonen, F. Kerr et al., "Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*," *Cell Metabolism*, vol. 11, no. 1, pp. 35–46, 2010.

[279] N. Anisimov, M. A. Zabezhinski, I. G. Popovich et al., "Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice," *Cell Cycle*, vol. 10, no. 24, pp. 4230–4236, 2011.

[280] S. Majumder, A. Caccamo, D. X. Medina et al., "Lifelong rapamycin administration ameliorates age-dependent cognitive deficits by reducing IL-1β and enhancing NMDA signaling," *Aging Cell*, vol. 11, no. 2, pp. 326–335, 2012.

[281] G. Payne, A. Laporte, R. Deber, and P. C. Coyte, "Counting backward to health care's future: using time-to-death modeling to identify changes in end-of-life morbidity and the impact of aging on health care expenditures," *The Milbank Quarterly*, vol. 85, no. 2, pp. 213–257, 2007.

[282] A. Yazdanyar and A. B. Newman, "The burden of cardiovascular disease in the elderly: morbidity, mortality, and costs," *Clinics in Geriatric Medicine*, vol. 25, no. 4, pp. 563–577, 2009.

[283] J. Mesterton, A. Wimo, Å. By, S. Langworth, B. Winblad, and L. Jönsson, "Cross sectional observational study on the societal costs of Alzheimer's disease," *Current Alzheimer Research*, vol. 7, no. 4, pp. 358–367, 2010.

[284] G. Atzmon, C. Schechter, W. Greiner, D. Davidson, G. Rennert, and N. Barzilai, "Clinical phenotype of families with longevity," *Journal of the American Geriatrics Society*, vol. 52, no. 2, pp. 274–277, 2004.

[285] G. Atzmon, M. Rincon, P. Rabizadeh, and N. Barzilai, "Biological evidence for inheritance of exceptional longevity," *Mechanisms of Ageing and Development*, vol. 126, no. 2, pp. 341–345, 2005.

[286] N. Barzilai and I. Gabriely, "Genetic studies reveal the role of the endocrine and metabolic systems in aging," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 10, pp. 4493–4500, 2010.

[287] M. C. Haigis and B. A. Yankner, "The aging stress response," *Molecular Cell*, vol. 40, no. 2, pp. 333–344, 2010.

[288] D. Nipič, A. Pirc, B. Banič, D. Šuput, and I. Milisav, "Preapoptotic cell stress response of primary hepatocytes," *Hepatology*, vol. 51, no. 6, pp. 2140–2151, 2010.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 601836, 8 pages
doi:10.1155/2012/601836

Review Article

Yeast Colonies: A Model for Studies of Aging, Environmental Adaptation, and Longevity

Libuše Váchová,[1,2] Michal Čáp,[2] and Zdena Palková[2]

[1] *Institute of Microbiology of the ASCR, v.v.i., 142 20 Prague 4, Czech Republic*
[2] *Department of Genetics and Microbiology, Faculty of Science, Charles University in Prague, 128 44 Prague 2, Czech Republic*

Correspondence should be addressed to Zdena Palková, zdenap@natur.cuni.cz

Received 1 June 2012; Accepted 9 July 2012

Academic Editor: Heinz D. Osiewacz

When growing on solid surfaces, yeast, like other microorganisms, develops organized multicellular populations (colonies and biofilms) that are composed of differentiated cells with specialized functions. Life within these populations is a prevalent form of microbial existence in natural settings that provides the cells with capabilities to effectively defend against environmental attacks as well as efficiently adapt and survive long periods of starvation and other stresses. Under such circumstances, the fate of an individual yeast cell is subordinated to the profit of the whole population. In the past decade, yeast colonies, with their complicated structure and high complexity that are also developed under laboratory conditions, have become an excellent model for studies of various basic cellular processes such as cell interaction, signaling, and differentiation. In this paper, we summarize current knowledge on the processes related to chronological aging, adaptation, and longevity of a colony cell population and of its differentiated cell constituents. These processes contribute to the colony ability to survive long periods of starvation and mostly differ from the survival strategies of individual yeast cells.

1. Introduction

The molecular basis of aging has been examined using numerous methods and organisms and is one of the main biological questions under investigation. A substantial amount of information on individual gene products, metabolic pathways, signal transduction cascades, environmental factors, and cellular mechanisms impacting aging is available, but a deeper insight into how all these components interact under specific conditions and how they contribute to the process of aging is still unavailable. In addition, aging is tightly linked with the process of adaptation, through which the cells adjust to a hostile environment and can reenter the proliferative state. With metazoans, this potentially leads to tumor formation and the subsequent death of the organism.

Yeast cells have been used for a long time as a tool for identifying the genes and pathways involved in basic cellular processes such as the cell cycle, aging, and stress response. Two types of lifespan, the replicative and the chronological, have been defined and studied in the yeast *Saccharomyces cerevisiae* [1]. While the replicative (mitotic) lifespan is characterized by the number of divisions that an individual yeast cell can undergo, the chronological (postmitotic) lifespan represents a period during which nondividing (stationary phase, G_0) yeast cells remain viable. Various genes and pathways have been identified as being involved in both types of lifespan in yeast. For example, several nutrient sensing pathways affect the chronological lifespan in a way that their inactivation leads to a longevity phenotype. This was shown for the TOR (target of rapamycin), protein kinase A, and Sch9p pathways, as deletions of *TOR1*, *RAS2*, *SCH9,* or rapamycin treatment increase chronological lifespan in yeast [2–5].

Moreover, metabolic products can also modulate chronological lifespan. Acetic acid-induced medium acidification was shown to be a cause of accelerated aging in glucose-grown yeast cultures [1, 6]. Importantly, these mechanisms are evolutionary conserved from yeast to mammals, as rapamycin treatment induces longevity in yeast, worms, fruit flies, and mice [7], and lactate-induced acidification induces

senescence in human tumor cells [8]. Various high-throughput screens performed to identify the genes responsible for long-living versus short-living yeast phenotypes led to the identification of large collections of genes, which highly differed between individual screens, however [4, 9]. These observations indicate that chronological lifespan is dependent on specific environmental conditions (e.g., nutrient sources), genetic background, and developmental program. Similarly, many recent results point to the conclusion that the properties of stationary-phase cells are also dependent on environmental conditions [10–15]. In other words, there are different types of chronologically aged (i.e., stationary-phase) yeast populations under different conditions. In addition, chronological lifespan analyses in liquid cultivations are complicated by the facts that cells are heterogeneous and mutual interactions between the cellular subpopulations are difficult to analyze.

Various findings suggested that different mechanisms and regulations can occur in liquid cultivations (where yeast cells behave as unicellular individuals) and in precisely structured multicellular colonies. During the past decade, studies on multicellular yeast colonies have developed in different directions and indicated that colonies represent a promising model for studying various aspects of microbial multicellularity. Thus, findings on colony communication, adaptation, and the differing longevity of cell subpopulations occupying different colony regions raised an attractive possibility of using colonies as a model for studies of processes such as stress defense, aging, adaptation, programmed cell death (PCD), and longevity, that is, of processes that are important for any organism and that are linked to each other. In addition, recent findings on the differentiation of *S. cerevisiae* laboratory strain smooth colonies and of structured biofilm colonies formed by natural isolates of *S. cerevisiae* showed that different developmental programs can be realized by yeast communities growing on solid media [10, 16]. These programs are dependent on both environmental factors such as medium composition, and, importantly, also on phenotypic switching between different colony types that is reversible and epigenetically regulated in some cases. Thus, very probably, processes and mechanisms related to aging and adaptation that have been already identified and that are discussed in this paper are only a fraction of the broad capabilities of yeast cells developing within differently organized colonies.

In this paper, we summarize current knowledge on aging-related processes that have been described in colonies of the two different types, those formed by laboratory strains and those formed by strains isolated from natural settings.

2. Cell Growth and Developmental Phases of Smooth Colonies Formed by *S. cerevisiae* Laboratory Strains

Yeast cells growing in shaken liquid media exhibit a typical grow profile. After lag phase (adaptation to growth medium), it begins with an exponential growth phase, which is characterized by rapid growth under conditions of nutrient abundance, followed by a diauxic phase, which is characterized by slower growth as a consequence of a limitation of the preferred nutrients and modulation of the cellular metabolism to utilize alternative nutrients. A typical example is the shift from fermentative to respiratory growth. These two phases of growth are followed by a stationary phase when the growth is significantly reduced or stopped. The duration of the respective phases differs depending on the type and amount of nutrient sources and on the yeast population doubling time. Similarly, early growth of smooth colonies of *S. cerevisiae* laboratory strains starts with an exponential growth phase. On complex glucose medium (YPD), this phase lasts about 42 hours with a doubling time that is independent of colony density and similar to the doubling time of cells growing in YPD liquid medium. The majority of the cells in a colony divide during this phase [17]. Later, the number of budding cells drops to about 15% and the colonies continue to grow more slowly. During this second "slow-growth" phase, the colony population starts to diversify. While cell growth continues mostly at the colony periphery stationary-phase cells appear in the colony center, as documented by the activation of the expression of the stationary-phase specific gene *SSA3* in the colony center and growth-specific gene *ACT1* at the colony margin. In addition to preferential cell growth at the colony periphery [17, 18], cells in the uppermost layers of the colony center continue to divide efficiently after 3 days of colony growth. Cells in the lower layers of the colony center enter a stationary phase at that time [10]. Slowly dividing cells are present in all marginal regions and in the central uppermost cell layers, even in older colonies (Figure 1(b)).

In contrast to the typical diauxic shift in liquid cultures caused by the change from fermentative to respiratory growth, the shift between the exponential and the slow-growth phase of colonies was independent of the carbon source used. Thus, colonies underwent these phases on both fermentative and respiratory medium, however, the exponential phase was shorter on fermentative (about 2 days) than on respiratory medium (about 3 days) where cells grow more slowly [17]. The slow growth phase continued even after 8 days of growth, while the diauxic slow growth in liquid cultures was almost stopped after 40 hours (or even earlier) [17]. These observations are in agreement with the finding that the biomass of *S. cerevisiae* giant colonies increases linearly when growing on complex respiratory medium during a relatively long interval from day 3 to day 16–18. In colonies older than 18 days the biomass accrual is reduced, but not stopped completely [18, 19]. This growth profile is probably a consequence of two factors: firstly, only a small subpopulation of cells located in specific colony regions undergo cell division and proliferate during colony growth, and secondly, nutrients only reach the cells by the slow process of diffusion, creating conditions of the slow continuous supply of low concentrations of nutrients.

Various data indicate that the entry of colony cell population to the slow growing/stationary phase is only the starting point of various metabolic and other changes that occur during subsequent colony development. Accordingly, cells within colonies survive much longer than a yeast population

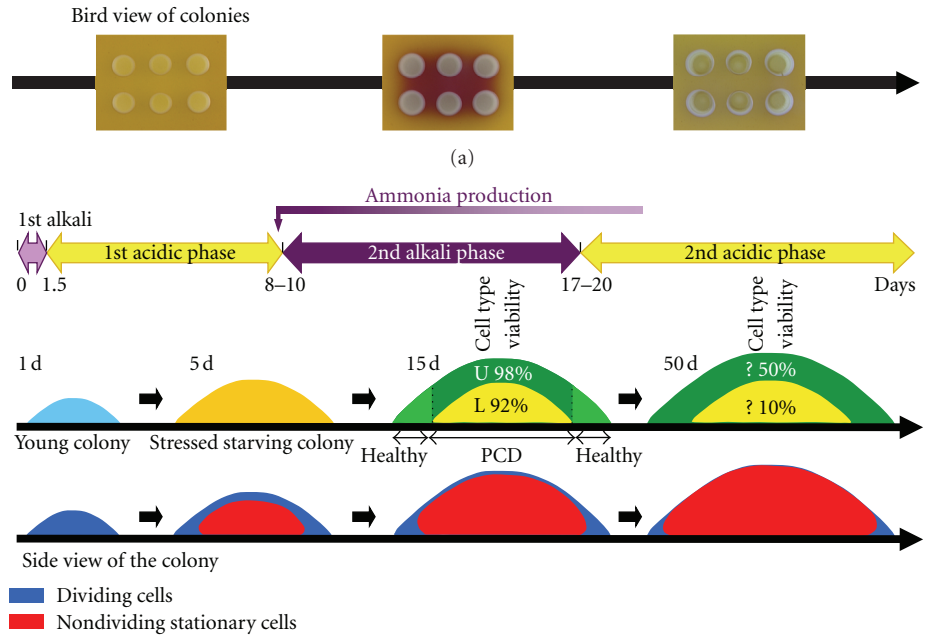


Figure 1: (a) Development of colonies formed by laboratory strain of *S. cerevisiae*. Colonies develop on complex glycerol agar with Bromocresol purple, pH dye indicator with pKa of 6.3, the color of which changes from yellow at acidic pH to purple at more alkali pH. (b) Upper part: process of colony differentiation to cells with specific metabolic properties localized in upper (U cells) and lower (L cells) layers of the colony center, respectively. Viability: percentage of colony-forming cells is given. PCD: programmed cell death. Lower part: presence of subpopulations of dividing and nondividing cells.

in shaken liquid cultivations. The number of colony forming cells (CFC) (i.e., the cells that regrow when replated on fresh media) decreased to about 2–5% after 10 days of cultivation in liquid complete synthetic glucose medium [20, 21] and to 10% and 30% after a 10-day cultivation in synthetic medium with ethanol and glycerol, respectively, as the carbon source [5]. Complex medium is much better for population survival, as 40–70% of the population survive after a 10-day cultivation in complex glucose medium (YPD) [22, 23], while respiratory complex medium shows the best results for long-term survival, as about 50–90% of cells are able to form colonies after 10 days in liquid glycerol complex medium [23, 24]. The population of the colony of a relatively long-living BY4742 strain grown on complex respiratory medium contained 90% CFC after 10 days of cultivation, which is almost double the value in liquid cultivations of the same strain grown in a similar medium [18, 19, 24]. Moreover, during prolonged cultivation the difference in survival between liquid cultivations and colonies becomes even more evident. Liquid cultures rapidly lose viability over time while a colonial population possesses a high fraction of CFC, even in very old colonies (75% on day 30, 40% on day 50, and 5% on day 135) [19]. Thus, the survival rate in any liquid cultivation does not reach the survival rate in colonies. In addition, it seems that cell survival within the colony is even better, as a subpopulation of resting cells resembling VBNC ("viable-but-non-culturable") cells of bacteria [25] emerges within the colony after day 20 of its development [19]. Such data indicate that different types of stationary-phase and slow-growing cells are present within colonies (see also below).

3. Metabolic Reprogramming and Stress Defense of Smooth Colonies

In addition to developmental changes related to cell division and colony growth, metabolic reprogramming has been observed during the development of both microcolonies and giant colonies of *S. cerevisiae* grown on complex respiratory medium. These metabolic changes are accompanied by changes in external pH, which shifts from near to alkali to acidic and *vice versa* (Figure 1(a)) [26–29]. The so-called alkali phases are linked with the production of volatile ammonia that functions as a signal influencing colony metabolic reprogramming and cell differentiation. The first alkali phase starts early after giant colonies are inoculated and lasts until about 24–35 hours, that is, it approximately correlates with the exponential growth phase described above. Later, in parallel to colony entry to the slow growth phase [17], colonies start to intensively acidify their surroundings [26]. At day 8–10 of colony growth, the second alkali phase is initiated, that lasts until approximately day 17–20 [30] and is accompanied by expressive metabolic changes. Linear biomass accrual of colonies continues during this alkali phase. Only later, when colonies enter the second acidic phase, linear biomass accrual gradually decreases. The

metabolic reprogramming that occurs during the second alkali phase includes the abrupt activation of some metabolic genes, including those of amino acid metabolism, peroxisomal functions (including fatty acid β-oxidation) and of some alternative branches of carbon metabolism (such as the methylglyoxylate cycle) [26]. Genes for various transporters including putative ammonium transporters (e.g., Ato1p) and transporters that may contribute to pH alterations (e.g., phosphate, sulphate and carboxylic acid transporters) are also strongly induced. In contrast, other cellular functions are gradually decreased, such as the expression of genes involved in mitochondrial oxidative respiration and those belonging to the group of environmental stress response genes [26]. Moreover, it was shown that the functionality of some of the stress defense enzymes such as cytosolic superoxide dismutase Sod1p, which is crucial for yeast longevity in liquid cultivations [31, 32], are dispensable in the healthy development and survival of a colony population [33]. These findings raised the intriguing possibility that chronologically aged colonies activate metabolic pathways that may allow them to exploit some previously released waste products and that are more economical than the metabolism of young colonies growing in nutrient abundance. This metabolism, paralleled with reduced mitochondrial phosphorylation, subsequently allows the cells to decrease the level of stress evoked by nutrient depletion within colonies and promotes population longevity. This adaptation, thus, subsequently makes stress eliminating systems (e.g., catalases and superoxide dismutases) unnecessary. Importantly, colonies formed by strains defective in ammonia signaling and in the activation of an adaptive metabolism decrease their vitality over long periods of starvation [33, 34]. The metabolic changes activated at the beginning of the second alkali phase partially persist in the second acidic phase and are supplemented by additional changes, the function of which is mostly unknown [30].

4. Smooth Colony Center-Margin Differentiation and PCD Contribute to Population Longevity

As indicated above, from the third day of colony growth on respiratory medium, the colony diversifies, and nondividing cells can be found in lower central colony areas (Figure 1(b)). Subsequently, the number of dividing cells gradually decreases and, for example, in 7-day old colonies dividing cells are present mostly at the colony margin and in the uppermost layer of the colony.

In addition to the localization of dividing/nondividing cells into distinct colony areas, colony stratification and the differentiation of chronologically aged nondividing cells has been discovered to occur later in aging colonies. Such differentiation is in terms of metabolic differences, stress-related characteristics and the survival of specifically localized colony cell subpopulations. In the late first acidic phase, nutrients seem to be exhausted and a level of reactive oxygen species (ROS) starts to increase in cells randomly distributed within the colony, including infant cells located at the colony margin. In parallel with the transition to the second alkali phase, the colony became differentiated both "horizontally" and "vertically" (and independently of whether cells are dividing or not).

When examined in the horizontal direction, central chronologically aging cells maintain higher stress-defense enzyme activities [30, 33] and a fraction of these cells undergoes PCD (Figure 1(b)) [18, 33]. On the other hand, relatively young infant cells at the colony margin activate the adaptive metabolism and acquire the phenotype of healthy cells with a low ROS level. This center-margin diversification is dependent on alkalization and ammonia signaling, as colonies that fail to enter the alkali phase and produce ammonia contain dying cells spread throughout the colony, including the margin region [18, 33]. Interestingly, the removal of central cells from a differentiated colony diminished accrual at the colony margin [18]. These data showed that a kind of PCD occurs within the central regions of an aged colony. PCD is regulated in parallel with medium alkalization and ammonia production, and is an important factor in the subsequent longevity and development of cells inhabiting more propitious areas at the colony margin.

Potentially, dying cells of chronologically aged central areas may release some compounds that may serve as nutrients for late colony growth. When compared with accidental (or regulated) cell lysis, PCD exhibiting apoptosis-like features prevents the release of various lytic enzymes (proteases and other hydrolases) that could destroy healthy cells located in the vicinities of dying cells. As shown by confocal microscopy, cells are tightly attached within laboratory yeast colonies [35]. Thus, PCD in colonies could have a similar purpose as the apoptosis of a fraction of the cells during the development of a real multicellular organism. On the other hand, while apoptosis in multicellular organisms depends on a group of evolutionary conserved effectors and pathways, PCD observed within the colonies is independent of the presence of the yeast metacaspase Mca1p and the yeast homologue of apoptosis-inducing factor Aif1p, which implies that programmed dying in yeast colonies is regulated independently of these evolutionary conserved factors. As alternative pathways involved in PCD in yeast are proposed [36, 37], these could regulate cell dying in yeast colonies. Such regulated dying contributes to the longevity of the whole colony by providing nutrients released by the death of one subpopulation to the benefit of the other subpopulations.

5. Differentiation of Chronologically Aged Cells in Distinct Smooth Colony Layers

A confocal microscopy study on the localization of GFP-labeled transporter Ato1p showed that the central colony population is not homogeneous and that Ato1p is exclusively produced in the upper cell layers of the colony, that is, this protein starts to be produced both in the colony margin and in upper central colony areas during colony entry to the second alkali phase [35]. The localization of Ato1p-producing cells in the upper layer of the colony center

corresponds to the localization of one of the two major cell subpopulations revealed by a recent study [10]. This study provided a complex view of the properties of these two cell subpopulations, the upper and the lower, that form the colony center. These subpopulations are formed by the differentiation of cells of late acidic-phase colonies (approximately 7-day-old colonies), that is, by the differentiation of a population composed mostly of chronologically aged cells with only a negligible fraction of slowly dividing cells in most upper colony regions. Colony stratification develops over about the next 3 days and both layers (upper and lower) are fully developed in colonies more than 10 days old. Thus, the population of chronologically aged stressed cells of the colony center differentiates into two prominent subpopulations that apparently differ from each other as well as from their ancestors (Figure 1(b)).

