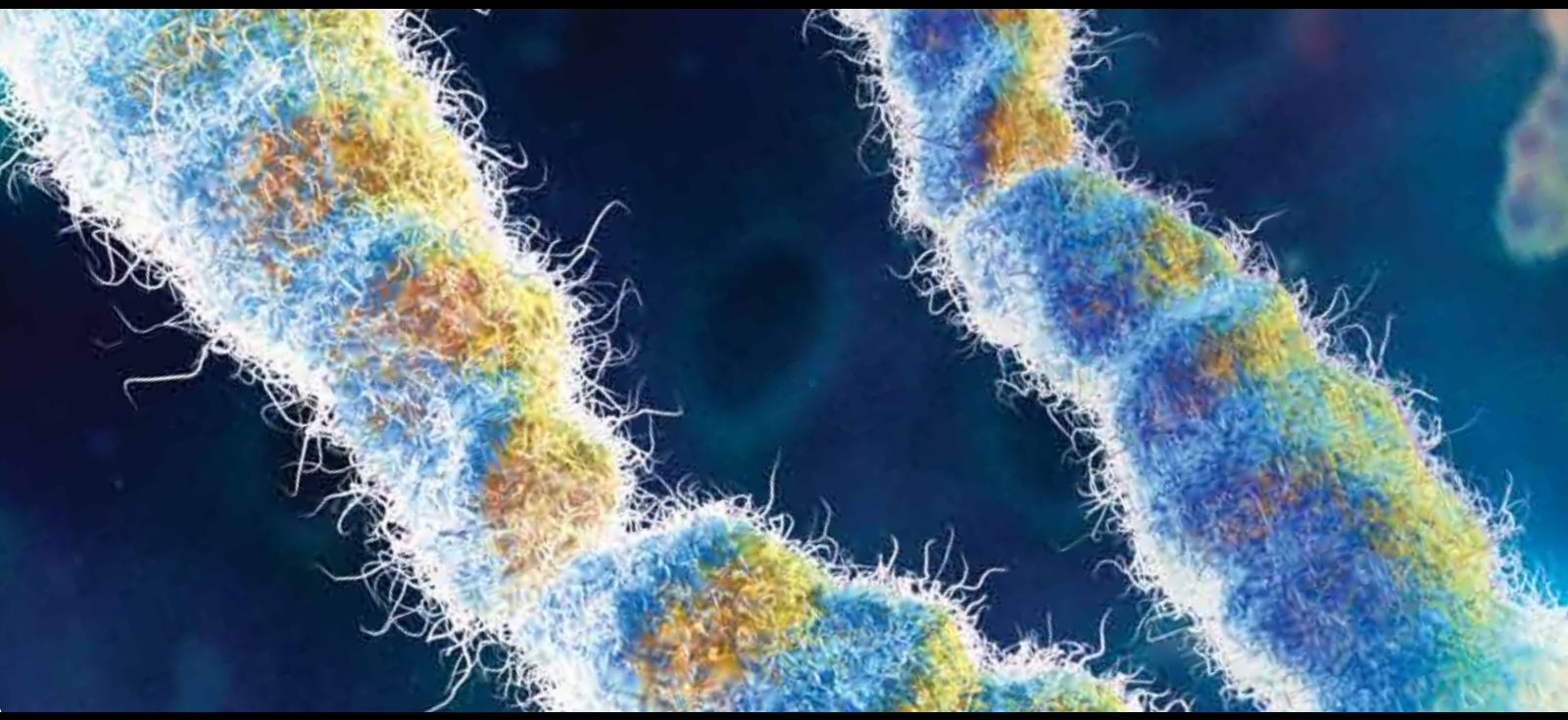
Journal of Aging Research

Cancer, Senescence, and Aging: Translation from Basic Research to Clinics

Guest Editors: Matilde E. LLeonart, Amancio Carnero, Rosanna Paciucci, Zhao-Qi Wang, and Noam Shomron



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Editorial Board

Contents

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Editorial

Cancer, Senescence, and Aging: Translation from Basic Research to Clinics

Matilde E. LLeonart,[1] Amancio Carnero,[2] Rosanna Paciucci,[1] Zhao-Qi Wang,[3] and Noam Shomron[4]