Cells in the upper colony layer (U cells) gain a high resistance to stress and exhibit a longevity phenotype, while cells in the lower layers (L cells) are sensitive to stress and die more rapidly [10]. This simple comparison would imply that U and L cells could resemble so called Q (quiescent) and NQ (nonquiescent) cells, respectively, described in stationary liquid cultures [38, 39]. However, transcriptomic, microscopic, and biochemical analyses revealed that the situation within a colony is more complicated. U cells, although resistant and long-living, are metabolically active cells that activate various metabolic pathways controlled by an unusual combination of nutrient sensing regulators. On the one hand, U cells activate pathways (including TOR pathway) that are usually active under nutrient-rich conditions. On the other hand, the activation of a group of amino acid metabolic genes indicates that the general amino acid control (GCN) pathway with transcription factor Gcn4p, a master regulator of amino acid biosynthetic genes, is active in U cells. Both TOR and GCN pathways monitor the availability of amino acids [40], but the GCN pathway usually opposes TOR pathway and is activated under conditions of nutrient shortage [41]. Thus, the expression of genes involved in glycolysis, pentose shunt, and translation indicates the active consumption of nutrients by U cells, but the expression of amino acid metabolic genes usually induced by amino acid starvation [41] indicates some degree of nutrient limitation in U cells. U cells also store glycogen and activate autophagy; both of these features are also found in cells entering the stationary phase. A surprising feature of U cells is the downregulation of the respiratory function of their mitochondria, which are swollen and contain only a few cristae. All of the above characteristics imply that U cells develop a unique metabolism that contributes to their longevity and stress resistance. This metabolism is dissimilar to that of Q cells identified in stationary liquid cultivations by Allen et al. [38]. These authors isolated two subpopulations of aged cells in stationary liquid cultures and named them Q cells and NQ cells. Q cells are highly respiring cells [42] and do not exhibit prominent features of U cells such as the activation of amino acid metabolic genes. Thus, similarly to the previous indications of the differing properties of cell populations from colonies and cells from liquid cultivations [33, 43], the mechanisms of aging and related metabolic reprogramming also seem to significantly differ in these two different yeast life-styles. What are the reasons for such expressive differences? In contrast to the mixed population of Q and NQ cell types within a liquid culture, cell subpopulations within colonies possess specific positions, can mutually interact with their neighbors, and can form gradients of nutrients and signaling compounds that affect remote colony subpopulations. Hence, in contrast to a liquid population, the colony behaves more like an organized primitive multicellular organism containing specialized mutually interacting cell types.

Interestingly, upper cells in 8-day-old colonies of diploid *S. cerevisiae* strains grown on sporulation acetate medium exhibit efficient meiosis and sporulation [44]. Sporulating cells are located in the interior of an unusual stalk-like structure formed by yeast cells growing from a cavity in the agar medium [45, 46]. The process of sporulation is tightly connected with limited nutrient availability and produces highly stress-resistant spores capable of long-term survival. Sporulation is thus one of the possible developmental programs after entering nutrient-limiting conditions, being an alternative to stationary-phase state.

As indicated above, U cells seem to activate metabolic pathways that allow them to consume nutrients, possibly including sugars, although they have developed from chronologically aged stressed cells of acidic-phase colonies, which grow on complete respiratory medium without glucose [10]. Features of the other subpopulation of a stratified colony, the L cells, could explain such a paradox. In contrast to U cells, L cells exhibit typical features of starving cells including a high level of ROS. These cells are able to effectively consume oxygen and they activate various degradative mechanisms, including proteasomal and vacuolar functions. The hypothesis that L cells could release nutrients to feed U cells has been therefore postulated and supported by the discovery of extracellular amino acids within a differentiated colony [10]. In addition to amino acids originating from protein degradation, carbohydrates arising from gluconeogenesis (activated in L cells) or partial cell wall degradation could be released from L cells.

6. Yeast Laboratory-Strain Colonies: A Model for Studies of Different Types of Chronologically Aged Cells

The above-mentioned discoveries indicate that the colonies of *S. cerevisiae* laboratory strains are an excellent model for studies of chronological aging. At least three different types of chronologically aged cells have been identified in these colonies: stressed cells of acidic-phase colonies, which are ancestors of the two other cell types: U cells (adapted, "healthy" chronologically aged cells of the upper colony layer) and L cells (cells with an enhanced stressed phenotype of the lower colony part) (Figure 1(b)). Interestingly, similar metabolic features to those exhibited by U cells (i.e., activation of glycolysis and decrease in mitochondrial respiration) were identified in early stationary populations of long-living deletants *tor1*Δ, *sch9*Δ and *ras2*Δ when compared to the wild type [5], indicating that the longevity phenotype

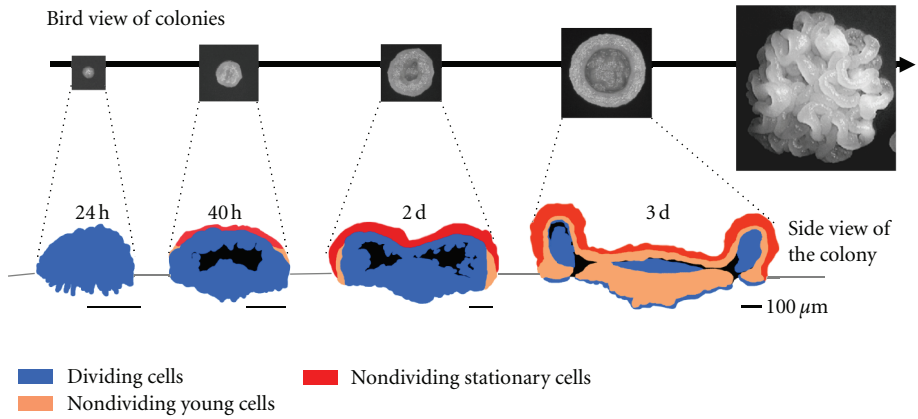


Figure 2: Development of colonies formed by wild strain of *S. cerevisiae*. Localization of dividing, young nondividing, and stationary cells within the colony is shown on vertical colony cross sections. Grey lines: surface of the agar.

is linked to specific metabolic changes. In the colonial model, however, this longevity phenotype is achieved not by a genetic manipulation, but rather in a "natural" way, that is, the process of aging and the formation of these different types of chronologically aged cells occurs during spontaneous aging and starvation of the entire colony population. The aging of colonial populations occurs within a precisely defined multicellular structure that exhibits the features of a primitive multicellular organism. The existence of the three major chronologically aged cell subpopulations enables the analysis of not only temporal but also spatial changes occurring during chronological aging and analyses of the interactions and signaling among the two aged U and L subpopulations including the formation and effects of gradients of metabolites and signaling compounds. It is also important that such types of chronologically aged cell subpopulations can be quickly isolated as relatively homogeneous populations, which enables the acquisition of strong conclusions based on measured data.

7. Structured Biofilm Colonies of *S. cerevisiae* Natural Strains

In contrast to most *S. cerevisiae* laboratory strains that form smooth colonies with the typical architecture described above, various natural *S. cerevisiae* isolates form biofilm colonies that are often noticeably structured [47]. These colonies resemble those formed by so-called nonconventional yeasts, such as those of the *Candida* and *Cryptococcus* genera. Studies of the prominent characteristics of structured *S. cerevisiae* colonies revealed that these colonies possess some features typical of yeast biofilms [16]. Such features include the production of a protective low-permeable extracellular matrix (ECM), the presence of adhesins, and production of specifically localized multidrug resistance transporters (MDR). In contrast to the above-mentioned laboratory strain colonies, relatively little is known about the aging of these structured colonies. However, a recent study on structured colony ultrastructure and differentiation indicated that the mechanisms regulating the transition from cell growth to stationary phase differ between smooth and structured colonies. In contrast to smooth colonies, where dividing cells are localized to the margin and to the uppermost colony layer (Figure 1(b)), dividing and stationary-phase cells are located differently within structured colonies [16] (Figure 2). Up to approximately 35 hours of growth, the whole of a structured colony is composed of dividing cells. This timing approximately correlates with the exponential growth phase of smooth colonies. Starting at about 40 hours of growth, a layer of stationary-phase cells appears at the top of the colony. As nutrients are spread very efficiently throughout the structured colonies [16], such a cell transition to the stationary phase is very likely unrelated to nutrient shortage, as colonies still grow in nutrient abundance. A 72-hour-old colony is composed of aerial and root structures with dividing cells detectable at precise locations in the colony interior and at the tips of the roots invading the agar (Figure 2). In contrast to smooth colonies, cells in the uppermost layers over the whole aerial part of the structured colony occur in stationary phase and new cell generations are formed within the internal colony cavity, where they are protected against the environment by various mechanisms including MDR transporters and the ECM.

When cultivated under laboratory conditions on rich nutrient media, the natural *S. cerevisiae* isolates forming structured colonies can domesticate, that is, they are capable of a phenotypic switch that results in smooth colony morphology. One can speculate that each of the colony life-styles (i.e., "biofilm-like" and "smooth") is adapted to a particular environment. In natural settings with many threats, nutrient scarcity and ever changing conditions, the protection of internal cells is the priority for the population as a whole. Cells in the outer regions of the colony are exposed to the hostile environment and enter the stationary phase state, which is characterized by high resistance to environmental impacts, as soon as possible. These colonies also invest resources and energy in the formation of an ECM that can form a scaffold that keeps distances between the cells (or cell groups) and helps to form the internal colony cavity that provides sufficient space for dividing cell progeny. As shown

in other microorganisms, ECMs can function as a sorptive sponge that sequesters organic molecules [48] and drugs [49]. Yeast ECMs also can have nutritional value [16, 50]. ECM in biofilm colony thus may play a dual function, a protective sequestration barrier and a nutrient pool for the cell progeny within the cavity. On the other hand, under stable conditions with rich initial nutrients such as those in the laboratory, the cells employ a different strategy. They do not produce an ECM scaffold, but rather form tightly packed colonies. Cells at the colony margin are those that can expand from the colony and reach nutrients in locations further away from the original colony position. In this case, stationary-phase cells are formed in the central colony regions, while cells at the margin are dividing. The question of whether the metabolic adaptive program identified in aged smooth colonies is also active in aged structured biofilm colonies remains to be elucidated.

8. Conclusions

Importantly, many genes and regulatory mechanisms are evolutionarily conserved so that the knowledge gained using primitive eukaryotes such as yeast can be often extrapolated to much more complex metazoans. Yeast multicellular colonies arising by the division of one or more of the original cells followed by a differentiation of nondividing cell population resemble multicellular organisms not only at the cellular level, but, to a limited extent, also at the level of tissue or even the whole organism. Similarly to metazoans, a significant part of the colony developmental program is likely to be determined genetically. On the other hand, environmental conditions can alter colony development and, in extreme cases, even induce a stable (genetic and/or epigenetic) modification of the developmental program. Colony "domestication," as an example of such modification, leads to the shift from structured to smooth colony morphology. This colony morphology switch is reflected in internal colony organization and differentiation, as well as in distinct ways of transition of specifically localized cell subpopulations to the stationary phase. In addition, further development of chronologically aged colonies composed of mostly stationary-phase cells is linked to diversification of these cells to various subpopulations differing in characteristic properties and in their subsequent fate. Presumably, development of these subpopulations is coordinated and includes formation and function of gradients of metabolites and signaling compounds between the subpopulations. This variability of developmental programs and diversity of different types of stationary-phase cells makes yeast colonies a perspective model for investigation of different pathways and signaling compounds involved in chronological aging, some of which may have their counterparts in metazoans.

Acknowledgments

This work was supported by the Grant Agency of the Czech Republic (204/08/0718), the Ministry of Education (MSM0021620858), Charles University in Prague (UNCE 204013), and RVO 61388971.

References

[1] M. Kaeberlein, "Lessons on longevity from budding yeast," *Nature*, vol. 464, no. 7288, pp. 513–519, 2010.

[2] P. Fabrizio, L. L. Liou, V. N. Moy et al., "SOD2 functions downstream of Sch9 to extend longevity in yeast," *Genetics*, vol. 163, no. 1, pp. 35–46, 2003.

[3] P. Fabrizio, F. Pozza, S. D. Pletcher, C. M. Gendron, and V. D. Longo, "Regulation of longevity and stress resistance by Sch9 in yeast," *Science*, vol. 292, no. 5515, pp. 288–290, 2001.

[4] R. W. Powers III, M. Kaeberlein, S. D. Caldwell, B. K. Kennedy, and S. Fields, "Extension of chronological life span in yeast by decreased TOR pathway signaling," *Genes and Development*, vol. 20, no. 2, pp. 174–184, 2006.

[5] M. Wei, P. Fabrizio, F. Madia et al., "Tor1/Sch9-regulated carbon source substitution is as effective as calorie restriction in life span extension," *PLoS Genetics*, vol. 5, no. 5, Article ID e1000467, 2009.

[6] C. R. Burtner, C. J. Murakami, B. K. Kennedy, and M. Kaeberlein, "A molecular mechanism of chronological aging in yeast," *Cell Cycle*, vol. 8, no. 8, pp. 1256–1270, 2009.

[7] M. A. McCormick, S. Y. Tsai, and B. K. Kennedy, "TOR and ageing: a complex pathway for a complex process," *Philosophical Transactions of the Royal Society B*, vol. 366, no. 1561, pp. 17–27, 2011.

[8] O. V. Leontieva and M. V. Blagosklonny, "Yeast-like chronological senescence in mammalian cells: phenomenon, mechanism and pharmacological suppression," *Aging*, vol. 3, no. 11, pp. 1078–1091, 2011.

[9] M. Matecic, D. L. Smith, X. Pan et al., "A microarray-based genetic screen for yeast chronological aging factors," *PLoS Genetics*, vol. 6, no. 4, Article ID e1000921, 2010.

[10] M. Cap, L. Stepanek, K. Harant, L. Vachova, and Z. Palkova, "Cell differentiation within a yeast colony: metabolic and regulatory parallels with a tumor-affected organism," *Molecular Cell*, vol. 46, no. 4, pp. 436–448, 2012.

[11] M. M. Klosinska, C. A. Crutchfield, P. H. Bradley, J. D. Rabinowitz, and J. R. Broach, "Yeast cells can access distinct quiescent states," *Genes and Development*, vol. 25, no. 4, pp. 336–349, 2011.

[12] J. Wu, N. Zhang, A. Hayes, K. Panoutsopoulo, and S. G. Oliver, "Global analysis of nutrient control of gene expression in *Saccharomyces cerevisiae* during growth and starvation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 9, pp. 3148–3153, 2004.

[13] L. G. Boender, M. J. Almering, M. Dijk et al., "Extreme calorie restriction and energy source starvation in *Saccharomyces cerevisiae* represent distinct physiological states," *Biochimica et Biophysica Acta*, vol. 1813, no. 12, pp. 2133–2144, 2011.

[14] L. G. Boender, A. J. van Maris, E. A. de Hulster et al., "Cellular responses of *Saccharomyces cerevisiae* at near-zero growth rates: transcriptome analysis of anaerobic retentostat cultures," *FEMS Yeast Research*, vol. 11, no. 8, pp. 603–620, 2011.

[15] D. Laporte, A. Lebaudy, A. Sahin et al., "Metabolic status rather than cell cycle signals control quiescence entry and exit," *The Journal of Cell Biology*, vol. 192, no. 6, pp. 949–957, 2011.

[16] L. Vachova, V. Stovicek, O. Hlavacek et al., "Flo11p, drug efflux pumps, and the extracellular matrix cooperate to form biofilm yeast colonies," *The Journal of Cell Biology*, vol. 194, no. 5, pp. 679–687, 2011.

[17] J. R. Meunier and M. Choder, "*Saccharomyces cerevisiae* colony growth and ageing: biphasic growth accompanied by changes in gene expression," *Yeast*, vol. 15, no. 12, pp. 1159–1169, 1999.

[18] L. Váchová and Z. Palková, "Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia," *The Journal of Cell Biology*, vol. 169, no. 5, pp. 711–717, 2005.

[19] Z. Palkova, L. Vachova, D. Gaskova, and H. Kucerova, "Synchronous plasma membrane electrochemical potential oscillations during yeast colony development and aging," *Molecular Membrane Biology*, vol. 26, no. 4, pp. 228–235, 2009.

[20] P. Fabrizio, L. Battistella, R. Vardavas et al., "Superoxide is a mediator of an altruistic aging program in *Saccharomyces cerevisiae*," *The Journal of Cell Biology*, vol. 166, no. 7, pp. 1055–1067, 2004.

[21] P. Fabrizio, S. Hoon, M. Shamalnasab et al., "Genome-wide screen in *Saccharomyces cerevisiae* identifies vacuolar protein sorting, autophagy, biosynthetic, and tRNA methylation genes involved in life span regulation," *PLoS Genetics*, vol. 6, no. 7, Article ID e1001024, 2010.

[22] L. Li, Y. Lu, L. X. Qin, Z. Bar-Joseph, M. Werner-Washburne, and L. L. Breeden, "Budding yeast *SSD1-V* regulates transcript levels of many longevity genes and extends chronological life span in purified quiescent cells," *Molecular Biology of the Cell*, vol. 20, no. 17, pp. 3851–3864, 2009.

[23] M. MacLean, N. Harris, and P. W. Piper, "Chronological lifespan of stationary phase yeast cells; a model for investigating the factors that might influence the ageing of postmitotic tissues in higher organisms," *Yeast*, vol. 18, no. 6, pp. 499–509, 2001.

[24] P. W. Piper, N. L. Harris, and M. MacLean, "Preadaptation to efficient respiratory maintenance is essential both for maximal longevity and the retention of replicative potential in chronologically ageing yeast," *Mechanisms of Ageing and Development*, vol. 127, no. 9, pp. 733–740, 2006.

[25] J. D. Oliver, "The viable but nonculturable state in bacteria," *Journal of Microbiology*, vol. 43, no. 1, pp. 93–100, 2005.

[26] Z. Palková, F. Devaux, M. Řičicová, L. Mináriková, S. Le Crom, and C. Jacq, "Ammonia pulses and metabolic oscillations guide yeast colony development," *Molecular Biology of the Cell*, vol. 13, no. 11, pp. 3901–3914, 2002.

[27] Z. Palková and J. Forstová, "Yeast colonies synchronise their growth and development," *Journal of Cell Science*, vol. 113, no. 11, pp. 1923–1928, 2000.

[28] Z. Palkova, B. Janderova, J. Gabriel, B. Zikanova, M. Pospisek, and J. Forstova, "Ammonia mediates communication between yeast colonies," *Nature*, vol. 390, no. 6659, pp. 532–536, 1997.

[29] Z. Palkova and L. Vachova, "Ammonia signaling in yeast colony formation," *International Review of Cytology*, vol. 225, pp. 229–272, 2003.

[30] L. Vachova, H. Kucerova, F. Devaux, M. Ulehlova, and Z. Palkova, "Metabolic diversification of cells during the development of yeast colonies," *Environmental Microbiology*, vol. 11, no. 2, pp. 494–504, 2009.

[31] E. B. Gralla and J. S. Valentine, "Null mutants of *Saccharomyces cerevisiae* Cu, Zn superoxide dismutase: characterization and spontaneous mutation rates," *Journal of Bacteriology*, vol. 173, no. 18, pp. 5918–5920, 1991.

[32] V. D. Longo, E. B. Gralla, and J. S. Valentine, "Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. Mitochondrial production of toxic oxygen species *in vivo*," *The Journal of Biological Chemistry*, vol. 271, no. 21, pp. 12275–12280, 1996.

[33] M. Čáp, L. Váchová, and Z. Palková, "Yeast colony survival depends on metabolic adaptation and cell differentiation rather than on stress defense," *The Journal of Biological Chemistry*, vol. 284, no. 47, pp. 32572–32581, 2009.

[34] L. Váchová, F. Devaux, H. Kučerová, M. Řičicová, C. Jacq, and Z. Palková, "Sok2p transcription factor is involved in adaptive program relevant for long term survival of *Saccharomyces cerevisiae* colonies," *The Journal of Biological Chemistry*, vol. 279, no. 36, pp. 37973–37981, 2004.

[35] L. Váchová, O. Chernyavskiy, D. Strachotová et al., "Architecture of developing multicellular yeast colony: spatio-temporal expression of Ato1p ammonium exporter," *Environmental Microbiology*, vol. 11, no. 7, pp. 1866–1877, 2009.

[36] S. Büttner, T. Eisenberg, E. Herker, D. Carmona-Gutierrez, G. Kroemer, and F. Madeo, "Why yeast cells can undergo apoptosis: death in times of peace, love, and war," *The Journal of Cell Biology*, vol. 175, no. 4, pp. 521–525, 2006.

[37] D. Carmona-Gutierrez, T. Eisenberg, S. Büttner, C. Meisinger, G. Kroemer, and F. Madeo, "Apoptosis in yeast: triggers, pathways, subroutines," *Cell Death and Differentiation*, vol. 17, no. 5, pp. 763–773, 2010.

[38] C. Allen, S. Büttner, A. D. Aragon et al., "Isolation of quiescent and nonquiescent cells from yeast stationary-phase cultures," *The Journal of Cell Biology*, vol. 174, no. 1, pp. 89–100, 2006.

[39] A. D. Aragon, A. L. Rodriguez, O. Meirelles et al., "Characterization of differentiated quiescent and nonquiescent cells in yeast stationary-phase cultures," *Molecular Biology of the Cell*, vol. 19, no. 3, pp. 1271–1280, 2008.

[40] B. Smets, R. Ghillebert, P. De Snijder et al., "Life in the midst of scarcity: adaptations to nutrient availability in *Saccharomyces cerevisiae*," *Current Genetics*, vol. 56, no. 1, pp. 1–32, 2010.

[41] A. G. Hinnebusch, "Translational regulation of *GCN4* and the general amino acid control of yeast," *Annual Review of Microbiology*, vol. 59, pp. 407–450, 2005.

[42] G. S. Davidson, R. M. Joe, S. Roy et al., "The proteomics of quiescent and nonquiescent cell differentiation in yeast stationary-phase cultures," *Molecular Biology of the Cell*, vol. 22, no. 7, pp. 988–998, 2011.

[43] M. Cap, L. Vachova, and Z. Palkova, "How to survive within a yeast colony?: change metabolism or cope with stress?" *Communicative & Integrative Biology*, vol. 3, no. 2, pp. 198–200, 2010.

[44] S. Piccirillo, M. G. White, J. C. Murphy, D. J. Law, and S. M. Honigberg, "The Rim101p/PacC pathway and alkaline pH regulate pattern formation in yeast colonies," *Genetics*, vol. 184, no. 3, pp. 707–716, 2010.

[45] D. Engelberg, A. Mimran, H. Martinetto et al., "Multicellular stalk-like structures in *Saccharomyces cerevisiae*," *Journal of Bacteriology*, vol. 180, no. 15, pp. 3992–3996, 1998.

[46] R. Scherz, V. Shinder, and D. Engelberg, "Anatomical analysis of *Saccharomyces cerevisiae* stalk-like structures reveals spatial organization and cell specialization," *Journal of Bacteriology*, vol. 183, no. 18, pp. 5402–5413, 2001.

[47] V. Šťovíček, L. Váchová, M. Kuthan, and Z. Palková, "General factors important for the formation of structured biofilm-like yeast colonies," *Fungal Genetics and Biology*, vol. 47, no. 12, pp. 1012–1022, 2010.

[48] A. W. Decho, "Microbial biofilms in intertidal systems: an overview," *Continental Shelf Research*, vol. 20, no. 10-11, pp. 1257–1273, 2000.

[49] J. E. Nett, H. Sanchez, M. T. Cain, and D. R. Andes, "Genetic basis of *Candida* Biofilm resistance due to drug-sequestering matrix glucan," *Journal of Infectious Diseases*, vol. 202, no. 1, pp. 171–175, 2010.

[50] L. M. Joubert, G. M. Wolfaardt, and A. Botha, "Microbial exopolymers link predator and prey in a model yeast biofilm system," *Microbial Ecology*, vol. 52, no. 2, pp. 187–197, 2006.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 680304, 10 pages
doi:10.1155/2012/680304

Review Article

Growth Culture Conditions and Nutrient Signaling Modulating Yeast Chronological Longevity

Júlia Santos,[1,2] Cecília Leão,[1,2] and Maria João Sousa[3]

[1] *Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, 4710-057 Braga, Portugal*
[2] *ICVS/3B's-PT Government Associate Laboratory, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, 4710-057 Braga, Portugal*
[3] *Department of Biology, Centre of Molecular and Environmental Biology (CBMA), University of Minho, 4710-057 Braga, Portugal*

Correspondence should be addressed to Maria João Sousa, mjsousa@bio.uminho.pt

Received 1 June 2012; Accepted 10 July 2012

Academic Editor: Vitor Costa

The manipulation of nutrient-signaling pathways in yeast has uncovered the impact of environmental growth conditions in longevity. Studies using calorie restriction show that reducing glucose concentration of the culture media is sufficient to increase replicative and chronological lifespan (CLS). Other components of the culture media and factors such as the products of fermentation have also been implicated in the regulation of CLS. Acidification of the culture media mainly due to acetic acid and other organic acids production negatively impacts CLS. Ethanol is another fermentative metabolite capable of inducing CLS reduction in aged cells by yet unknown mechanisms. Recently, ammonium was reported to induce cell death associated with shortening of CLS. This effect is correlated to the concentration of NH_4^+ added to the culture medium and is particularly evident in cells starved for auxotrophy-complementing amino acids. Studies on the nutrient-signaling pathways regulating yeast aging had a significant impact on aging-related research, providing key insights into mechanisms that modulate aging and establishing the yeast as a powerful system to extend knowledge on longevity regulation in multicellular organisms.

1. Cellular Pathways Modulating Aging

Aging is common to most living organisms ranging from bacteria, a unicellular prokaryotic organism, to multicellular eukaryotic organisms like humans. It is a complex biological process that involves accumulation of damage at diverse components of the organism leading ultimately to the loss of function and demise [1–3]. In the aging process, cellular activities compromised are modulated by a network of nutrient and energy sensing signaling pathways that are highly conserved among organisms. These pathways include the insulin/insulin-like growth factor 1 (Ins/IGF-1), the protein kinase/target of rapamycin (TOR), and adenylate cyclase/protein kinase A (AC/PKA) pathways [4]. Pioneering studies using mutations in key genes of these pathways have shown an increase by threefold or more in the lifespan of model organisms like yeast [5, 6], fruit flies [7], worms [8, 9], and mice [10, 11]. Many of these mutations which extend lifespan decrease the activity of the nutrient-signaling pathways mimicking a starvation state during which oxidative stress responses are induced, reducing the levels of reactive oxygen species (ROS) and oxidative damage to macromolecules [12]. Accordingly, it has been shown in different aging models (yeast, flies, worms, fish, rodents, and rhesus monkeys) that reducing growth factors/nutrients intake has profound positive effects in extension of lifespan and also improves overall health by delaying or reducing age-related diseases in mammals including diabetes, cancer and cardiovascular diseases (reviewed in [4]). One of the first models to implicate growth- /nutrient-sensing signaling with longevity was the nematode worm *Caenorhabditis elegans*. In this model it was shown that the recessive mutation in *age-I* coding for phosphatidylinositol 3-Kinase (PI3K) extends lifespan significantly [8]. Also in *C. elegans*, the insulin/IGF-1 pathway was linked to longevity by the discovery that mutating the gene coding for an insulin/IGF-1 receptor ortholog,

DAF-2, doubled its lifespan [9]. This lifespan extension was dependent on the reduction of activity of Daf-2 and consequently of its downstream effector PI3K (encoded by *age-1*), and the subsequent activation of Daf-16, a Forkhead FoxO family transcription factor (FOXO), which regulates several genes involved in stress response, antimicrobial activity, and detoxification of xenobiotics and free radicals [4, 9, 13]. Another pathway involved in longevity regulation in *C. elegans* is the conserved TOR-S6K (Ribosomal S6 protein Kinase) pathway. This pathway interacts with the insulin/IGF-1 pathway converging on the worm ortholog of regulatory associated protein of mTOR, Daf-15, to regulate larval development, metabolism, and longevity [14] and so downregulation of its activity results in extended lifespan [4].

The fruit fly *Drosophila melanogaster* is a more complex model, allowing studies based on sex differences. As in *C. elegans*, reducing the activity of the insulin/IGF-1 pathway mediates cellular protection mechanisms and the extension of lifespan in this organism. Mutations in the insulin-like receptor favour the extension of lifespan yielding dwarf sterile flies with females showing up to 85% extension of adult longevity [15]. Downregulation of the TOR pathway in flies, similarly to *C. elegans*, was shown to increase lifespan when inactivated pharmacologically with rapamycin or with overexpression of dominant-negative forms of S6K or TSC1 or TSC2, which encode negative regulators of TOR [16, 17].

Identical outcomes for genetic or pharmacologic manipulation of insulin/IGF-1 and TOR pathways and for dietary restriction regimes were observed in *D. melanogaster* and *C. elegans*, as well as in yeasts, establishing the evolutionary conserved roles of these pathways in determining lifespan and implicating them as mediators of the protective effects of dietary restriction in different species [4, 18].

In mammals, hormones of the endocrine system, the growth hormone, insulin-like growth factor-1 (IGF-1), and insulin pathways are key players in the hormonal control of aging in association with an increase of antioxidant defenses and increased stress resistance (reviewed in [19]). Deficiency in levels of circulating growth hormone has been shown to enhance antioxidant defenses and stress resistance, reduce tumor burden, and to increase insulin sensitivity (reviewed in [20]). Enhanced insulin sensitivity is a common feature of long-living mutant mice and in humans, studies of centenarian populations strongly correlates this increase in insulin sensitivity with longevity [21, 22]. IGF-1 and insulin also modulate TOR activity through Akt kinase which is a downstream effector of the insulin/IGF-1 pathway [23], and inhibition of mTOR pathway by rapamycin [24] or deletion of its downstream effector S6K, increases mice lifespan [25]. The lifespan extension due to the deletion of S6K was accompanied by slower progression of age-related pathologies and in particular slower loss of insulin sensitivity [25].

Another pathway involved in longevity regulation is the AC/PKA pathway that is conserved from yeast to mammals. Downregulation of the Ras/AC/PKA pathway was first shown in yeast to have a major effect on lifespan extension [4, 6, 26]. Only recently, studies correlating AC/PKA pathway with aging and age-related diseases started to emerge in mammals. Deletion of the mouse AC type 5, which mediates PKA activity by modulating cAMP levels, was reported to significantly increase lifespan, as it does in yeast [27], and improve cardiac stress resistance [28]. Likewise as described for yeast [26], deletions of PKA subunits in mice have recently been shown to increase lifespan while protecting against age-related deleterious changes such as weight gain, hypertrophic liver, and cardiac dysfunction [29]. Although the subunits deleted are the regulatory subunits (RIIβ) and not the catalytic subunits like in yeast (TPK), loss of RIIβ in mice causes a concomitant and compensatory decrease in catalytic subunits showing a mechanistic association between loss of these subunits and lifespan extension [29]. This converging result in such divergent models suggests a highly conserved role for PKA in longevity and opens the possibility for new therapeutic targets for aging and obesity.

In mammals the Ras proteins do not directly signal to PKA through AC [30] as it occurs in yeast [31]; however, a recent study reported that homozygous deletion of Ras-GRF1 promotes both median and maximum longevity in mice [32]. Ras-GRF1 is a guanine nucleotide exchange factor (GEF) responsible for activating Ras by favouring its GTP-bound state [33], suggesting that the cause of longevity extension of the Ras-GRF1 deletion could be the reduction of Ras activity [34]. Therefore, the Ras pathway appears as a conserved pathway in the aging process from yeast to mammals [34].

Yeast has emerged as a highly exploited model to study the environmental and genetic factors affecting longevity. In particular, the genetic tools now available make yeast one of the best established experimental model organisms for screening genes involved in the regulation of fundamental cellular process including the pathways controlling lifespan. In the following sections we focus on the particular case of the lifespan in yeast and its modulation triggered by extrinsic culture medium factors. We start with basic aspects of both replicative and chronological lifespan in yeast. The subsequent sections are dedicated to an overview highlighting the impact of culture medium and products of fermentation on the yeast chronological lifespan.

2. Chronological and Replicative Lifespan in Yeast

Two yeast lifespan models have been characterized: replicative lifespan (RLS) and chronological lifespan (CLS) (reviewed in [35]). RLS is defined as the total number of times a single mother cell can undergo a mitotic event and originate daughter cells before senescence [36]. RLS is accurately measured by moving and counting small daughters away from the mothers via microscopic manipulation and simulates aging of mitotically active mammalian cells [35, 37, 38]. On the other hand, CLS defines the length of time non-dividing yeast cells remain viable [39] thus simulating aging

of the postmitotic mammalian cells [40, 41]. This viability is assessed by cells reentering the cell cycle after transfer from the depleted medium or water to nutrient complete-medium [35]. In CLS, two types of metabolic yeast cells can be studied: postdiauxic or stationary phase cells. Both metabolic state cells are grown in synthetic complete (SDC) medium but while postdiauxic cells are kept in the culture medium, the stationary phase ones are transferred to water (extreme calorie restriction) after 3 days of growth. Some protocols for postdiauxic cells could also use cells grown in YPD (yeast extract, peptone, dextrose) medium [35, 42] instead of SDC medium.

The two paradigms of aging in yeast, CLS and RLS, have become useful tools to compare the aging process in proliferating and nonproliferating cells as well as to serve as models to study the mechanics of the aging process in mitotic and postmitotic cells of multicellular organisms [38].

The yeast *S. cerevisiae* divides by budding and therefore undergoes asymmetrical cell division, with the mother cell retaining more volume than the daughter cell. In this asymmetric division mother cells retain most of the age-associated damage, thus sacrificing individual replicative capacity while daughter cells retain full replicative potential [43, 44]. One of the aging factors affecting RLS is the accumulation of extrachromosomal ribosomal DNA circles (ERCs) [45]. These circular DNA molecules are self-replicating units formed in the nucleus by homologous recombination between adjacent rDNA repeats which segregate asymmetrically to the mother-cell nucleus during cell division. During each division ERCs replicate leading to an exponential accumulation in the mother cell and consequently to cell senescence [44, 45]. This finding in yeasts came in large part from the study of important age-related proteins called sirtuins. Sirtuins are NAD^+-dependent protein deacetylases involved in chromatin silencing and known to mediate longevity in yeast, nematodes, flies, and mammals [40, 44, 46]. Deletion of *SIR2* decreases RLS and its overexpression increases RLS showing that Sir2p mediates RLS in yeast most probably by regulating rDNA recombination and ERCs formation [47]. ERCs appear to be an aging factor specific to yeast, although without relevance in nondividing yeast cells (CLS) and so far without a role in aging of multicellular eukaryotes [46, 48].

Another factor known to decrease longevity both in CLS as RLS is accumulation of oxidative damage due to the production of reactive oxygen species (ROS). Deletion of the yeast antioxidant defense enzymes superoxide dismutases (SOD), reduces significantly CLS [39] and RLS [49]. However, overexpression of cytosolic (*SOD1*) and mitochondrial (*SOD2*) superoxide dismutases increased longevity of nondividing cells [50] while it decreased RLS. Although studies show several similarities but also major differences between CLS and RLS mechanistic regulation, these two models are interconnected as RLS decreases in chronologically aged cells [48]. In addition, both aging models are regulated by the nutrient-signaling kinases, as screenings for long-lived mutants identified the same gene mutations in both paradigms [27, 51–53].

3. Glucose-Signaling Pathways Involved in Yeast Longevity

The most common dietary regimes used to study the interaction between nutrient-signaling pathways and longevity include: dietary restriction (DR) in which the intake of nutrients, but not necessarily calories, is reduced without causing malnutrition; calorie restriction (CR), a regime in which only calories are reduced without compromising other nutrients, for instance amino acids and vitamins [54, 55].

In yeast, when studying both replicative lifespan (RLS) and chronological lifespan (CLS), several results correlating environmental growth conditions and longevity emerged. Many studies, including those using calorie restriction (CR), showed that reducing the glucose or amino acids concentrations of the culture media is sufficient to increase replicative and chronological lifespan [56–60]. The composition of culture media has proven to be an extrinsic factor affecting chronological lifespan but this is still giving rise to different interpretations on longevity regulation.

The manipulation of nutrient-signaling pathways for the study of aging regulating mechanisms, as previously mentioned, can be accomplished by genetic manipulations of key components of these pathways or by dietary (DR) and calorie restriction (CR). In yeast, the vast majority of protocols for CR are based on the decrease of the glucose concentration in the medium from the standard 2% to 0.5 or 0.05%. The latter (0.05% glucose) is considered extreme calorie restriction as well as the one achieved by transferring cells grown in 2% glucose to water [35, 61].

The first studies to report glucose as an agent affecting lifespan in yeast were conducted by Granot and coworkers who showed that addition of glucose to stationary-phase cells previously transferred to water leads to a reduction of CLS [62]. The authors further demonstrated that glucose, in the absence of other complementing nutrients, induces apoptotic cell death accompanied by an increase in ROS production [63]. Further studies in yeast have revealed that the major nutrient-signaling pathways TOR, SCH9, and Ras/AC/PKA are all involved in longevity regulation by glucose [6, 46, 58]. These pathways promote cell division and growth in response to nutrients while inhibiting the general stress response and autophagy. *SCH9* was one of the first genes to be implicated in CLS [27]. Sch9p is the yeast closest homolog of the mammalian AKT/PKB and S6K, and its deletion leads to an increase in both CLS and RLS [27, 53]. Sch9p is a kinase that mediates PKA activation in the fermentable-growth-medium-(FGM)-induced pathway and also mediates many of the TOR complex 1 (TORC1) controlled processes [64–66]. Sch9p was first described as having a partially redundant role with PKA pathway, since deletion of *SCH9* could be compensated by increased activity of PKA and vice versa [67], and later as a direct target of TORC1 regulation [65]. More recently, TORC1 was also identified as a target for regulating longevity in both CLS and RLS [51, 52]. The TOR pathway responds to nitrogen and carbon sources, mainly to control cell growth, through the regulation of processes such as translation initiation, ribosome biogenesis, mRNA and amino acid permeases stability, transcription of

nutrient-regulated genes and stress response genes, and actin cytoskeleton organization and autophagy [68, 69]. Reduction of TORC1-Sch9p signaling was shown to promote longevity by increasing the expression of stress-response genes in a Rim15p-dependent manner [58], as *RIM15* deletion reduced the lifespan extension of the long-lived *sch9*Δ cells [27]. Alternatively, a recent study proposes a Rim15p-independent mechanism for lifespan extension in reduced TORC1-Sch9p signaling [70]. This study shows that in *tor1*Δ cells, CLS is reduced if mitochondrial respiration is uncoupled. The authors suggest that during growth, mitochondrial ROS signaling downregulates both the mitochondrial membrane potential and ROS accumulation of stationary phase cells to promote their longevity [70]. This is in agreement with previous data showing that preadaptation to respiratory growth can also promote extension of CLS [71]. CR also promoted CLS extension by doubling the lifespan of the long-lived *sch9*Δ and *tor1*Δ cells by a Rim15p partially dependent mechanism. In fact, cells with a triple mutation in Rim15p downstream transcription factors (*msn2*Δ *msn4*Δ *gis1*Δ) do not display a reduction of CR promoted CLS extension when compared to the long-lived *sch9*Δ and *tor1*Δ cells suggesting the involvement of additional Rim15p independent transcriptional factors [58].

Another pathway involved in aging is the other major nutrient-signaling pathway Ras/AC/PKA, responsible for the link between glucose availability and the control of growth, proliferation, metabolism, stress resistance, and longevity [6, 27, 50, 72]. Deletion of *RAS2* or a reduced activity of adenylate cyclase (Cyr1p), which is activated by the Ras proteins, causes lifespan extension and stress resistance [50]. Mutation in the *CYR1* gene increases both RLS and CLS while deletion of *RAS2* decreases RLS [53, 73]. Rim15p also mediates *ras2*Δ lifespan extension by enhancing cellular protection against oxidative stress through the activation of *SOD2* [50], indicating that Rim15p is a common denominator of the pathways Ras/AC/PKA, Sch9p, and TOR. In addition, deletion of *MSN2/4* in *ras2*Δ cells leads to lifespan reduction indicating that Msn2p/4p and Gis1p transcription factors controlled by Rim15p are also required for CLS extension. Nevertheless, the Rim15p downstream transcription factors (Msn2p, Msn4p, and Gis1p) appear to have different roles in *sch9*Δ and *ras2*Δ cells given that only the abrogation of *GIS1*, and not of *MSN2/4*, was shown to almost completely abolish the lifespan expansion of *sch9*Δ cells [27, 58]. Therefore, Sch9p and Ras2p seem to differentially modulate the common downstream effectors, which is also corroborated by the higher stress resistance and increased CLS exhibited by *ras2*Δ *sch9*Δ double knockout cells in comparison to the single deletion mutants [58, 74].

More recently, the correlation between glucose signaling, oxidative stress and aging was further addressed in a study showing that increasing glucose from the standard 2% to 10%, promotes a shortening of CLS accompanied by increased levels of intracellular superoxide anion ($O_2^{\bullet -}$), decreased levels of hydrogen peroxide (H_2O_2), reduced efficiency of stationary phase G_0/G_1 arrest, and activation of DNA damage [12]. On the other hand, CR by reducing glucose or by deletion of *SCH9* or *TOR1* extends CLS and diminishes superoxide anion levels promoting at the same time a more efficient G_0/G_1 arrest. These and other results point to superoxide levels as one of the key factors regulating aging [75], which is in agreement with the aforementioned results showing that reduction of signaling pathways leads to the activation of oxidative stress responses mediated by Rim15p [12]. Nevertheless, an alternative activation of oxidative stress responses independent of Rim15p [58] and mediated by H_2O_2 has been also reported [12]. Furthermore, high levels of H_2O_2, which respond to glucose in an inversely dose-dependent manner, promotes activation of SODs, leading to a reduction in superoxide anion levels and therefore to CLS extension [12, 76].