[1] *Oncología y Patología Grupo, Vall d'Hebron Institut de Recerca, 08035 Barcelona, Spain*
[2] *Unitat de Recerca Biomédica, Instituto de Biomedicina de Sevilla, 41013 Sevilla, Spain*
[3] *Genomic Stability Laboratory, Leibniz Institute for Age Research, 07745 Jena, Germany*
[4] *Genome High-Throughput Sequencing Laboratory, Department of Cell and Developmental Biology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel*

Correspondence should be addressed to Matilde E. LLeonart, melleona@ir.vhebron.net

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Unravelling the molecular basis of malignancy is a challenging process of great priority, as cancer rates are increasing worldwide and because certain cancer types are still incurable. The urgent need for novel treatment modalities based upon recent discoveries at the genetic and epigenetic level necessitates a strong collaboration between researchers and clinicians to work toward a common aim: the control of the carcinogenic process in order to ultimately achieve a 100% cure rate.

In the last 20 years, a myriad of discoveries at the molecular level have been accomplished, especially with the completion of the "Human Genome Project." This special issue focuses on how senescence affects tumourigenesis and how novel senescence-related therapeutic approaches could be used to benefit tumour regression and eradication efforts.

Mammalian cells have developed complex defence mechanisms, such as apoptosis, growth or cell cycle arrest, and senescence, to combat uncontrolled proliferation caused by external stimuli (e.g., carcinogenic agents). Replicative senescence occurs when somatic cells spontaneously decline their growth rate in continuous culture due to an increasing number of population doublings, eventually terminating in a quiescent but viable state. Importantly, senescence has been observed in patients with premalignant tumours but has not been detected in malignant tumours. Clear evidence points to a crucial role of cellular senescence in counteracting malignant transformation. Therefore, in order to eradicate cancer, key molecules (proteins, microRNAs, etc...) and processes important in senescence could be targets for therapeutic intervention.

The papers collected in this issue deal with the above-mentioned key senescence factors.

(1) All important oncogenes and tumour suppressor genes crucial in this process are shown. How and to what extent their targeting would be effective in cancer therapeutics is reviewed.

(2) Recently, a specific group of proteins called Sirtuins, which are part of an evolutionarily conserved family of NAD-dependent protein deacetylases/ADP-ribosyltransferases, have been identified as key components in senescence and aging. The relationship of Sirtuins with genomic instability and their influence on telomerase and tumourigenesis is discussed.

(3) DNA-damage response and its link with self-renewal and senescence are reviewed as determinant processes in which senescent cells may decide how their genetic backgrounds and protein statuses can promote or prevent carcinogenesis.

(4) MicroRNAs, short noncoding RNA molecules of ~22 nucleotides, are key post-transcriptional regulators of gene expression. MicroRNAs have an important role in tumour development, progression, chemosensitivity and cellular senescence. Decoding microRNA function is required for the development of novel

therapies, such as restoring tumour suppressor-microRNAs and targeting onco-microRNAs with anti-miR technology. All of these approaches are extensively reviewed.

Moreover, abundant evidence suggests that senescence plays an important role in aging. The paradoxical role of senescence as a protective mechanism against the eradication of cancer might be detrimental to the possible contribution of senescence to aging. The molecular regulation of senescence in cancer and aging is discussed.

Overall, the potential of cellular senescence to be used as a target for anticancer therapy is a close reality in the clinical practice. In this issue, therapeutic strategies are fully considered, and their applications in each case are proposed.

Matilde E. LLeonart
Amancio Carnero
Rosanna Paciucci
Zhao-Qi Wang
Noam Shomron

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Review Article

MicroRNAs Regulate Key Effector Pathways of Senescence

Andrea Feliciano,[1] Beatriz Sánchez-Sendra,[1] Hiroshi Kondoh,[2] and Matilde E. LLeonart[1]

[1] *Oncology and Molecular Pathology Group, Pathology Department, Fundacil Institut de Recerca Hospital Vall d'Hebron, Passeig Vall d'Hebron 119-129, 08035 Barcelona, Spain*
[2] *Department of Geriatric Medicine, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*

Correspondence should be addressed to Matilde E. LLeonart, melleona@ir.vhebron.net

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MicroRNAs (miRNAs) are small (approximately 22 nt) noncoding endogenous RNA molecules that regulate gene expression and protein coding by base pairing with the 3′ untranslated region (UTR) of target mRNAs. miRNA expression is associated with cancer pathogenesis because miRNAs are intimately linked to cancer development. Senescence blocks cell proliferation, representing an important barrier that cells must bypass to reach malignancy. Importantly, certain miRNAs have been shown to have an important role during cellular senescence, which is also involved in human tumorigenesis. Therefore, therapeutic induction of senescence by drugs or miRNA-based therapies is a potential method to treat cancer by inducing a persistent growth arrest in tumors.