4. Amino Acid Metabolism in the Regulation of the Yeast Chronological Lifespan

In nature, yeast cells enter a resting or quiescent state in the absence of favorable nutritional conditions. When inadequate carbon, nitrogen, sulfur, or phosphorus levels are sensed by yeast cells, growth ceases and cell cycle is arrested as a survival strategy. In natural environments, yeast are prototrophs capable of synthetizing most of their metabolites from simple carbon and nitrogen sources, whereas laboratory strains commonly have auxotrophic markers that confer a nutrient-limiting growth phenotype useful for genetic manipulation. These markers are usually genes involved in the biosynthesis of specific amino acids or nucleotides. Amino acids are important nutrients that can also be recycled by autophagy. This recycling process maintains amino acid homeostasis and is crucial for cell survival under nitrogen starvation leading to rapid loss of viability in autophagy-defective mutants [77] and therefore has been implicated in CLS regulation. Curiously, it was demonstrated that prototrophic and auxotrophic strains display different responses to nutrient starvation [78]. Starvation of "natural" nutrients leads to an arrest in G_0/G_1 cell cycle phase of prototrophics cells, while auxotrophic cells failed to arrest the cell cycle upon starvation of "supplemental" nutrients (auxotrophic nutrients) [78]. It was also observed that auxotrophic cells limited for leucine or uracil consume glucose at a much faster rate, exhausting it from the medium, than prototrophic cells limited for phosphate, sulfate, or ammonium that spare glucose [79]. These findings clearly reveal a failure of auxotrophic cells in regulating nutrient sensing in response to starvation of "supplemental" nutrients [80]. Furthermore, limiting levels of auxotrophy-complementing amino acids, in the growth medium, induce an early arrest in G_2/M phase, negatively affecting chronological longevity and leading to a premature aging phenotype [81]. In accordance, reduction of total amino acid levels, including essential ones, in the medium also decreases CLS [82]. Starvation for leucine in nondividing leucine auxotrophic cells induces a rapid loss of viability [80]. Nevertheless, this phenotype is partially dependent on the carbon source present in the starvation medium but not in that used in the growth medium. For example the presence of ethanol/glycerol or galactose in the starvation medium increases CLS in contrast to starvation

in glucose [80]. However, not all essential amino acids have the same effect on CLS. In fact, methionine starvation of methionine auxotrophic cells has no effect on viability [83]. Another study also reported that from the auxotrophic-complementing amino acids, lysine, histidine, and leucine, the latter has a more pronounced negative effect in CLS in both autophagy-competent and autophagy-deficient strains [84]. The authors pointed out that the enhanced sensitivity of yeast cells to leucine starvation is correlated to the high levels of leucine codon, the most frequent amino acid codon [84]. CLS is extended by the presence of nonessential amino acids, particularly isoleucine, and its precursors threonine and valine, via the general amino acid control (GAAC) pathway. The authors proposed a mechanism for CLS regulation by the branched side chain amino acids (BCAA) leucine, isoleucine and valine, in which low levels of these amino acids induce the GAAC pathway therefore shortening CLS and vice versa [84].

Starvation for nonessential amino acids was reported to extend RLS [56] and starvation for preferred amino acids such as asparagine- or glutamate- induced CLS extension in direct proportion to the nature of the amino acid removed [52].

5. Impact of Products of Fermentation in the Yeast Chronological Lifespan

Ethanol is the main product resulting from alcoholic fermentation and it is used as a carbon source during the diauxic shift and postdiauxic phase. Nevertheless, ethanol is known to negatively affect the metabolic activity of the yeast cells by inhibiting cell growth and fermentation. It is also known to cause among others the damage of cell membranes by increasing membrane fluidity [85, 86] and the inhibition of transport systems across the plasma membrane [87, 88]. The severity of the effects is dependent on the alcohol concentration and at high ethanol levels it results in cell death [89]. Recently, ethanol was described as an apoptotic inducer [90] and has also been implicated as an extrinsic factor in aging, significantly decreasing CLS of severely calorie restricted strains (CR in water), known for their lifespan extension in this condition [91]. In contrast to wild type cells, long-lived *sch9*Δ cells consume all the ethanol from the medium during chronological aging, further supporting ethanol as a modulator of aging [91].

A recent study on the genetic expression profile of long-lived *tor1*Δ, *sch9*Δ, and *ras2*Δ cells revealed an upregulation of genes involved in the metabolism of glycerol. In contrast to wild type cells that accumulate ethanol and rapidly deplete glycerol, those long-lived mutant cells accumulate glycerol whereas ethanol was early depleted. These observations suggest that inhibition of Tor1p/Sch9p mediates a metabolic switch from biosynthesis and release of ethanol to activation of glycerol biosynthesis and its consequent release [74]. Glycerol, unlike glucose and ethanol [58, 61, 91], does not promote aging or cell death and so this metabolic change extends CLS [74, 92].

In calorie restricted cells, ethanol is completely consumed before the beginning of viability decline. Conversely, noncalorie restricted cells were unable to completely consume ethanol before viability decline. The authors suggested a correlation between ethanol accumulation and loss of peroxisome function in noncalorie restricted cells since ethanol suppresses the synthesis of certain proteins localized to peroxisomes [93].

Acetic acid is a byproduct of fermentative metabolism in yeast accumulating in the medium during fermentation of glucose to ethanol and is also one factor described to affect CLS [94, 95]. After sugar is depleted in 2% glucose standard conditions, a shift in metabolism occurs from fermentation to respiration and the metabolization of ethanol also leads to the production and accumulation of acetic acid. Acetic acid is a well-known inducer of apoptotic cell death leading to ROS production [96, 97]. In a recent study, Burtner and coworkers identified acetic acid as an important extracellular factor affecting CLS in SDC medium [94]. The authors showed that cells grown for 48 hours under extreme calorie restriction conditions (0.05% glucose concentration), known to extend CLS, rapidly loss viability if transferred to cell-free supernatants of 2% standard glucose-depleted medium, indicating that cell-extrinsic aging factors were present in the SDC depleted medium [94]. Although several other organic acids also accumulate in the culture medium during chronological aging, only acetic acid was identified as being sufficient to cause chronological aging [94]. In the same study it was also shown that buffering of aging cultures to pH 6 is sufficient to increase CLS, neutralizing the toxic effect of acetic acid. Actually, the acetate anion is not readily taken up from the environment by glucose-grown yeast cells, but the protonated acetic acid can cross the plasma membrane resulting in intracellular acidification [98]. This negative effect of acetic acid in CLS was diminished by mutational inactivation of conserved signaling pathways, namely deletion of *SCH9* and *RAS2,* conferring resistance via unknown mechanisms [94]. *SCH9* and *RAS2* mutant cells are known to have a more frequent growth arrest in G_1 phase when compared to the wild-type, promoted by the reduction in growth signaling in these mutants [99]. In accordance, nutrient-depleted stationary phase cells are continuously subjected to acetic acid-induced growth signals, even in the absence of glucose, that promote cell cycle progression and consequently replication stress due to the lack of favorable conditions [100]. These and other results show that acetic acid, as glucose, activates Sch9p and RAS pathways and seems to mediate cell death by promoting the accumulation of superoxide anion ($O_2^{\bullet -}$) in consequence of downregulation of SODs and other oxidative stress defenses by the activated pathways [12]. The long-lived *ade4*Δ cells (Ade4p is involved in the purine *de novo* biosynthetic pathway) do not accumulate acetic acid in the culture medium when compared to the wild type cells, while the short-lived *atg16*Δ cells (Atg16p is involved in the autophagic process) accumulate acetic acid at higher concentrations than the wild type cells, inversely correlating the amount of acid release from cells and the extension of CLS [101]. Buffering the growth media to pH 6.0 of

the short-lived *atg16*Δ cells and the wild type strain, also dramatically increase CLS to the same levels obtained for the CR growth condition and for the long-lived *ade4*Δ cells, indicating that pH neutralized the toxic effects of acetic acid. Overall the results demonstrate that acetic acid can have an important impact on CLS through a cell extrinsic mechanism that is dependent on media pH.

6. Ammonium Toxicity in Aging Yeast Cells

Ammonium (NH_4^+) toxicity has been well described in animals and plant systems [102]. In yeast, NH_4^+ is commonly used as nitrogen source for growth and it is usually not toxic having a central role in nitrogen metabolism both in degradative and biosynthetic pathways [103]. In yeast, nitrogen sources, prior to their use, need to be converted into glutamate and glutamine. However not all nitrogen sources are equally preferred and yeast can select the nitrogen sources through nitrogen catabolite repression (NCR) mechanism also known as nitrogen discrimination pathway (NDP). This pathway enables yeast to repress genes that code for proteins required for the use of poor nitrogen sources, when in the presence of sufficient quantities of rich nitrogen sources like glutamine [104].

Production of ammonia in yeast colonies has even been described as a mechanism of protection from cell death during colony development [105]. An excess of ammonium was found to be toxic for *S. cerevisiae*, under potassium limitation, resulting in amino acid excretion similar to the detoxifying mechanism found in mammals [106]. The authors described that ammonium toxicity in yeast is related to a "leak current" of ammonium ions that enter the cell through potassium channels, in limiting potassium conditions, and this influx causes an excess of internal ammonium that becomes toxic for the cell. To cope with this ammonium excess, cells excrete amino acids possibly through the Ssy1p-Ptr3p-Ssy5p (SPS)-system of amino acid transporters, which were found to be strongly upregulated in this condition, or by directly excreting ammonium via the Ato (Ammonium Transporter Outward) transporters [106].

Recently, we have reported that ammonium is toxic for aging cells and acts as an extrinsic factor affecting CLS [107]. In this study, it was shown that decreasing the concentration of NH_4^+ in the culture medium increases yeast CLS in amino acid restriction conditions. In contrast, when the initial $(NH_4)_2SO_4$ concentration in the culture medium, either with or without restriction of amino acids, was increased from 0.5% to 1%, there was a decrease in cell survival, demonstrating that the toxic effects of ammonium are correlated with its concentration in the culture medium. Moreover, after transferring cultured cells to extreme calorie restriction conditions in water, the addition of ammonium drastically decreases the CLS, indicating that ammonium alone could also induce loss of cell viability as observed in culture media, and providing, for the first time, a role for ammonium in chronological longevity regulation [107].

Cells starved for auxotrophic-complementing amino acids are particularly sensitive to ammonium-induced cell death [107]. Death induced by ammonium in these cells is mediated through the regulation of the evolutionary conserved pathways PKA, TOR, and SCH9 and is accompanied by an initial apoptotic cell death followed by a fast secondary necrosis. Autophagy, which has been described as essential for cell survival during nitrogen starvation and regulating amino acid homeostasis [84], does not seem to have a role in ammonium-induced cell death.

The ammonium effects were also not dependent on its metabolism as activity of enzymes involved in the metabolism of ammonium showed no correlation with ammonium toxicity and the use of a nonmetabolizable analog produced the same outcome as ammonium [107]. Even though, ammonium signaling is capable of activating the PKA pathway in agreement with previous results showing that the addition of ammonium, in nitrogen starvation medium, directly signals PKA activation through Mep2p [108]. In contrast, in aging yeast, although Mep2p is involved in ammonium-induced death it does not appear to have a major role in PKA activation. Tor1p and Sch9p were shown to be necessary for ammonium-induced PKA activation in amino acid-starvation conditions as deletion of *TOR1* and *SCH9* resulted in a decrease of PKA activation. Ammonium action on both pathways, resulting in the over-activation of PKA and TOR pathways and inhibition of Sch9p, culminates in the shortening of CLS [107].

Altogether results support that ammonium induces cell death in aging cultures through the regulation of evolutionary conserved pathways. They also show that the study of ammonium toxicity in yeast aging may be a powerful system to understand longevity regulation in multicellular organisms.

7. Final Remarks

Yeast has emerged as one of the most important model organisms to study the environmental and genetic factors affecting longevity, and its exploitation has made huge contributions to the progress in understanding aging. Although some aspects of aging in yeast are specific to this organism, many of the most important features reveled in yeast proved to be evolutionarily conserved in higher eukaryotic organisms. The two paradigms of aging in yeast, chronological and replicative lifespans, are useful tools to compare the aging process in proliferating and nonproliferating cells and to study the aging process in mitotic and postmitotic cells. The pathways controlling yeast lifespan occur through complex signaling cascades, allowing cells to stimulate proliferation in optimal conditions and also to induce cell cycle arrest and enter into a quiescent state in nutrient exhaustion conditions. In the present paper, we focused on the particular case of the lifespan in yeast and its modulation triggered by extrinsic culture medium factors. A scheme illustrating the current scenario on the regulation of CLS by different nutrient/energy signalling pathways in yeast described herein is presented in Figure 1. Major advances in this research field have come from dietary regimes that have been shown to increase longevity in organisms ranging

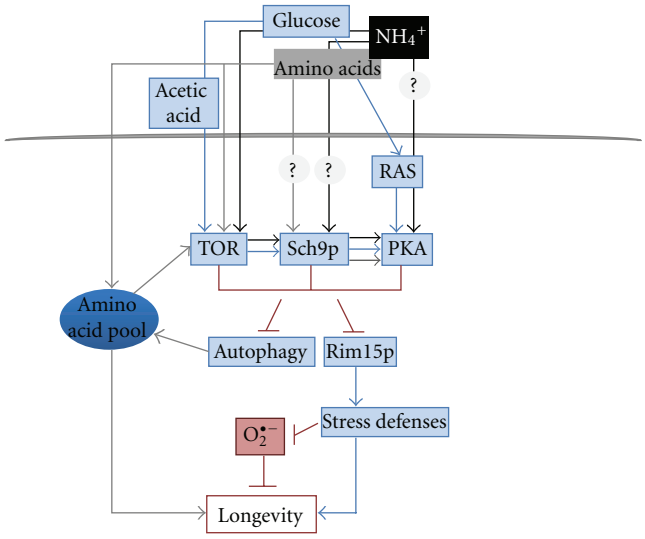


Figure 1: Scheme illustrating the current scenario on the regulation of chronological lifespan (CLS) in yeast by the different nutrient/energy signalling pathways described in the present paper (the detailed description and abbreviations are given in the text).

from yeast to mammals. Altogether, data presented clearly establishes that the carbon and the nitrogen sources as well as the products of fermentation, are among the main extrinsic factors modulating yeast chronological longevity. Loss of cell viability induced by these environmental factors in aging cultures is regulated through evolutionary conserved pathways and their study can provide key insights into pathways that modulate aging in mammals, being a powerful system to understand longevity regulation in multicellular organisms.

Authors' Contribution

M. J. Sousa and C. Leão contributed equally to this work.

Acknowledgments

This work was supported by Fundação para a Ciência e Tecnologia (FCT), Portugal Grant PTDC/AGR-ALI/102608/2008. J. Santos received a fellowship from FCT (SFRH/BD/33314/2008).

References

[1] J. M. G. Gómez, "Aging in bacteria, immortality or not-a critical review," *Current Aging Science*, vol. 3, no. 3, pp. 198–218, 2010.

[2] E. L. Greer and A. Brunet, "Signaling networks in aging," *Journal of Cell Science*, vol. 121, no. 4, pp. 407–412, 2008.

[3] S. D. Narasimhan, K. Yen, and H. A. Tissenbaum, "Converging pathways in lifespan regulation," *Current Biology*, vol. 19, no. 15, pp. R657–R666, 2009.

[4] L. Fontana, L. Partridge, and V. D. Longo, "Extending healthy life span-from yeast to humans," *Science*, vol. 328, no. 5976, pp. 321–326, 2010.

[5] V. D. Longo, "Mutations in signal transduction proteins increase stress resistance and longevity in yeast, nematodes, fruit flies, and mammalian neuronal cells," *Neurobiology of Aging*, vol. 20, no. 5, pp. 479–486, 1999.

[6] V. D. Longo, "The Ras and Sch9 pathways regulate stress resistance and longevity," *Experimental Gerontology*, vol. 38, no. 7, pp. 807–811, 2003.

[7] J. Sun and J. Tower, "FLP recombinase-mediated induction of Cu/Zn-superoxide dismutase transgene expression can extend the life span of adult Drosophila melanogaster flies," *Molecular and Cellular Biology*, vol. 19, no. 1, pp. 216–228, 1999.

[8] D. B. Friedman and T. E. Johnson, "A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility," *Genetics*, vol. 118, no. 1, pp. 75–86, 1988.

[9] C. Kenyon, J. Chang, E. Gensch, A. Rudner, and R. Tabtiang, "A C. elegans mutant that lives twice as long as wild type," *Nature*, vol. 366, no. 6454, pp. 461–464, 1993.

[10] H. M. Brown-Borg, K. E. Borg, C. J. Meliska, and A. Bartke, "Dwarf mice and the ageing process," *Nature*, vol. 384, no. 6604, p. 33, 1996.

[11] K. T. Coschigano, D. Clemmons, L. L. Bellush, and J. J. Kopchick, "Assessment of growth parameters and life span of GHR/BP gene-disrupted mice," *Endocrinology*, vol. 141, no. 7, pp. 2608–2613, 2000.

[12] M. Weinberger, A. Mesquita, T. Caroll et al., "Growth signaling promotes chronological aging in budding yeast by inducing superoxide anions that inhibit quiescence.," *Aging*, vol. 2, no. 10, pp. 709–726, 2010.

[13] C. Kenyon, "The plasticity of aging: insights from long-lived mutants," *Cell*, vol. 120, no. 4, pp. 449–460, 2005.

[14] K. Jia, D. Chen, and D. L. Riddle, "The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span," *Development*, vol. 131, no. 16, pp. 3897–3906, 2004.

[15] M. Tatar, A. Kopelman, D. Epstein, M. P. Tu, C. M. Yin, and R. S. Garofalo, "A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function," *Science*, vol. 292, no. 5514, pp. 107–110, 2001.

[16] I. Bjedov, J. M. Toivonen, F. Kerr et al., "Mechanisms of life span extension by rapamycin in the fruit fly drosophila melanogaster," *Cell Metabolism*, vol. 11, no. 1, pp. 35–46, 2010.

[17] L. Partridge, N. Alic, I. Bjedov, and M. D. W. Piper, "Ageing in Drosophila: the role of the insulin/Igf and TOR signalling network," *Experimental Gerontology*, vol. 46, no. 5, pp. 376–381, 2011.

[18] P. Kapahi, D. Chen, A. N. Rogers et al., "With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging," *Cell Metabolism*, vol. 11, no. 6, pp. 453–465, 2010.

[19] H. M. Brown-Borg, "Hormonal regulation of longevity in mammals," *Ageing Research Reviews*, vol. 6, no. 1, pp. 28–45, 2007.

[20] A. Bartke and H. Brown-Borg, "Life extension in the dwarf mouse," *Current Topics in Developmental Biology*, vol. 63, pp. 189–225, 2004.

[21] T. Kojima, H. Kamei, T. Aizu et al., "Association analysis between longevity in the Japanese population and polymorphic variants of genes involved in insulin and insulin-like growth factor 1 signaling pathways," *Experimental Gerontology*, vol. 39, no. 11-12, pp. 1595–1598, 2004.

[22] D. Van Heemst, M. Beekman, S. P. Mooijaart et al., "Reduced insulin/IGF-1 signalling and human longevity," *Aging Cell*, vol. 4, no. 2, pp. 79–85, 2005.

[23] A. J. Levine, Z. Feng, T. W. Mak, H. You, and S. Jin, "Coordination and communication between the p53 and IGF-1-AKT-TOR signal transduction pathways," *Genes and Development*, vol. 20, no. 3, pp. 267–275, 2006.

[24] D. E. Harrison, R. Strong, Z. D. Sharp et al., "Rapamycin fed late in life extends lifespan in genetically heterogeneous mice," *Nature*, vol. 460, no. 7253, pp. 392–395, 2009.

[25] C. Selman, J. M. A. Tullet, D. Wieser et al., "Ribosomal protein S6 kinase 1 signaling regulates mammalian life span," *Science*, vol. 326, no. 5949, pp. 140–144, 2009.

[26] S. J. Lin, P. A. Defossez, and L. Guarente, "Requirement of NAD and SIR2 for life-span extension by calorie restriction in saccharomyces cerevisiae," *Science*, vol. 289, no. 5487, pp. 2126–2128, 2000.

[27] P. Fabrizio, F. Pozza, S. D. Pletcher, C. M. Gendron, and V. D. Longo, "Regulation of longevity and stress resistance by Sch9 in yeast," *Science*, vol. 292, no. 5515, pp. 288–290, 2001.

[28] L. Yan, D. E. Vatner, J. P. O'Connor et al., "Type 5 adenylyl cyclase disruption increases longevity and protects against stress," *Cell*, vol. 130, no. 2, pp. 247–258, 2007.

[29] L. C. Enns, J. F. Morton, P. R. Treuting et al., "Disruption of protein kinase A in mice enhances healthy aging," *PLoS ONE*, vol. 4, no. 6, Article ID e5963, 2009.

[30] N. Gerits, S. Kostenko, A. Shiryaev, M. Johannessen, and U. Moens, "Relations between the mitogen-activated protein kinase and the cAMP-dependent protein kinase pathways: comradeship and hostility," *Cellular Signalling*, vol. 20, no. 9, pp. 1592–1607, 2008.

[31] R. Dechant and M. Peter, "Nutrient signals driving cell growth," *Current Opinion in Cell Biology*, vol. 20, no. 6, pp. 678–687, 2008.

[32] C. Borras, M. Daniel, L. Raul et al., "RasGrf1 deficiency delays in mice," *Aging*, vol. 3, no. 3, pp. 262–276, 2011.

[33] A. Wittinghofer and N. Nassar, "How Ras-related proteins talk to their effectors," *Trends in Biochemical Sciences*, vol. 21, no. 12, pp. 488–491, 1996.

[34] M. G. Mirisola and V. D. Longo, "Conserved role of Ras-GEFs in promoting aging: from yeast to mice," *Aging*, vol. 3, no. 4, pp. 340–343, 2011.

[35] P. Fabrizio and V. D. Longo, "The chronological life span of Saccharomyces cerevisiae.," *Aging Cell*, vol. 2, no. 2, pp. 73–81, 2003.

[36] R. K. Mortimer and J. R. Johnston, "Life span of individual yeast cells," *Nature*, vol. 183, no. 4677, pp. 1751–1752, 1959.

[37] K. J. Bitterman, O. Medvedik, and D. A. Sinclair, "Longevity regulation in saccharomyces cerevisiae: linking metabolism, genome stability, and heterochromatin," *Microbiology and Molecular Biology Reviews*, vol. 67, no. 3, pp. 376–399, 2003.

[38] I. Dilova, E. Easlon, and S. J. Lin, "Calorie restriction and the nutrient sensing signaling pathways," *Cellular and Molecular Life Sciences*, vol. 64, no. 6, pp. 752–767, 2007.

[39] V. D. Longo, E. B. Gralla, and J. S. Valentine, "Superoxide dismutase activity is essential for stationary phase survival in Saccharomyces cerevisiae: mitochondrial production of toxic oxygen species in vivo," *Journal of Biological Chemistry*, vol. 271, no. 21, pp. 12275–12280, 1996.

[40] V. D. Longo and B. K. Kennedy, "Sirtuins in aging and age-related disease," *Cell*, vol. 126, no. 2, pp. 257–268, 2006.

[41] M. MacLean, N. Harris, and P. W. Piper, "Chronological lifespan of stationary phase yeast cells; a model for investigating the factors that might influence the ageing of postmitotic tissues in higher organisms," *Yeast*, vol. 18, no. 6, pp. 499–509, 2001.

[42] P. Fabrizio and V. D. Longo, "Chronological aging-induced apoptosis in yeast," *Biochimica et Biophysica Acta*, vol. 1783, no. 7, pp. 1280–1285, 2008.

[43] B. K. Kennedy, N. R. Austriaco Jr, and L. Guarente, "Daughter cells of Saccharomyces cerevisiae from old mothers display a reduced life span," *Journal of Cell Biology*, vol. 127, no. 6, pp. 1985–1993, 1994.

[44] K. A. Steinkraus, M. Kaeberlein, and B. K. Kennedy, "Replicative aging in yeast: the means to the end," *Annual Review of Cell and Developmental Biology*, vol. 24, pp. 29–54, 2008.

[45] D. A. Sinclair and L. Guarente, "Extrachromosomal rDNA circles—a cause of aging in yeast," *Cell*, vol. 91, no. 7, pp. 1033–1042, 1997.

[46] M. Kaeberlein, "Lessons on longevity from budding yeast," *Nature*, vol. 464, no. 7288, pp. 513–519, 2010.

[47] M. Kaeberlein, M. McVey, and L. Guarente, "The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms," *Genes and Development*, vol. 13, no. 19, pp. 2570–2580, 1999.

[48] K. Ashrafi, D. Sinclair, J. I. Gordon, and L. Guarente, "Passage through stationary phase advances replicative aging in Saccharomyces cerevisiae," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 16, pp. 9100–9105, 1999.

[49] J. Wawryn, A. Krzepiłko, A. Myszka, and T. Biliński, "Deficiency in superoxide dismutases shortens life span of yeast cells," *Acta Biochimica Polonica*, vol. 46, no. 2, pp. 249–253, 1999.

[50] P. Fabrizio, L. L. Liou, V. N. Moy et al., "SOD2 functions downstream of Sch9 to extend longevity in yeast," *Genetics*, vol. 163, no. 1, pp. 35–46, 2003.

[51] M. Kaeberlein, R. W. Powers, K. K. Steffen et al., "Cell biology: regulation of yeast replicative life span by TOR and Sch9 response to nutrients," *Science*, vol. 310, no. 5751, pp. 1193–1196, 2005.

[52] R. W. Powers, M. Kaeberlein, S. D. Caldwell, B. K. Kennedy, and S. Fields, "Extension of chronological life span in yeast by decreased TOR pathway signaling," *Genes and Development*, vol. 20, no. 2, pp. 174–184, 2006.

[53] M. Kaeberlein, K. T. Kirkland, S. Fields, and B. K. Kennedy, "Genes determining yeast replicative life span in a long-lived genetic background," *Mechanisms of Ageing and Development*, vol. 126, no. 4, pp. 491–504, 2005.

[54] A. A. Goldberg, V. R. Richard, P. Kyryakov et al., "Chemical genetic screen identifies lithocholic acid as an anti-aging compound that extends yeast chronological life span in a TOR-independent manner, by modulating housekeeping longevity assurance processes.," *Aging*, vol. 2, no. 7, pp. 393–414, 2010.

[55] M. D. W. Piper and A. Bartke, "Diet and aging," *Cell Metabolism*, vol. 8, no. 2, pp. 99–104, 2008.

[56] J. C. Jiang, E. Jaruga, M. V. Repnevskaya, and S. M. Jazwinski, "An intervention resembling caloric restriction prolongs life span and retards aging in yeast," *FASEB Journal*, vol. 14, no. 14, pp. 2135–2137, 2000.

[57] M. Kaeberlein, C. R. Burtner, and B. K. Kennedy, "Recent developments in yeast aging," *PLoS Genetics*, vol. 3, no. 5, p. e84, 2007.

[58] M. Wei, P. Fabrizio, J. Hu et al., "Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9.," *PLoS Genetics*, vol. 4, no. 1, p. e13, 2008.

[59] M. Kaeberlein, K. T. Kirkland, S. Fields, and B. K. Kennedy, "Sir2-independent life span extension by calorie restriction in yeast," *PLoS Biology*, vol. 2, no. 9, 2004.

[60] S. J. Lin, M. Kaeberlein, A. A. Andalis et al., "Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration," *Nature*, vol. 418, no. 6895, pp. 344–348, 2002.

[61] D. L. Smith Jr, J. M. McClure, M. Matecic, and J. S. Smith, "Calorie restriction extends the chronological lifespan of Saccharomyces cerevisiae independently of the Sirtuins," *Aging Cell*, vol. 6, no. 5, pp. 649–662, 2007.

[62] D. Granot and M. Snyder, "Glucose induces cAMP-independent growth-related changes in stationary-phase cells of Saccharomyces cerevisiae," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 13, pp. 5724–5728, 1991.

[63] D. Granot, A. Levine, and E. Dor-Hefetz, "Sugar-induced apoptosis in yeast cells," *FEMS Yeast Research*, vol. 4, no. 1, pp. 7–13, 2003.

[64] J. Roosen, K. Engelen, K. Marchal et al., "PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability," *Molecular Microbiology*, vol. 55, no. 3, pp. 862–880, 2005.

[65] J. Urban, A. Soulard, A. Huber et al., "Sch9 Is a Major Target of TORC1 in Saccharomyces cerevisiae," *Molecular Cell*, vol. 26, no. 5, pp. 663–674, 2007.

[66] M. Crauwels, M. C. V. Donaton, M. B. Pernambuco, J. Winderickx, J. H. De Winde, and J. M. Thevelein, "The Sch9 protein kinase in the yeast Saccharomyces cerevisiae controls cAPK activity and is required for nitrogen activation of the fermentable-growth-medium-induced (FGM) pathway," *Microbiology*, vol. 143, no. 8, pp. 2627–2637, 1997.

[67] T. Toda, S. Cameron, P. Sass, and M. Wigler, "SCH9, a gene of Saccharomyces cerevisiae that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits.," *Genes & Development*, vol. 2, no. 5, pp. 517–527, 1988.

[68] J. L. Crespo and M. N. Hall, "Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae," *Microbiology and Molecular Biology Reviews*, vol. 66, no. 4, pp. 579–591, 2002.

[69] C. De Virgilio and R. Loewith, "The TOR signalling network from yeast to man," *International Journal of Biochemistry and Cell Biology*, vol. 38, no. 9, pp. 1476–1481, 2006.

[70] Y. Pan, E. A. Schroeder, A. Ocampo, A. Barrientos, and G. S. Shadel, "Regulation of yeast chronological life span by TORC1 via adaptive mitochondrial ROS signaling," *Cell Metabolism*, vol. 13, no. 6, pp. 668–678, 2011.

[71] P. W. Piper, N. L. Harris, and M. MacLean, "Preadaptation to efficient respiratory maintenance is essential both for maximal longevity and the retention of replicative potential in chronologically ageing yeast," *Mechanisms of Ageing and Development*, vol. 127, no. 9, pp. 733–740, 2006.

[72] J. M. Thevelein and J. H. De Winde, "Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast Saccharomyces cerevisiae," *Molecular Microbiology*, vol. 33, no. 5, pp. 904–918, 1999.

[73] J. Sun, S. P. Kale, A. M. Childress, C. Pinswasdi, and S. M. Jazwinski, "Divergent roles of RAS1 and RAS2 in yeast longevity," *Journal of Biological Chemistry*, vol. 269, no. 28, pp. 18638–18645, 1994.

[74] M. Wei, P. Fabrizio, F. Madia et al., "Tor1/Sch9-regulated carbon source substitution is as effective as calorie restriction in life span extension," *PLoS Genetics*, vol. 5, no. 5, Article ID e1000467, 2009.

[75] P. Fabrizio, L. Battistella, R. Vardavas et al., "Superoxide is a mediator of an altruistic aging program in Saccharomyces cerevisiae," *Journal of Cell Biology*, vol. 166, no. 7, pp. 1055–1067, 2004.

[76] A. Mesquita, M. Weinberger, A. Silva et al., "Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H2O2 and superoxide dismutase activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 34, pp. 15123–15128, 2010.

[77] M. Tsukada and Y. Ohsumi, "Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae," *FEBS Letters*, vol. 333, no. 1-2, pp. 169–174, 1993.

[78] A. J. Saldanha, M. J. Brauer, and D. Botstein, "Nutritional homeostasis in batch and steady-state culture of yeast," *Molecular Biology of the Cell*, vol. 15, no. 9, pp. 4089–4104, 2004.

[79] M. J. Brauer, C. Huttenhower, E. M. Airoldi et al., "Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast," *Molecular Biology of the Cell*, vol. 19, no. 1, pp. 352–367, 2008.

[80] V. M. Boer, S. Amini, and D. Botstein, "Influence of genotype and nutrition on survival and metabolism of starving yeast," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 19, pp. 6930–6935, 2008.

[81] P. Gomes, B. Sampaio-Marques, P. Ludovico, F. Rodrigues, and C. Leão, "Low auxotrophy-complementing amino acid concentrations reduce yeast chronological life span," *Mechanisms of Ageing and Development*, vol. 128, no. 5-6, pp. 383–391, 2007.

[82] C. J. Murakami, C. R. Burtner, B. K. Kennedy, and M. Kaeberlein, "A method for high-throughput quantitative analysis of yeast chronological life span," *Journals of Gerontology A*, vol. 63, no. 2, pp. 113–121, 2008.

[83] M. W. Unger and L. H. Hartwell, "Control of cell division in Saccharomyces cerevisiae by methionyl tRNA," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 73, no. 5, pp. 1664–1668, 1976.

[84] A. L. Alvers, L. K. Fishwick, M. S. Wood et al., "Autophagy and amino acid homeostasis are required for chronological longevity in Saccharomyces cerevisiae," *Aging Cell*, vol. 8, no. 4, pp. 353–369, 2009.

[85] C. Leão and N. Van Uden, "Effects of ethanol and other alkanols on passive proton influx in the yeast Saccharomyces cerevisiae," *Biochimica et Biophysica Acta*, vol. 774, no. 1, pp. 43–48, 1984.

[86] T. M. Swan and K. Watson, "Membrane fatty acid composition and membrane fluidity as parameters of stress tolerance in yeast," *Canadian Journal of Microbiology*, vol. 43, no. 1, pp. 70–77, 1997.

[87] H. Cardoso and C. Leão, "Sequential inactivation of ammonium and glucose transport in Saccharomyces cerevisiae during fermentation," *FEMS Microbiology Letters*, vol. 94, no. 1-2, pp. 155–160, 1992.

[88] C. Leão and N. Van Uden, "Effects of ethanol and other alkanols on the general amino acid permease of Saccharomyces cerevisiae," *Biotechnology and Bioengineering*, vol. 26, no. 4, pp. 403–405, 1984.

[89] D. Stanley, A. Bandara, S. Fraser, P. J. Chambers, and G. A. Stanley, "The ethanol stress response and ethanol tolerance of Saccharomyces cerevisiae," *Journal of Applied Microbiology*, vol. 109, no. 1, pp. 13–24, 2010.

[90] H. Kitagaki, Y. Araki, K. Funato, and H. Shimoi, "Ethanol-induced death in yeast exhibits features of apoptosis mediated by mitochondrial fission pathway," *FEBS Letters*, vol. 581, no. 16, pp. 2935–2942, 2007.

[91] P. Fabrizio, C. Gattazzo, L. Battistella et al., "Sir2 blocks extreme life-span extension," *Cell*, vol. 123, no. 4, pp. 655–667, 2005.

[92] P. Fabrizio, S. Hoon, M. Shamalnasab et al., "Genome-wide screen in Saccharomyces cerevisiae identifies vacuolar protein sorting, autophagy, biosynthetic, and tRNA methylation genes involved in life span regulation," *PLoS Genetics*, vol. 6, no. 7, pp. 1–14, 2010.

[93] A. A. Goldberg, S. D. Bourque, P. Kyryakov et al., "Effect of calorie restriction on the metabolic history of chronologically aging yeast," *Experimental Gerontology*, vol. 44, no. 9, pp. 555–571, 2009.

[94] C. R. Burtner, C. J. Murakami, B. K. Kennedy, and M. Kaeberlein, "A molecular mechanism of chronological aging in yeast," *Cell Cycle*, vol. 8, no. 8, pp. 1256–1270, 2009.

[95] I. Pinto, H. Cardoso, C. Leão, and N. van Uden, "High enthalpy and low enthalpy death in Saccharomyces cerevisiae induced by acetic acid," *Biotechnology and Bioengineering*, vol. 33, no. 10, pp. 1350–1352, 1989.

[96] P. Ludovico, F. Rodrigues, A. Almeida, M. T. Silva, A. Barrientos, and M. Côrte-Real, "Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in Saccharomyces cerevisiae," *Molecular Biology of the Cell*, vol. 13, no. 8, pp. 2598–2606, 2002.

[97] P. Ludovico, M. J. Sousa, M. T. Silva, C. Leão, and M. Côrte-Real, "Saccharomyces cerevisiae commits to a programmed cell death process in response to acetic acid," *Microbiology*, vol. 147, no. 9, pp. 2409–2415, 2001.

[98] M. Casal, H. Cardoso, and C. Leão, "Mechanisms regulating the transport of acetic acid in Saccharomyces cerevisiae," *Microbiology*, vol. 142, no. 6, pp. 1385–1390, 1996.

[99] M. Weinberger, L. Feng, A. Paul et al., "DNA replication stress is a determinant of chronological lifespan in budding yeast.," *PloS one*, vol. 2, no. 1, p. e748, 2007.

[100] W. C. Burhans and M. Weinberger, "Acetic acid effects on aging in budding yeast: are they relevant to aging in higher eukaryotes?" *Cell Cycle*, vol. 8, no. 14, pp. 2300–2302, 2009.

[101] M. Matecic, D. L. Smith, X. Pan et al., "A microarray-based genetic screen for yeast chronological aging factors," *PLoS Genetics*, vol. 6, no. 4, Article ID e1000921, 2010.

[102] N. von Wirén and M. Merrick, "Regulation and function of ammonium carriers in bacteria, fungi, and plants," in *Molecular Mechanisms Controlling Transmembrane Transport*, pp. 95–120, Springer, Berlin, Germany , 2004.

[103] E. G. Ter Schure, N. A. W. Van Riel, and C. T. Verrips, "The role of ammonia metabolism in nitrogen catabolite repression in Saccharomyces cerevisiae," *FEMS Microbiology Reviews*, vol. 24, no. 1, pp. 67–83, 2000.

[104] B. Smets, R. Ghillebert, P. De Snijder et al., "Life in the midst of scarcity: adaptations to nutrient availability in Saccharomyces cerevisiae," *Current Genetics*, vol. 56, no. 1, pp. 1–32, 2010.

[105] L. Váchová and Z. Palková, "Physiological regulation of yeast cell death in multicellular colonies is triggered by ammonia," *Journal of Cell Biology*, vol. 169, no. 5, pp. 711–717, 2005.

[106] D. C. Hess, W. Lu, J. D. Rabinowitz, and D. Botstein, "Ammonium toxicity and potassium limitation in yeast.," *PLoS Biology*, vol. 4, no. 11, p. e351, 2006.

[107] J. Santos, M. J. Sousa, and C. Leão, "Ammonium is toxic for aging yeast cells, inducing death and shortening of the chronological lifespan," *PLoS One*, vol. 7, no. 5, Article ID e37090, 2012.

[108] A. Van Nuland, P. Vandormael, M. Donaton et al., "Ammonium permease-based sensing mechanism for rapid ammonium activation of the protein kinase A pathway in yeast," *Molecular Microbiology*, vol. 59, no. 5, pp. 1485–1505, 2006.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2012, Article ID 785743, 13 pages
doi:10.1155/2012/785743

Research Article

Inhibition of Mitochondrial Cytochrome *c* Release and Suppression of Caspases by Gamma-Tocotrienol Prevent Apoptosis and Delay Aging in Stress-Induced Premature Senescence of Skin Fibroblasts

Suzana Makpol,[1] Norhazira Abdul Rahim,[1] Chua Kien Hui,[2] and Wan Zurinah Wan Ngah[1]

[1] *Department of Biochemistry, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*
[2] *Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*

Correspondence should be addressed to Suzana Makpol, suzanamakpol@yahoo.com

Received 3 March 2012; Revised 16 May 2012; Accepted 24 May 2012

Academic Editor: Paula Ludovico

In this study, we determined the molecular mechanism of γ-tocotrienol (GTT) in preventing cellular aging by focusing on its anti-apoptotic effect in stress-induced premature senescence (SIPS) model of human diploid fibroblasts (HDFs). Results obtained showed that SIPS exhibited senescent-phenotypic characteristic, increased expression of senescence-associated β-galactosidase (SA β-gal) and promoted G_0/G_1 cell cycle arrest accompanied by shortening of telomere length with decreased telomerase activity. Both SIPS and senescent HDFs shared similar apoptotic changes such as increased Annexin V-FITC positive cells, increased cytochrome *c* release and increased activation of caspase-9 and caspase-3 ($P < 0.05$). GTT treatment resulted in a significant reduction of Annexin V-FITC positive cells, inhibited cytochrome *c* release and decreased activation of caspase-9 and caspase-3 ($P < 0.05$). Gene expression analysis showed that GTT treatment down regulated BAX mRNA, up-regulated BCL2A1 mRNA and decreased the ratio of Bax/Bcl-2 protein expression ($P < 0.05$) in SIPS. These findings suggested that GTT inhibits apoptosis by modulating the upstream apoptosis cascade, causing the inhibition of cytochrome *c* release from the mitochondria with concomitant suppression of caspase-9 and caspase-3 activation. In conclusion, GTT delays cellular senescence of human diploid fibroblasts through the inhibition of intrinsic mitochondria-mediated pathway which involved the regulation of pro- and anti-apoptotic genes and proteins.

1. Introduction

Replicative senescence is the final *in vitro* state reached by all primary cells in culture and is characterized by decreased total cell numbers and loss of proliferation capacity, in which they remain alive and metabolically active [1]. It has been widely used to identify aging-associated molecular changes in human cells and correlated with the *in vivo* aging process [2]. Recently, the term stress-induced premature senescence (SIPS) was introduced to represent an irreversible growth arrest of proliferative cells induced by exogenous oxidative agents.

The imbalance between cellular oxidants and antioxidants leads to oxidative damage to cellular macromolecules thus contributing to the decline in cellular functions and finally progressive organism aging [3]. Vitamin E in particular alpha-tocopherol protects biological membrane from oxidation by acting as a radical chain-breaking molecule. Other biological functions of vitamin E that are unrelated to its antioxidant properties include its roles in cellular signaling, gene expression, immune response, and apoptosis are now considered to be of importance [4].

Recently, an isomer of vitamin E, the tocotrienols have gained increasing interest due to their stronger antioxidant

effects and a nonantioxidant activity profile that differs from tocopherols [5]. Tocotrienols were found not only to have antioxidant function, but also modulate signal transduction and gene expression. In cellular aging, tocotrienols have been observed to decrease biomarkers of senescent cells, reduced damaged DNA, decreased cells in G_0/G_1 phase, and promoted cells to enter S phase [6]. However, the mechanism of action in delaying aging is not fully understood.

Other reports have also shown that tocotrienol administration reduced oxidative protein damage and consequently extended the mean life span of *C. elegans* [7]. γ-Tocotrienol at low concentration protected against H_2O_2-induced cell loss in fibroblast cell lines derived from different age groups. γ-Tocotrienol was also able to protect against H_2O_2-induced telomere shortening and telomerase activity loss [8].

Accumulating evidence suggested that dysregulation of apoptosis is associated with the aging process. Oxidative stress and DNA damage, both being important factors in the aging process, induce apoptosis. Previous study had shown that apoptosis was upregulated during aging in various cells [9]. Other report showed that apoptotic changes were increased in senescent endothelial cells [10].

In the present study, we determined the binding of Annexin V-FITC to phosphatidylserine (PS) on plasma membrane. FITC-conjugated Annexin V is widely used to detect apoptotic cells in a diverse range of cell types and in response to many different proapoptotic stimuli [11]. It has been reported that PS translocation to the outer leaflet of plasma membrane is the early and widespread event during apoptosis of cells from numerous lineages and is inhibited by overexpression of apoptosis repressor proteins such as Bcl-2 [11, 12].

Caspases are a family of proteases that play major roles in apoptosis process. Caspase-3, caspase-6, and caspase-7, known as executioner caspases, are located down stream of the caspase-8 or caspase-9 activation cascade. Activation of executioner caspases is responsible for the proteolytic cleavage of a broad range of proteins [13].