1. Introduction

miRNAs are small noncoding RNAs (approximately 22 nt) that regulate gene expression by interfering with protein translational machinery and/or inducing degradation of target mRNAs [1]. Hundreds of microRNA (miRNA) genes have been found in animals, plants, and viruses [2–4] making them one of the largest gene families.

Recent studies revealed the key roles of miRNAs in diverse regulatory pathways including development timing control, hematopoietic cell differentiation, apoptosis, cell proliferation, and organ development [2]. miRNAs and their targets constitute remarkably complex regulatory networks because a single miRNA can bind to and regulate many different mRNA targets, and conversely, several different miRNAs can bind to and cooperatively control a single mRNA target [5]. In general, miRNAs repress protein expression at the posttranscriptional level through base pairing with the 3′-UTR leading to reduced translation, or in some cases, degradation. However, some miRNAs have been shown to bind to the open reading frame or to the 5′-UTR of the target mRNAs. In some cases, miRNAs have been shown to activate rather than inhibit gene expression [6–8].

miRNAs are involved in many aspects of cell biology including physiological modulation and pathological disruption of basic pathways. In this regard, miRNAs are key mediators in cancer where they regulate many aspects of tumorigenesis and tumor progression from the initiating steps to metastasis formation and chemosensitivity [9, 10]. Therefore, miRNA expression may be deregulated in cancer because abnormal miRNA activity may lead to tumorigenesis. It has been shown that human tumors exhibit distinctive miRNA expression signatures [11]. miRNA expression is tissue specific, and certain cancer histotypes can be classified based on miRNA expression profiles [12]. Some miRNAs have been found to be upregulated or downregulated in cancer. An overexpressed miRNA that downregulates a tumor suppressor gene is defined as an oncomir, and a downregulated miRNA that normally downregulates the expression of an oncogene is defined as a tumor suppressor miR (TS-miR). However, some miRNAs may function as oncogenes in some cell types and as tumor suppressors in other cell types [13]. Aberrant miRNA expression in cancer due to chromosomal abnormalities, polymorphism, and/or epigenetic changes has a direct impact on miRNA biogenesis. In this study, we reviewed the potential impact of miRNAs in senescence and cancer. We suggest that the biological

function of miRNAs is extensively studied in the near future to identify their potential clinical applications.

2. MicroRNA Biogenesis

miRNA biogenesis has been studied by many scientists. A schematic overview of miRNA biogenesis is shown in Figure 1. Most of the miRNA genes exist in clusters in the genome and are polycistronically expressed from their own promoter. Other miRNA genes are found in intronic regions and are transcribed as a part of annotated genes. The transcription of most miRNA genes is mediated by RNA polymerase II (Pol II), producing long primary miRNAs (pri-miRNAs) that are capped at the 5′ end and polyadenylated at the 3′ end. These pri-miRNAs contain a stem of approximately 33 base pairs, a terminal loop, and flanking ssRNA segments [14]. However, a small group of miRNAs associated with Alu repeats can be transcribed by Pol III [15]. Two steps of ribonuclease pro-cessing reactions are required to generate mature miRNAs. The first step occurs in the nucleus and involves the release of a 70 nt intermediate hairpin structure (pre-miRNA) from the RNA duplex in the pri-miRNA by the RNase III-type protein, Drosha [16]. Moreover, Drosha-mediated pri-miRNA processing requires the cofactor, DiGeorge syndrome critical region gene 8 (DGCR8). Together with DGCR8, Drosha forms a large complex known as the microprocessor complex, which is approximately 650 kDa in humans [17, 18]. Specifically, DGCR8 interacts with pri-miRNAs through the ssRNA segments and the stem (approximately 33 bp), and DGCR8 assists Drosha to cleave the substrate (approximately 11 bp) from the ssRNA/dsRNA junction [19, 20]. The resulting pre-miRNA is transported out of the nucleus and into the cytoplasm by Exportin-5 and its cofactor, Ran-GTP [21].