Bax, a member of the Bcl-2 protein family, is involved in controlling apoptotic events. High level of Bcl-2 inhibits apoptosis by preventing cytochrome *c* release while high level of Bax induces apoptosis by binding to the mitochondrial membrane and increases membrane permeability allowing the release of cytochrome *c* [14]. The mitochondrial outer membrane permeability induced either by Bax- or Bak-dependent proapoptotic drugs, or by H_2O_2 results in the intracytosolic release of cytochrome *c* but the subsequent caspase activation is required to induce the translocation of apoptosis-inducing factor (AIF) into the cytosol. Therefore, mitochondrial response to several proapoptotic stimuli was suggested as a selective process leading to a hierarchical ordering of the effectors involved in cell death induction [15].

To date, there have been very few studies on the antiapoptotic effects of tocotrienol on normal fibroblasts *in vitro*. In the present study, we first established the SIPS model of primary HDFs by comparing the cellular senescence markers between SIPS and replicative senescent HDFs. We then determined the molecular apoptotic events involved in SIPS and the effects of γ-tocotrienol in modulating the apoptosis pathway and thus delaying cellular aging.

2. Results

2.1. Characterization of HDFs Stress-Induced Premature Senescence (SIPS). Control young HDFs displayed the normal spindle shape characteristic of fibroblast cells, while prolonged exposure to 20 μM H_2O_2 resulted in HDFs exhibiting senescence features such as cells were enlarged and flattened with increased size of nucleus. Less spindle-shaped cells were present and accumulation of granular cytoplasmic inclusions was observed in SIPS and control senescent HDFs (Figure 1(a)). SA-β-galactosidase staining was also positive in SIPS and control senescent cells (Figure 1(b)). The percentage of SA-β-gal positive cells was markedly increased in SIPS and control senescent cells as compared to control young HDFs ($P < 0.05$) (Figure 1(c)).

Analysis on cell cycle profile showed that HDFs population in G_0/G_1 phase was significantly increased while S phase and G_2/M phase cells decreased in SIPS as compared to control young HDFs (Figures 2(a) and 2(b)) ($P < 0.05$). Similar increase and decrease in G_0/G_1 phase and S phase cells, respectively, were observed in control senescent HDFs ($P < 0.05$).

Shortening of telomere length was observed in SIPS and control senescent HDFs as compared to young HDFs ($P < 0.05$) (Figures 3(a) and 3(b)). Similarly, decreased telomerase activity was observed both in SIPS and control senescent HDFs (Figures 4(a) and 4(b)).

2.2. Apoptotic Changes Detected by Annexin V-FITC. Figure 5(a) shows the contour diagram of FITC-Annexin V/PI double staining by flow cytometry analysis. The three quadrants represent different cells conditions: the upper right quadrant (R1) indicates nonviable, late apoptotic, and necrotic cells ($FITC^+/PI^+$), lower left quadrant (R2) indicates viable cells ($FITC^-/PI^-$), and lower right quadrant (R3) indicates early apoptotic cells ($FITC^+/PI^-$) which is demonstrated by Annexin V binding and cytoplasmic membrane integrity. The percentage of cells at early apoptotic stage was significantly increased in SIPS and control senescent cells as compared to control young HDFs ($P < 0.05$) (Figure 5(b)). GTT treatment in SIPS significantly decreased the percentage of early apoptotic cells as compared to SIPS alone ($P < 0.05$).

2.3. Activation of Caspase-3, Caspase-8, and Caspase-9. Significant activation of caspase-8 and caspase-9 was observed in SIPS and control senescent cells as compared to control young and GTT-treated control HDFs ($P < 0.05$) (Figure 6(b)) while caspase-3 was significantly activated in SIPS, but not in control senescent cells. GTT treatment of SIPS inhibited caspase-3 and caspase-9 activation with no notable inhibition observed in caspase-8 activation ($P < 0.05$). In addition, GTT-treated control HDFs showed suppression of caspase-9 activation as compared to untreated young HDFs ($P < 0.05$).

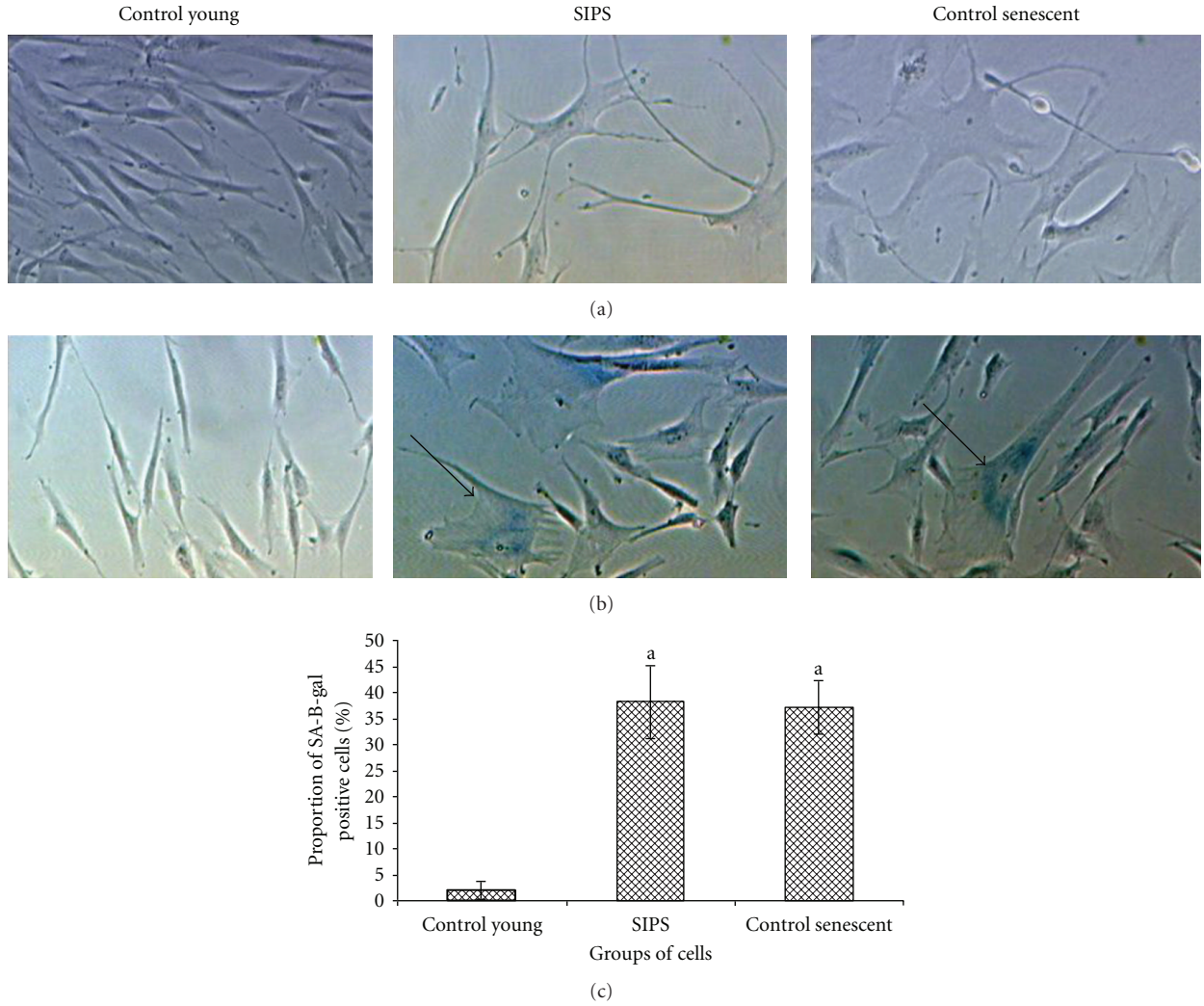


Figure 1: Morphological changes in HDFs in culture (a). Senescence-associated β-galactosidase staining. Positive blue stains of SA β-gal appeared in SIPS and control senescent HDFs as indicated by arrows. Micrographs are shown at 100x magnification (b). Quantitative analysis of positive β-galactosidase stained cells (c). Data are expressed as means ± SD, $n = 6$. [a]denotes $P < 0.05$ compared to control young.

2.4. Cytochrome c Release. Cytochrome *c* release was increased in SIPS and control senescent cells as compared to control young cells and GTT-treated control HDFs ($P < 0.05$) (Figure 7). SIPS cells released 47.7% of cytochrome *c* from the mitochondria into the cytosol as compared to control young HDFs (1%) and control senescent cells (79.5%). Cytochrome *c* release decreased significantly to 36.5% in SIPS treated with GTT as compared to SIPS alone ($P < 0.05$).

2.5. Analysis of Apoptotic-Associated Genes Expression. Proapoptotic gene, BAX was markedly increased in SIPS and control senescent cells as compared to control young and GTT-treated control HDFs ($P < 0.05$) (Figure 8(a)). SIPS treated with GTT; however, showed a significant downregulation of BAX ($P < 0.05$). No significant difference was observed in the expression of another proapoptotic gene, BID in all treatment groups (Figure 8(b)).

Analysis of antiapoptotic genes showed that GTT treatment to control HDFs caused a significant upregulation of BCL2A1 mRNA ($P < 0.05$) while no change was observed in other treatment groups (Figure 9(a)). Analysis on the expression of another antiapoptotic gene, BCL2L1 showed no significant change observed in all treatment groups (Figure 9(b)).

2.6. Analysis of Bax and Bcl-2 Protein Expression. The ratio of Bax/Bcl-2 protein expression in SIPS and control senescent cells was markedly increased as compared to untreated control young and GTT-treated control HDFs ($P < 0.05$) (Figure 10). Treatment with GTT in SIPS considerably reversed the effect of H_2O_2 in Bax/Bcl-2 protein expression whereby the ratio was significantly decreased as compared to SIPS alone ($P < 0.05$).

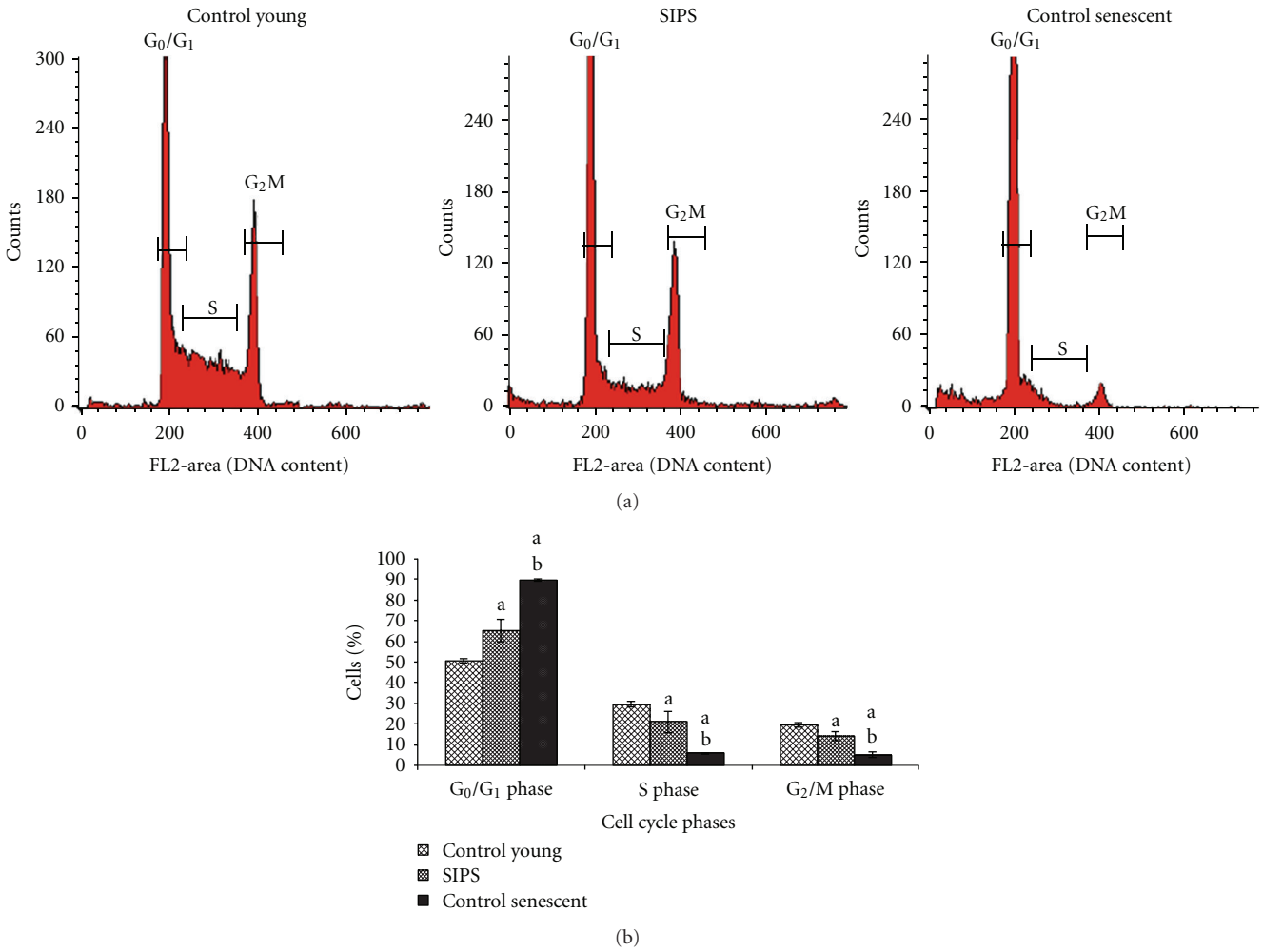


Figure 2: Flow cytometry analysis of cell cycle progression in young, SIPS, and control senescent HDFs (a). Quantitative analysis of cell cycle progression in control young HDFs, SIPS, and control senescent HDFs. Cell population in the G_0/G_1 phase was significantly increased while S phase cells decreased in SIPS and control senescent HDFs (b). [a]denotes $P < 0.05$ compared to control young HDFs, [b]$P<0.05$ compared to SIPS. Comparison was done between HDFs in the same phase of cell cycle. Data is presented as mean ± SD, $n = 6$.

3. Discussion

The present study explored the molecular mechanism of GTT in inhibiting apoptosis and delaying cellular aging in H_2O_2-induced premature senescence (SIPS) of HDFs. Our results confirmed that SIPS demonstrated senescence characteristics as evidenced by senescent-like morphological changes, a significant increase in senescence-associated marker SA-β-galactosidase activity, promoted G_0/G_1 cell cycle arrest, shortening of telomere length, and decreased telomerase activity. Similar changes were observed for cultures at late stages (replicative senescent cells).

Both SIPS and senescent HDFs shared similar apoptotic changes as shown by increase in Annexin V-FITC positive cells, cytochrome *c* release, and activation of caspase-9 and caspase-3. These findings were supported by previous studies which reported that premature senescence of fibroblasts can be triggered by induction with acute sublethal doses of H_2O_2 [16, 17] or prolonged low dose of H_2O_2 [1].

In this study, we exposed young HDFs to prolonged low dose of H_2O_2 which mimics the oxidative stress *in vivo* to induce premature senescence state instead of acute induction with sublethal doses of H_2O_2. Exposure of prolonged low dose of H_2O_2 to the embryonic human lung HDFs was found not only induced irreversible cell cycle arrest and senescent-like morphology, but also caused accumulation of damaged DNA accompanied by telomere shortening [1].

The initial step to develop SIPS model is to find doses that are subcytotoxic to the cells. We found that doses of H_2O_2 30–80 μM slowed down cells growth rate which leads to cell death after 1 week culture while concentrations of H_2O_2 above 100 μM triggered cell death within 24 h culture (data not shown). Thus, the higher H_2O_2 concentration used, the higher the proportion of cells undergoing apoptosis and cell death was observed. Our results confirmed that induction of H_2O_2 for 2 weeks induced the premature senescence state of young HDFs, where they demonstrated senescence characteristics as evidenced by senescent-like morphological

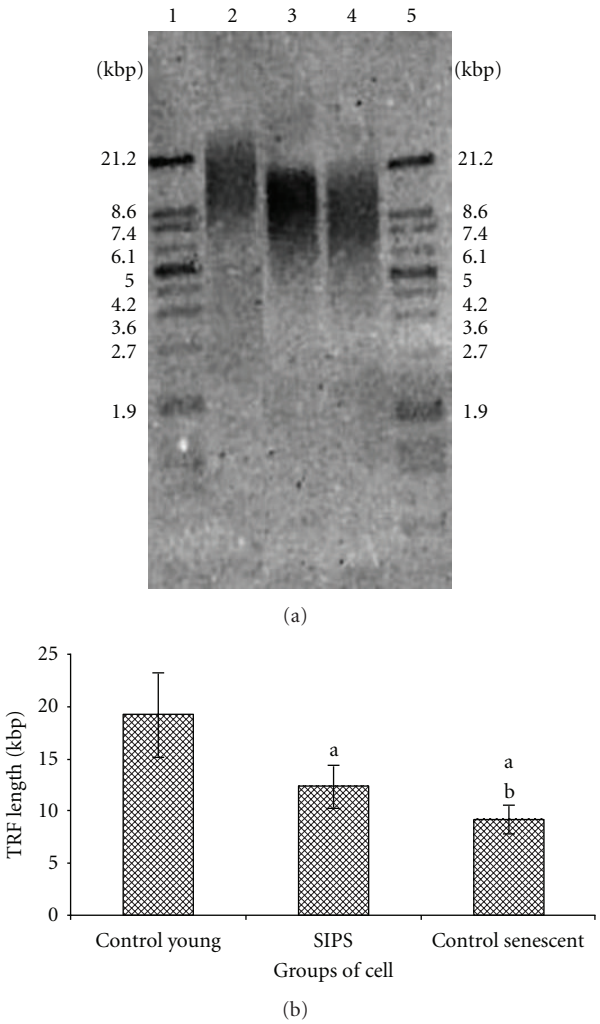


Figure 3: Representative Southern blot analysis of control young HDFs, SIPS, and control senescent HDFs. Telomeric DNA was shown as wide smears in all lanes. The telomere length was represented by mean terminal restriction fragment (TRF) value. Lane 1; molecular weight marker, lane 2; control young HDFs, lane 3; SIPS, lane 4; senescent HDFs, lane 5; molecular weight marker (a). Terminal restriction fragment, (TRF length) of young HDFs, SIPS, and control senescent HDFs. Shortening of telomere length was observed in SIPS and control senescent HDFs (b). [a]denotes $P < 0.05$ compared to young HDFs, [b]$P<0.05$ compared to SIPS. Data is presented as mean ± SD, $n = 6$.

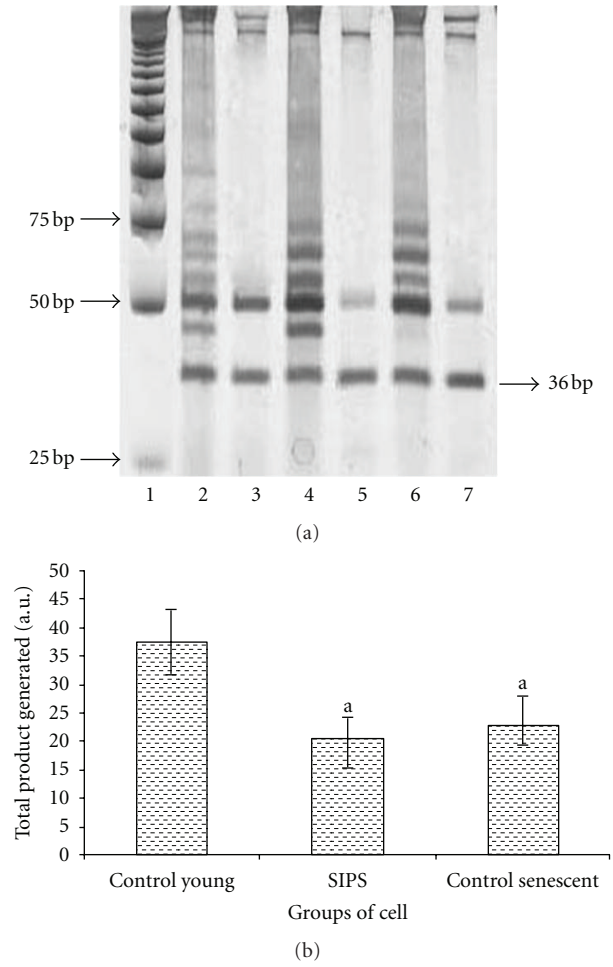


Figure 4: Representative PCR analysis for telomerase activity of control young, SIPS, and control senescent HDFs. Lane 1, molecular weight marker, lane 2, control young, lane 3, control young (heat treated), lane 4, SIPS, lane 5, SIPS (heat treated), lane 6, control senescent, and lane 7, control senescent (heat treated). Band at 36 bp represents the internal control for the assay (a). Telomerase activity (Total Product Generated, TPG) of control young, SIPS, and control senescent HDFs. Reduction in telomerase activity was observed in SIPS and control senescent HDFs (b). [a]denotes $P < 0.05$ compared to control young HDFs. Data is presented as mean ± SD, $n = 6$.

changes and a significant increase in senescence-associated marker SA-β-galactosidase activity which was similar to replicative senescent cells. Previously, human foreskin fibroblasts which were exposed to H_2O_2 for 7 days demonstrated positive staining for SA-β-gal [18] indicating cellular senescence. Therefore, based on these findings we decided to use the dose of 20 μM H_2O_2 for 2 weeks to induce SIPS in HDFs.

One of the mechanisms mediating the development of senescent phenotype is telomere shortening. In this study, we found that both SIPS and senescent cells demonstrated shortening of telomere length with concomitant reduction in telomerase activity. We further determined cell cycle progression which showed that SIPS cells were mainly at G_0/G_1 phase and decrease cell population in S phase indicating they might enter the irreversible growth arrest state followed by apoptosis. The reduction in S phase cells in SIPS suggested a slowing down of cellular proliferation which was similar to that of senescent cells. Previous *in vitro* study reported that H_2O_2-induced premature senescence and spontaneous senescent fibroblasts activated the apoptosis pathway as evidenced by activation of caspases in both groups of fibroblasts [19]. Human fibroblasts showed increased number of dead cells with increasing passage and increase in DNA fragmentation and mitochondrial dysfunction represented by loss of mitochondrial membrane potential, increased caspase-3, and increased cytochrome *c* positive cells [20]. Further identification of the molecular

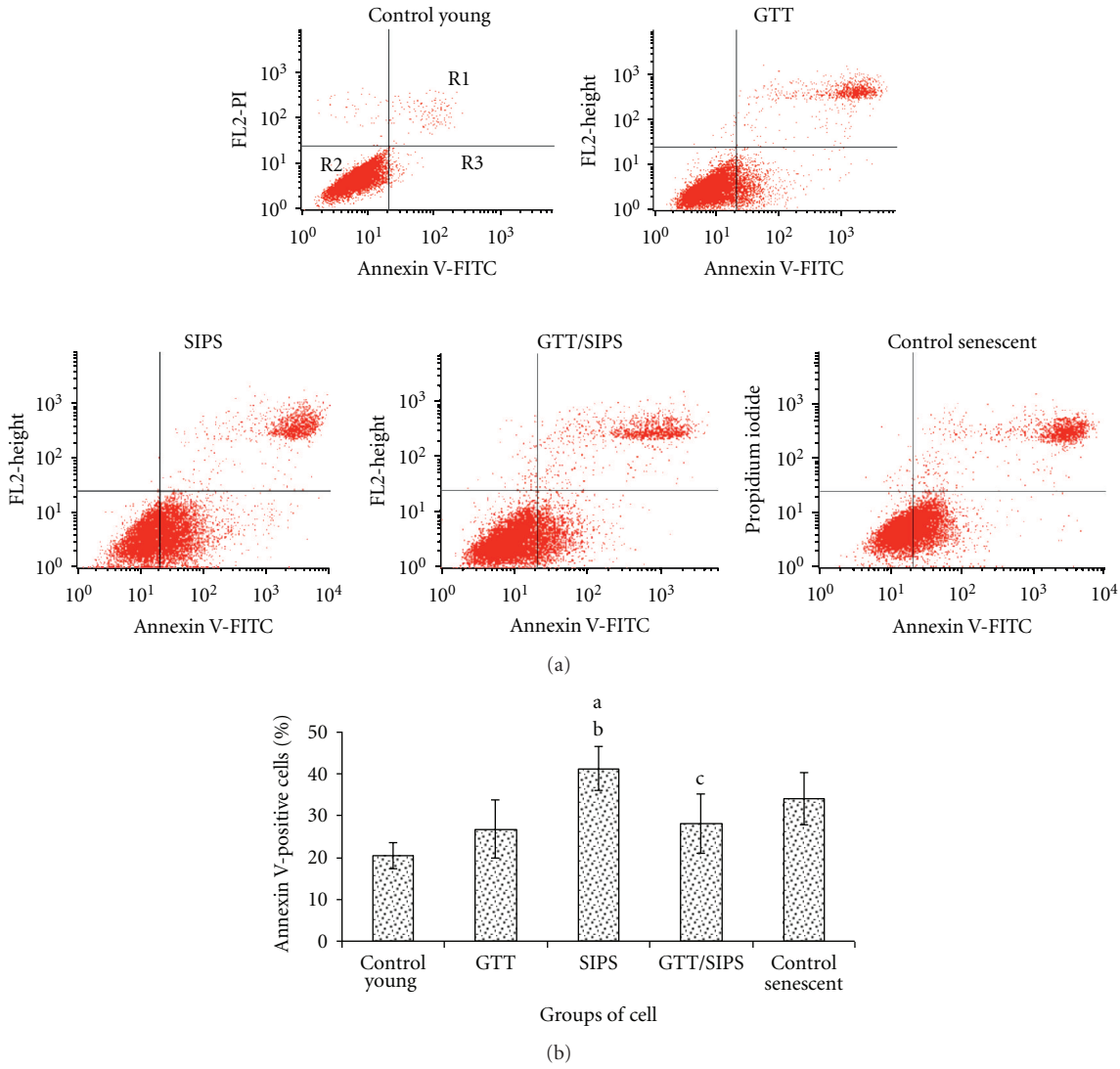


Figure 5: Contour diagram of FITC-Annexin V/PI double staining by flow cytometry. The three quadrants represent different cells conditions: the upper right quadrant (R1); nonviable, late apoptotic and necrotic cells ($FITC^+/PI^+$), lower left quadrant (R2); viable cells ($FITC^-/PI^-$), and lower right quadrant (R3); early apoptotic cells ($FITC^+/PI^-$) (a). Percentage of cells at early apoptotic stage demonstrated by $FITC^+/PI^-$ (b). Data are expressed as means ± SD, $n = 6$. [a]denotes $P < 0.05$ compared to control young, [b]$P<0.05$ compared to GTT, and [c]$P<0.05$ compared to SIPS.

apoptotic changes in SIPS and senescent HDFs showed that Annexin V-FITC positive cells were increased both in SIPS and replicative senescent HDFs confirming early apoptotic changes occurred in both groups of cells.

Characterization of the apoptotic events in stress-induced premature senescence of HDFs showed that in SIPS, caspase-3 was activated not only by the activation of caspase-9 but also by caspase-8. These findings are consistent with a study done by Nagase et al. [21] and suggested that apoptosis pathway is activated during stress-induced cellular aging of HDFs. The activation of caspase-3, -8, and-9 indicated that both cell-surface-death receptor pathway and the intrinsic mitochondrial pathway are involved in inducing cell death during stress-induced premature senescence of HDFs [19].

We further investigated the involvement of mitochondrion-initiated pathway for both SIPS and senescent HDFs by determining the percentage of cytochrome *c* release from the mitochondria to the cytosol. We found that both SIPS and replicative senescent HDFs displayed significantly high percentage of cytochrome *c* release.

The induction of cytochrome *c* release can be achieved through various mechanisms, including the involvement of pro- and antiapoptotic proteins such as Bcl-2 family proteins. The 2 classes of proteins are localized to intracellular membranes, particularly mitochondria, and have been shown to interact with each other [22]. Activation of Bax protein resulted in rapid cell death, and this can be inhibited by death-inhibiting proteins; Bcl-2 and Bcl-xL.

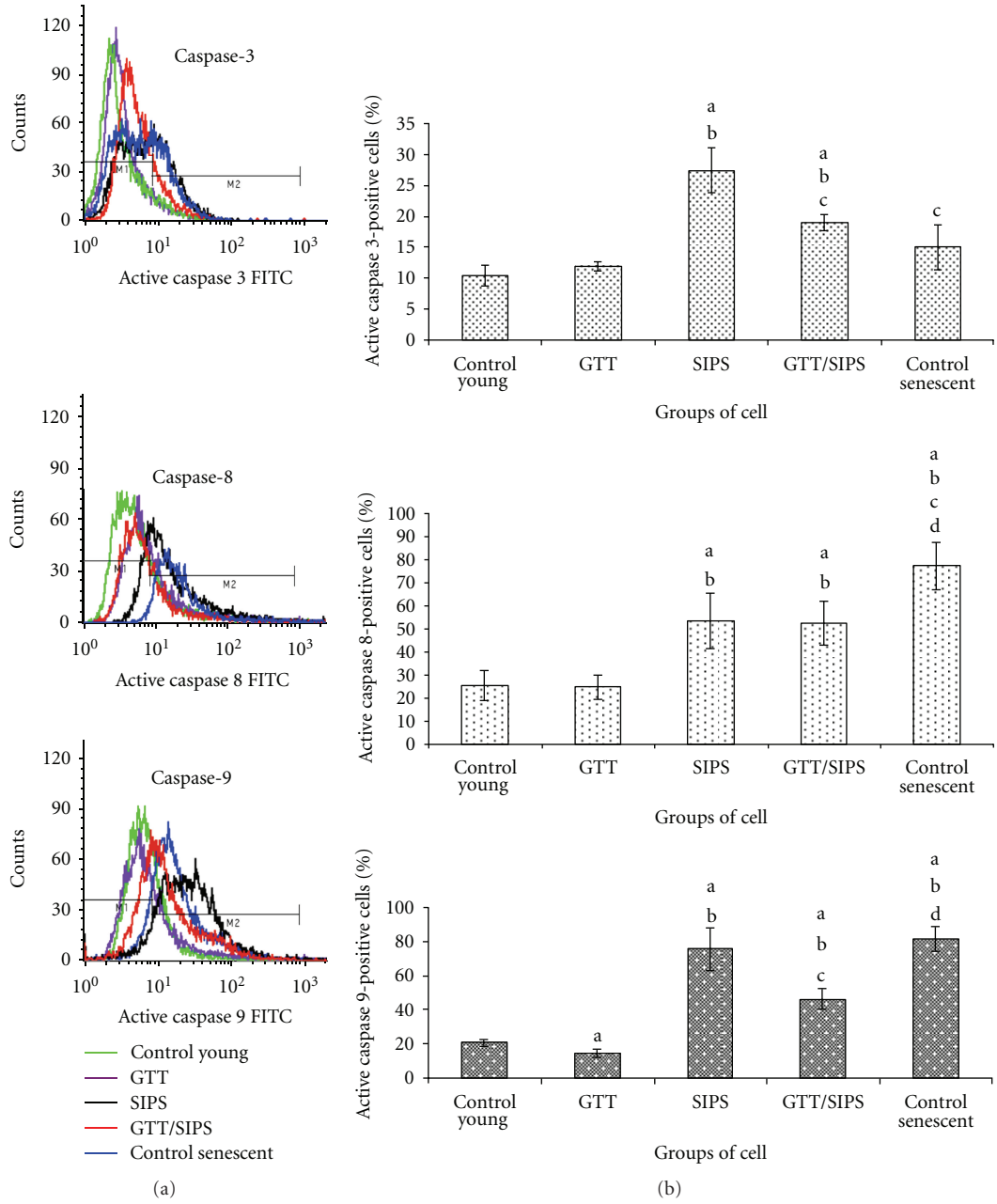


Figure 6: Flow cytometry analysis of active caspase-3, caspase-8, and caspase-9 with FITC/PI double staining in HDFs (a). Quantitative analysis of apoptotic cells population for active caspase-3, caspase-8, and caspase-9. (b). Data are expressed as means ± SD, $n = 6$. [a]denotes $P < 0.05$ compared to control young, [b]$P < 0.05$ compared to GTT, [c]$P < 0.05$ compared to SIPS, and [d]$P < 0.05$ compared to control senescent cells.

Further evaluation of the transcriptional and translational processes involving the expression of Bax and Bcl-2 proteins, the regulators of apoptotic pathway showed that Bax/Bcl-2 protein ratio was increased in SIPS and senescent HDFs indicating activation of apoptotic pathway and inhibition of antiapoptotic pathway with H_2O_2 induction.

Our data on gene expression analysis showed that BAX expression was significantly increased in SIPS and senescent HDFs while another key proapoptotic gene, BID was not affected by H_2O_2 induction. As for the antiapoptotic genes BCL2A1 and BCL2L1, our results did not show any significant changes in the expression of both genes in SIPS and cellular senescence of HDFs.

Based on these findings we suggested that both SIPS and replicative senescent HDFs undergo apoptosis. Therefore, further study was carried out to determine the specific stage that GTT intervenes in the apoptosis cascade and thus delaying aging.

Flow cytometry analysis showed that GTT treatment decreased the percentage of Annexin V-FITC positive cells

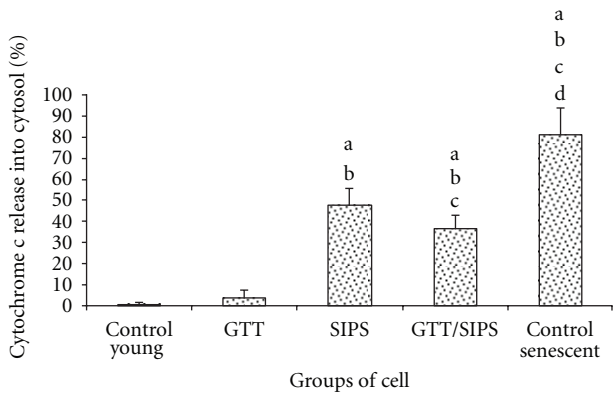


Figure 7: Percentage of mitochondria cytochrome *c* release in HDFs. Data are presented as means ± SD; $n = 6$. [a]denotes $P < 0.05$ compared to control young, [b]$P < 0.05$ compared to GTT, [c]$P < 0.05$ compared to SIPS, and [d]$P < 0.05$ compared to control senescent cells.

suggesting the prevention of PS translocation to the outer layer of cell membrane, thus inhibiting the early apoptotic changes of HDFs. Besides, GTT treatment to SIPS was found to inhibit the activation of caspase-9 and subsequently caspase-3. Similar effects however were not observed for caspase-8. These results indicated that GTT possessed antiapoptotic effects in preventing stress-induced premature senescence of HDFs which attenuated the key elements of intrinsic caspases in the mitochondrion-initiated pathway.

The involvement of mitochondrion-initiated pathway was further investigated to determine the specific stage that GTT intervenes in the apoptosis cascade. Our results showed a reduction of cytochrome *c* release with GTT treatment indicating inhibition of stress-induced apoptosis by GTT was probably at the upstream stage in the cascade of events leading to apoptosis. In addition, GTT may also be involved in the regulation of pro- and/or antiapoptotic proteins which induced cytochrome *c* release from the mitochondria.

Gene expression analysis showed that BCL2A1 was markedly upregulated in control young HDFs treated with GTT. Furthermore, GTT was also found to downregulate BAX in SIPS cells. These findings demonstrated the involvement of GTT in regulating the apoptotic genes expression particularly BAX and BCL2A1 mRNA in normal diploid fibroblast cells.

Treatment of SIPS with GTT was also found to decrease Bax/Bcl-2 protein ratio, suggesting that exogenous antioxidants can modulate Bax and Bcl-2 expression in HDFs exposed to oxidant and thus prevents apoptosis. Several studies have shown that antioxidants may have antiapoptotic or proapoptotic properties, depending on the type of cells and the apoptosis-inducing signal involved, but the trend showed that antioxidants can prevent apoptosis in healthy cells and may promote apoptosis of transformed cells [22, 23].

Previously, it had been demonstrated that high levels of Bcl-2 can prevent the release of cytochrome *c* and thus caspase activation in response to a number of apoptosis-inducing stimuli such as UVB, staurosporine and etoposide [24, 25]. Therefore, the decrease in Bax/Bcl-2 protein ratio with GGT treatment is suggestive of its action in preventing apoptosis in HDFs subjected to oxidative stress. The anti-apoptotic effects of GTT may be attributable not only to its potent antioxidant properties but may also attributed to its nonantioxidant functions.

It is shown that GTT inhibited the apoptosis cascade leading to the prevention of cell death in SIPS. The ability of GTT to inhibit caspases and cytochrome *c* release upon prolong exposure of H_2O_2 suggests the underlying mode of action of this compound in preventing oxidative stress-induced apoptosis.

4. Materials and Methods

4.1. Cell Culture. This research has been approved by the Ethics Committee of the Universiti Kebangsaan Malaysia (Approval Project Code: FF-313-2009). Primary HDFs were derived from foreskins of three 9- to 12-year-old boys after circumcision. Written informed consents were obtained from parents of all subjects. The samples were aseptically collected and washed several times with 75% alcohol and phosphate buffered saline (PBS) containing 1% antibiotic-antimycotic solution (PAA, Austria). After removing the epidermis layer, the pure dermis was cut into small pieces and transferred into centrifuge tubes containing 0.03% collagenase type I solution (Worthington Biochemical Corporation, USA). The cells were rinsed with PBS before being cultured in Dulbecco's Modified Essential Medium (DMEM) (Flowlab, Australia) supplemented with 10% bovine fetal serum (FBS) (PAA, Austria) and 1% antibiotic-antimycotic solution at 37°C in a humidified atmosphere at 5% CO_2. Cultures were grown to 80–90% confluency in 75-mm culture flasks (Nunc, Denmark) with expansion degree 1 : 4. For subsequent experiments, cells were used at passage 4 (young cells, population doubling; PD < 12) and passage 30 (senescent cell, PD > 55).

4.2. Treatment Protocols. Stress-induced premature senescence model was established by culturing the early passage cells (passage 6) with culture medium containing 20 μM H_2O_2 for 2 weeks which was replaced every 3 days. Treated cells were preincubated with 1 μM γ-tocotrienol (Malaysian Palm Oil Board, Malaysia) at passage 4 followed by H_2O_2 induction at passage 6 for 2 weeks. Untreated control cells were cultured without any treatment until similar population doublings were reached.

At the end of the treatment duration, the treated culture medium was replaced by fresh medium containing 10% serum for 3 days to ensure that the observed changes to the cells were not due to lack of mitogenic stimulations [26].

4.3. Morphology Analysis and Senescence-Associated β-Galactosidase (SA-β-gal) Staining. HDFs positive for SA-β-gal activity was determined as described by Dimri et al. [27]. SA-β-gal staining was performed with a senescent cell staining kit (Sigma, USA) according to the manufacturer's instructions. A total of 1×10^5 cells were seeded

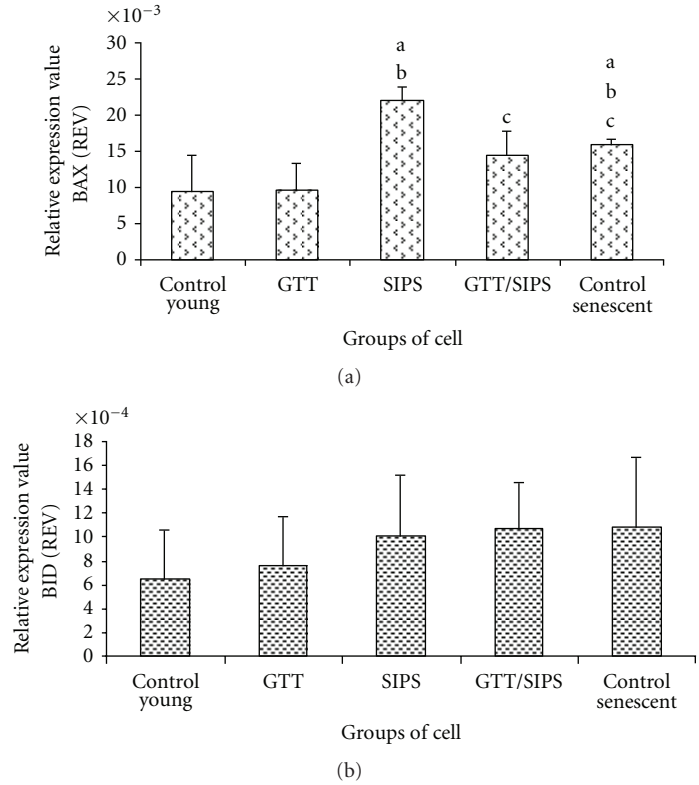


Figure 8: Relative expression value of proapoptotic genes BAX (a) and BID (b). Data are expressed as means ± SD, $n = 6$. [a]denotes $P < 0.05$ compared to control young, [b]$P < 0.05$ compared to GTT, and [c]$P < 0.05$ compared to SIPS.

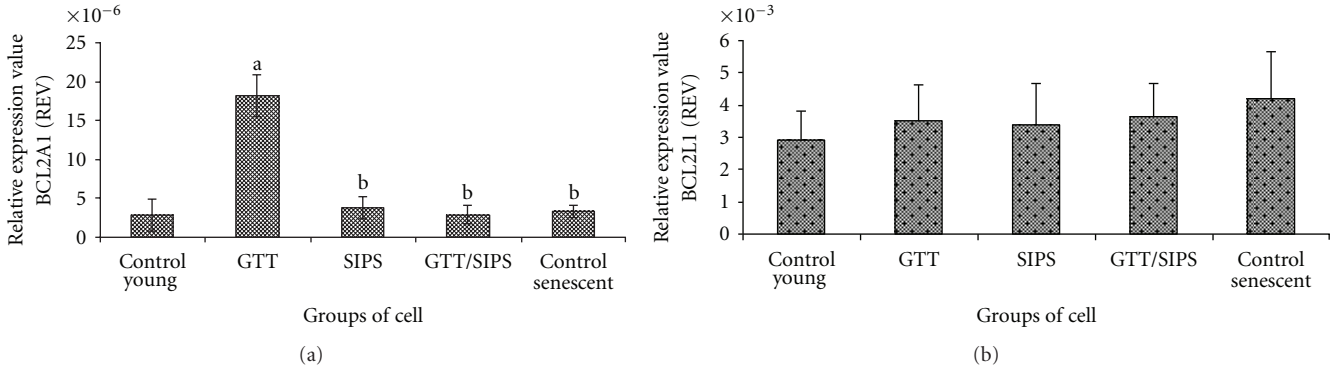


Figure 9: Relative expression value of antiapoptotic genes BCL2A1 (a) and BCL2L1 (b). Data are expressed as means ± SD, $n = 6$. [a]denotes $P < 0.05$ compared to control young and [b]$P < 0.05$ compared to GTT.

in six-well plates and incubated with fixation buffer (2% formaldehyde/0.2% glutaraldehyde) for 6–7 min at room temperature. Cells were then rinsed three times with PBS and incubated with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside at 1 mg/mL in a buffer containing 40 mM citric acid/phosphate (pH 6.0), 5 mM K_3FeCN_6, 5 mM K_4FeCN_6, 150 mM NaCl, and 2 mM $MgCl_2$ for 4 h at 37°C in the absence of CO_2. Blue staining was visible after incubation, and the percentage of blue cells observed in 100 cells under a light microscope was calculated.

4.4. Cell Cycle Analysis by Flow Cytometry. HDFs were harvested at desired time points after trypsinization and were rinsed 3 times with buffer solution with adjusted concentration 1×10^6 cells/mL and prepared using CycleTEST PLUS DNA Reagent Kit (Becton Dickinson, USA) according to the manufacturer's instruction. Cell cycle status was analyzed by flow cytometer using propidium iodide (PI) as a specific fluorescent dye probe. The PI fluorescence intensity of 10,000 cells was measured for each sample using a Becton–Dickinson FACS Calibur Flow Cytometer.

Table 1

Gene definition	Gene symbol	Accession no.	Base pair sequences (RefSeq)	PCR product size (bp)
Homo sapiens BCL2-associated X protein, transcript variant alpha, and mRNA	BAX	NM_138761	F: 5′acgaactggacagtaacatggag 3′ R: 5′cagtttgctggcaaagtagaaaag 3′	158 bp
Homo sapiens BH3 interacting domain death agonist, transcript variant 1, and mRNA	BID	NM_197966	F: 5′tgtgaaccaggagtgagtcg 3′ R: 5′ctttggaggaagccaaacac 3′	122 bp
Homo sapiens BCL2-related protein A1, transcript variant 1, and mRNA	BCL2A1	NM_004049	F: 5′tccaaaaagaagtggaaaagaatc 3′ R: 5′gctgtcgtagaagtttcttgatga 3′	189 bp
BCL2-like 1 nuclear gene encoding mitochondrial protein, transcript variant 1, and mRNA	BCL2L1	NM_138578	F: 5′gcatatcagagctttgaacaggt 3′ R: 5′taggtggtcattcaggtaagtgg 3′	180 bp
Glyceraldehyde-3- phosphate dehydrogenase, and mRNA	GAPDH	BC_020308	F: ccaagatgccacagatgattg R: actccttgggtccacctggta	217 bp

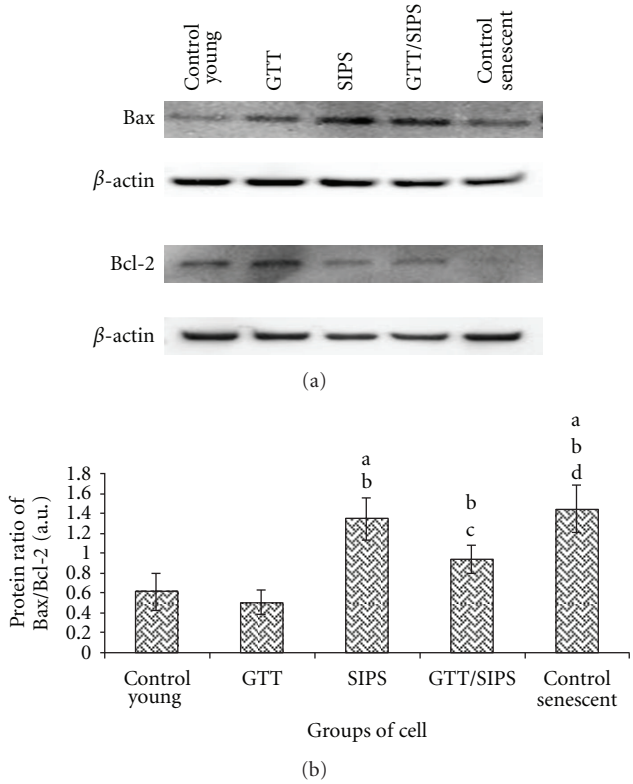


Figure 10: Representative Western blot of proapoptotic protein (Bax) and antiapoptotic protein (Bcl-2) in HDFs (a). Comparison of Bax/Bcl-2 protein ratio and in different treatment groups (b). Data are expressed as means ± SD, $n = 6$. [a]denotes $P < 0.05$ compared to control young, [b]$P < 0.05$ compared to GTT, [c]$P < 0.05$ compared to SIPS and [d]$P < 0.05$ compared to control senescent cells.

4.5. Estimation of Telomere Length. Genomic DNA was isolated using Wizard Genomic Purification Kit (Promega, USA) and the assay was carried out using TeloTAGGG Telomere Length Assay kit (Roche, Germany). Three μg of DNA was digested with 20 units each of *Hinf*I and *Rsa*1 for 2 h at 37°C, electrophoresed on 0.8% agarose gel, and transferred onto nylon membrane Hybond-N^+ (Amersham, UK) using 20X SSC (3 M $NaCl_2$, 0.3 M sodium citrate, pH 7.0) transfer buffer. After overnight blotting, DNA was cross-linked to membrane by UV light (150 mJ) and prehybridized for 45 min and hybridized for 3 h at 42°C with digoxigenin (DIG)-labeled telomeric DNA probes. Detection of telomeric restriction fragments (TRFs) was visualized by alkaline phosphatase metabolizing CDP-Star, a highly sensitive chemiluminescence substrate. Smears on the membrane were detected by chemiluminescence signal using Fluorchem FC2 gel documentation system (Alpha Innotech, USA). Densitometry analysis was carried out using Image-Master TotalLab v1.11 software (Amersham, Germany) by comparing signals relative to DNA molecular weight marker. The mean TRF length was calculated for each sample by integrating the signal density above background over the entire TRF distribution as a function of TRF length, using the formula below:

$$L = \frac{\sum(\mathrm{OD}_i \times L_i)}{\sum(\mathrm{OD}_i)}, \quad (1)$$

where OD_i and L_i are, respectively, the signal intensity and TRF length at position i on the gel image, as described in the manufacturer's recommendations.

4.6. Estimation of Telomerase Activity. The TRAPeze telomerase detection kit (Chemicon, USA) was used as recommended by the manufacturer with minor modifications. For the PCR reaction, 2 μL of extract (corresponding to 100–1000 cells) was added to 48 μL reaction mixture and 2 units of Taq DNA polymerase (Qiagen, USA). A negative control with an enzyme-inactivated sample (2 μL heat-treated cell extract, 10 min at 65°C) was performed parallel to the non-inactivated samples to confirm specificity of the products. PCR was performed as follows: primer elongation (30 min, 30°C), telomerase inactivation (4 min, 94°C), and product amplification for 35 cycles (94°C for 30 s, 59°C for 30 s,

72°C for 1 min). The PCR products were electrophoresed on 10% polyacrylamide gel using 0.5X TBE buffer. The gel was further analysed and quantitated using the ImageMaster TotalLab v1.11 software (Amersham, Germany). Telomerase activity (total product generated; TPG) was determined as the ratio of the intensity of telomerase ladders to the intensity of the 36 bp internal standard based on the formula below:

$$\mathrm{TPG(unit)} = \frac{(X - X_0)/C \times 100}{(r - r_0)/C_R}, \quad (2)$$

where, X is TRAP product ladder (noninactivated sample); X_0 is TRAP product ladder (enzyme-inactivated sample); r is quantification control TSR 8 (0.5 μL); r_0 is quantification control TSR 8 (1.0 μL); C is assay internal control/S-IC; C_R is negative control.

4.7. Detection of Caspase-3 Activation. Caspase-3 activation was determined using the FITC Active Caspase-3 Apoptosis kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. The cells were resuspended in BD Cytofix/Cytoperm buffer in the provided solution at a concentration of 2×10^6 cells/mL and were incubated 20 min in ice. After incubation, pellets were washed twice with BD Perm/Wash buffer and were stained with fluorescein isothiocyannate (FITC)-conjugated with 20 μL antiactive caspase-3 antibody for 30 min at room temperature and subsequently analyzed by FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA, USA). Fluorescence was detected with a FL1 detector and histogram data was generated with the CellQuest software.

4.8. Measurement of Active Caspase-8 and Caspase-9. Caspase-8 and caspase-9 activation was analyzed using Caspase-8 Detection Kit and Caspase-9 Detection Kit (Calbiochem, Germany), respectively, according to the manufacturer's instructions. Cells were harvested and incubated with 1 μL fluorescein-labeled caspase inhibitor (FITC-IETD-FMK for caspase-8 and FITC-LEHD-FMK for caspase-9) for 1 h at 37°C in a humidified incubator with 5% CO_2. After incubation, cells were centrifuged at 3000 rpm for 5 min. Pellets were then washed with wash buffer and subsequently analyzed by FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA, USA). Fluorescence was detected with a FL1 detector and histogram data was generated with the CellQuest software.

4.9. Measurement of Annexin V-FITC. Externalization of phosphatidylserine (PS) at the cell surface of HDFs as an early apoptotic event was assessed by Annexin V-FITC Apoptosis Detection Kit II (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. This method based on cell staining by FITC-labeled Annexin-V (green flourescein) and propidium iodide (PI) which distinguished between early apoptotic (Annexin V^+/PI^-) and late apoptotic or necrotic cells (Annexin V^+/PI^+) [28]. Cells were washed twice with cold PBS and resuspended in 1X binding buffer. Then 5 μL of Annexin V-FITC and 5 μL of PI staining solution were added, followed by incubation for 15 min in the dark at room temperature (25°C). Finally, cells were suspended in 1X binding buffer and analyzed within 1 h by FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA, USA).

4.10. Measurement of Mitochondria Cytochrome c Release. The InnoCyte Flow Cytometric Cytochrome *c* Release Kit (Calbiochem, Germany) was used to quantify mitochondria-associated cytochrome *c*. This method selectively permeabilized the plasma membrane without injuring the mitochondrial membrane. Cells were resuspended with 300 μL permeabilization buffer and incubated for 10 min in ice. Then, cells were fixed by adding 300 μL 8% paraformaldehyde in PBS, incubated for 20 min at room temperature and were washed with 1 mL 1X wash buffer for 3 times. Cells were centrifuged and pellets were incubated in 250 μL blocking buffer for 1 h at room temperature. Anticytochrome *c* WS (250 μL) was added and cells were incubated for 1 h at room temperature followed by incubation with 500 μL anti-IgG FITC WS in the dark for 1 h at room temperature. Finally, cells were resuspended in 500 μL wash buffer and analysed by FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA, USA).

4.11. Primer Design. Primers for human GAPDH, BAX, BID, BCL2A1, and BCL2L1 were designed with Primer 3 software and blasted with GeneBank database sequences in order to obtain primers with high specificity. The efficiency and specificity of each primer set were confirmed with standard curve (Ct value versus serial dilution of total RNA) and melting profile evaluation. Primer sequences for quantitative gene expression analysis are shown in Table 1.

4.12. Total RNA Extraction. Total RNA from HDFs in different groups was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instruction. Polyacryl Carrier (Molecular Research Center) was added in each extraction to precipitate the total RNA. Extracted total RNA pellet was then washed with 75% ethanol and dried before being dissolved in RNase and DNase-free distilled water. Total RNA was stored at −80°C immediately after extraction. Yield and purity of the extracted RNA were determined by Nanodrop (Thermo Scientific, USA).

4.13. Quantitative Real-Time RT-PCR. The expression level of BAX, BID, BCL2A1, and BCL2L1 was determined using iScript One-Step RT-PCR Kit with SYBR Green (Biorad, USA) and iQ5 Multicolor Real-Time PCR system (Biorad, USA) with the following reaction profile; cDNA synthesis at 50°C for 20 min, iScript reverse transcriptase inactivation at 95°C for 4 min, followed by 38 cycles of denaturation at 95°C for 10 sec and primer annealing and extension at 61°C for 30 sec. Melt curve analysis was carried out at 95°C for 1 min, 55°C for 1 min, and 60°C for 10 sec (70 cycles, increase set point temperature after cycle 2 by 0.5°C). The expression level of each targeted gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as an internal

reference [29]. Agarose gel electrophoresis was performed for confirmation of the PCR products. Relative expression value of target genes was calculated based on the $2^{-\Delta\Delta Ct}$ method of relative quantification [30] by the following equation:

$$\text{Relative expression value} = 2^{\text{Ct value of GAPDH} - \text{Ct value gene of interest.}} \quad (3)$$

4.14. Western Blotting Analysis. Determination of protein expression was carried out by Western blotting using NuPAGE electrophoresis system (Invitrogen, CA, USA). Protein (25 μg) from each sample was loaded on a 4–12% NuPAGE Novex Bis-Tris gel using NuPAGE MES running buffer followed by dry-blotting of protein using iBlot Transfer Device for 7 min. WesternBreeze Chemiluminescent Kit was used to detect the primary antibodies. After transferring onto nitrocellulose membranes, the membranes were blocked for 30 min with blocking solution and probed with antibodies against Bax (1 : 200, overnight), Bcl-2 (1 : 200, overnight), and β-actin (1 : 1000, 1 h) as an internal control. All antibodies were purchased from Santa Cruz Biotechnology Inc., CA, USA. After 3 washing steps of antibody wash, membranes were incubated with secondary antibody solution (anti-mouse-IgG) for 1 h. Blots were developed using chemiluminescence substrate with enhancer for 7 min. The autoradiographies were scanned using gel documentation system Fluorchem FC2 (Alpha Innotech, USA) and band intensities were quantified by Image Master TotalLab software (Amersham Biosciences, UK).

4.15. Statistical Analysis. Each experiment was carried out in triplicates. Data were reported as means ±SD. Comparison between groups was made by Student t-test (two-tailed). $P < 0.05$ was considered statistically significant

5. Conclusions

In summary, GTT was found to inhibit the translocation of PS into the outer membrane which is an early event in the apoptosis pathway. The antiapoptotic effect of GTT was through the mitochondria-dependent pathway as shown by GGT preventing the activation of caspase-3 and caspase-9 and reduced the release of cytochrome *c* from the mitochondria. Genes and proteins expression analysis suggested that GTT stimulated the signaling in the regulation of apoptotic-related genes expression, prevented H_2O_2-induced apoptosis in HDFs, and delayed cellular aging.

In conclusion, GTT delays cellular senescence of human diploid fibroblasts through the inhibition of intrinsic mitochondria-mediated pathway which involved the regulation of pro- and antiapoptotic genes and proteins.

Acknowledgments

This study was financially supported by Universiti Kebangsaan Malaysia Fundamental Funds (FF-144-2007 and FF-313-2009) and Ministry of Higher Education Grant (UKM-FF-03 FRGS0036-2010).

References

[1] J. Duan, J. Duan, Z. Zhang, and T. Tong, "Irreversible cellular senescence induced by prolonged exposure to H_2O_2 involves DNA-damage-and-repair genes and telomere shortening," *International Journal of Biochemistry and Cell Biology*, vol. 37, no. 7, pp. 1407–1420, 2005.

[2] J. Campisi, "Cancer, aging and cellular senescence," *In Vivo*, vol. 14, no. 1, pp. 183–188, 2000.

[3] L. A. Loeb, D. C. Wallace, and G. M. Martin, "The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 52, pp. 18769–18770, 2005.

[4] R. Brigelius-Flohé, "Vitamin E: the shrew waiting to be tamed," *Free Radical Biology and Medicine*, vol. 46, no. 5, pp. 543–554, 2009.

[5] S. Schaffer, W. E. Müller, and G. P. Eckert, "Tocotrienols: constitutional effects in aging and disease," *Journal of Nutrition*, vol. 135, no. 2, pp. 151–154, 2005.

[6] S. Makpol, L. W. Durani, K. H. Chua, Y. A. Mohd Yusof, and W. Z. Wan Ngah, "Tocotrienol-rich fraction prevents cell cycle arrest and elongates telomere length in senescent human diploid fibroblasts," *Journal of Biomedicine and Biotechnology*, vol. 2011, Article ID 506171, 2011.

[7] H. Adachi and N. Ishii, "Effects of tocotrienols on life span and protein carbonylation in Caenorhabditis elegans," *Journals of Gerontology A*, vol. 55, no. 6, pp. B280–B285, 2000.

[8] S. Makpol, A. Z. Abidin, K. Sairin, M. Mazlan, G. M. Top, and W. Z. W. Ngah, "γ-tocotrienol prevents oxidative stress-induced telomere shortening in human fibroblasts derived from different aged individuals," *Oxidative Medicine and Cellular Longevity*, vol. 3, no. 1, pp. 35–43, 2010.

[9] Y. Higami and I. Shimokawa, "Apoptosis in the aging process," *Cell and Tissue Research*, vol. 301, no. 1, pp. 125–132, 2000.

[10] H.-Y. Shan, X. J. Bai, and X. M. Chen, "Apoptosis is involved in the senescence of endothelial cells induced by angiotensin II," *Cell Biology International*, vol. 32, no. 2, pp. 264–270, 2008.

[11] S. J. Martin, D. M. Finucane, G. P. Amarante-Mendes, G. A. O'Brien, and D. R. Green, "Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity," *Journal of Biological Chemistry*, vol. 271, no. 46, pp. 28753–28756, 1996.

[12] G. Koopman, C. P. M. Reutelingsperger, G. A. M. Kuijten, R. M. J. Keehnen, S. T. Pals, and M. H. J. Van Oers, "Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis," *Blood*, vol. 84, no. 5, pp. 1415–1420, 1994.

[13] U. Fischer, R. U. Jänicke, and K. Schulze-Osthoff, "Many cuts to ruin: a comprehensive update of caspase substrates," *Cell Death and Differentiation*, vol. 10, no. 1, pp. 76–100, 2003.

[14] P. A. Parone, D. James, and J. C. Martinou, "Mitochondria: regulating the inevitable," *Biochimie*, vol. 84, no. 2-3, pp. 105–111, 2002.

[15] D. Arnoult, P. Parone, J. C. Martinou, B. Antonsson, J. Estaquier, and J. C. Ameisen, "Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome *c* release in response to several proapoptotic stimuli," *Journal of Cell Biology*, vol. 159, no. 6, pp. 923–929, 2002.

[16] J. P. de Magalhães, F. Chainiaux, J. Remacle, and O. Toussaint, "Stress-induced premature senescence in BJ and hTERT-BJ1 human foreskin fibroblasts," *Federation of European Biochemical Societies*, vol. 523, no. 1-2, pp. 157–162, 2002.

[17] S. Zdanov, J. Remacle, and O. Toussaint, "Establishment of H_2O_2-induced premature senescence in human fibroblasts concomitant with increased cellular production of H 2O2," *Annals of the New York Academy of Sciences*, vol. 1067, no. 1, pp. 210–216, 2006.

[18] N.-C. Yang and M. L. Hu, "The limitations and validities of senescence associated-β- galactosidase activity as an aging marker for human foreskin fibroblast Hs68 cells," *Experimental Gerontology*, vol. 40, no. 10, pp. 813–819, 2005.

[19] S. Ohshima, "Apoptosis in stress-induced and spontaneously senescent human fibroblasts," *Biochemical and Biophysical Research Communications*, vol. 324, no. 1, pp. 241–246, 2004.

[20] T. Mammone, D. Gan, and R. Foyouzi-Youssefi, "Apoptotic cell death increases with senescence in normal human dermal fibroblast cultures," *Cell Biology International*, vol. 30, no. 11, pp. 903–909, 2006.

[21] M. Nagase, T. Shiota, A. Tsushima et al., "Molecular mechanism of satratoxin-induced apoptosis in HL-60 cells: activation of caspase-8 and caspase-9 is involved in activation of caspase-3," *Immunology Letters*, vol. 84, no. 1, pp. 23–27, 2002.

[22] J. C. Reed, "Double identity for proteins of the Bcl-2 family," *Nature*, vol. 387, no. 6635, pp. 773–776, 1997.

[23] P. Palozza, S. Serini, A. Torsello et al., "Mechanism of activation of caspase cascade during β-carotene-induced apoptosis in human tumor cells," *Nutrition and Cancer*, vol. 47, no. 1, pp. 76–87, 2003.

[24] G. Lu, I. Shimizu, X. Cui et al., "Antioxidant and antiapoptotic activities of idoxifene and estradiol in hepatic fibrosis in rats," *Life Sciences*, vol. 74, no. 7, pp. 897–907, 2004.

[25] J. Yang, X. Liu, K. Bhalla et al., "Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked," *Science*, vol. 275, no. 5303, pp. 1129–1132, 1997.

[26] P. Dumont, M. Burton, Q. M. Chen et al., "Induction of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblast," *Free Radical Biology and Medicine*, vol. 28, no. 3, pp. 361–373, 2000.

[27] G. P. Dimri, X. Lee, G. Basile et al., "A biomarker that identifies senescent human cells in culture and in aging skin in vivo," *Proceeding of the National Academy of Sciences of the United States of America*, vol. 92, no. 20, pp. 9363–9367, 1995.

[28] I. Vermes, C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger, "A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V," *Journal of Immunological Methods*, vol. 184, no. 1, pp. 39–51, 1995.

[29] A. Zainuddin, K. H. Chua, N. A. Rahim, and S. Makpol, "Effect of experimental treatment on GAPDH mRNA expression as a housekeeping gene in human diploid fibroblasts," *BMC Molecular Biology*, vol. 11, p. 59, 2010.

[30] L. Carthagena, A. Bergamaschi, J. M. Luna et al., "Human TRIM gene expression in response to interferons," *PLoS ONE*, vol. 4, no. 3, Article ID e4894, pp. 1–13, 2009.