Apart from the canonical miRNA pathway, an alternative nuclear pathway for miRNA biogenesis has been recently described in invertebrates [22, 23] and mammals [24]. This noncanonical pathway involves short introns with hairpin potential, which are termed mirtrons. Mirtrons bypass are processed by the microprocessor. Therefore, they are processed by splicing and debranching. Debranched mirtrons access the canonical miRNA pathway during nuclear export. The debranched mirtrons are cleaved by Dicer and incorporated into silencing complexes [22, 23].

The miRNA maturation process in the cytoplasm is carried out by Dicer, which is a highly conserved RNase III-type endoribonuclease present in almost all eukaryotic organisms. PremiRNAs are cleaved near the terminal loop by Dicer-releasing miRNA duplexes (approximately 22 nt) [25]. Human Dicer interacts with proteins, such as TAR RNA binding protein (TRBP; also known as TARBP2) [26, 27]. However, current studies have, suggested that they are not required for miRNA processing but that they contribute to the formation of the RNA-induced silencing complex (RISC) [28]. Following Dicer cleavage, the 22-nt RNA duplex binds to Argonaute (Ago) proteins to generate the effector complex, RISC. One strand of the 22-nt RNA duplex remains in the Ago complex as a mature miRNA (the guide strand or miRNA), and the other strand (the passenger strand or miRNA*) is degraded [25]. Next, the miRNA guides RISC to specifically recognize and repress target mRNAs. In most cases, miRNAs repress protein expression through base pairing with the 3′-UTRs of the target mRNA [28]. Perfect complementarity, which is rare in animal miRNA/mRNA base pairing, allows Ago-catalyzed cleavage of the mRNA strand. In contrast, central mismatches exclude cleavage and promote repression of mRNA translation.

The specificity of miRNA targeting is defined by Watson-Crick complementarities between positions 2 to 8 from the 5′-miRNA (also known as the seed) with the 3′-UTR of the target mRNAs. When miRNAs and their target mRNA sequence have perfect complementarities, RISC induces mRNA degradation. When an imperfect miRNA/mRNA target pairing occurs, protein translation is blocked. Regardless which of these two events occurs, the net result is a decrease in the amount of proteins encoded by the mRNA targets.

3. miRNAs Involved in Senescence

Cellular senescence was originally described in primary cells as a process that limits the replicative potential of human diploid fibroblasts in culture. This type of senescence is called replicative senescence. Senescence is an important block to cell cycle progression during the aging of cells in culture and is a fundamental barrier that cells must bypass during carcinogenesis. Senescent cells are characterized by the expression of β-galactosidase, overexpression of plasminogen activator protein 1 (PAI-1), and altered cell morphology characterized by a giant cell size, increased cytoplasmic granularity, and a single large nucleus [29].

Cellular senescence is a process that is triggered by several types of stresses as follows: telomeric erosion resulting from repeated cell division (replicative senescence); DNA damage; oxidative stress resulting from mitochondrial deterioration; overexpression of oncogenes; loss of tumor suppressors such as PTEN and VHL (oncogene-induced senescence; OIS) [29, 30]. OIS was first observed when an oncogenic form of Ras (e.g., Ras^{G12V}), which is a cytoplasmic transducer of mitogenic signals, was expressed in normal human fibroblasts [31]. Other members of the Ras signaling pathway, such as v-raf-1 murine leukemia viral oncogene homolog 1 (RAF), mitogen-activated protein kinase kinase 1 (MEK), v-mos Moloney murine sarcoma viral oncogene homolog (MOS), and v-raf murine sarcoma viral oncogene homolog B1 (BRAF), in addition to pro-proliferative nuclear proteins, such as the E2F transcription factor, can also induce a senescence response upon overexpression [32]. Moreover, OIS can be caused by the loss of the tumor suppressors that function upstream of oncogenes, such as phosphatase and tensin homolog (PTEN), von Hippel-Lindau tumor suppressor (VHL), and neurofibromin 1 (NF1), resulting in an increase of oncogenic signaling that leads to senescence [30]. In general terms, OIS has similar characteristics to replicative senescence including the presence of β-galactosidase-positive cells, induction of cell cycle inhibitory proteins, and phenotypic morphology of giant cells.

The major pathways that regulate cellular senescence are the $p53/p21^{Cip1}$ and $p16^{INK4A}$-pRB tumor suppressor

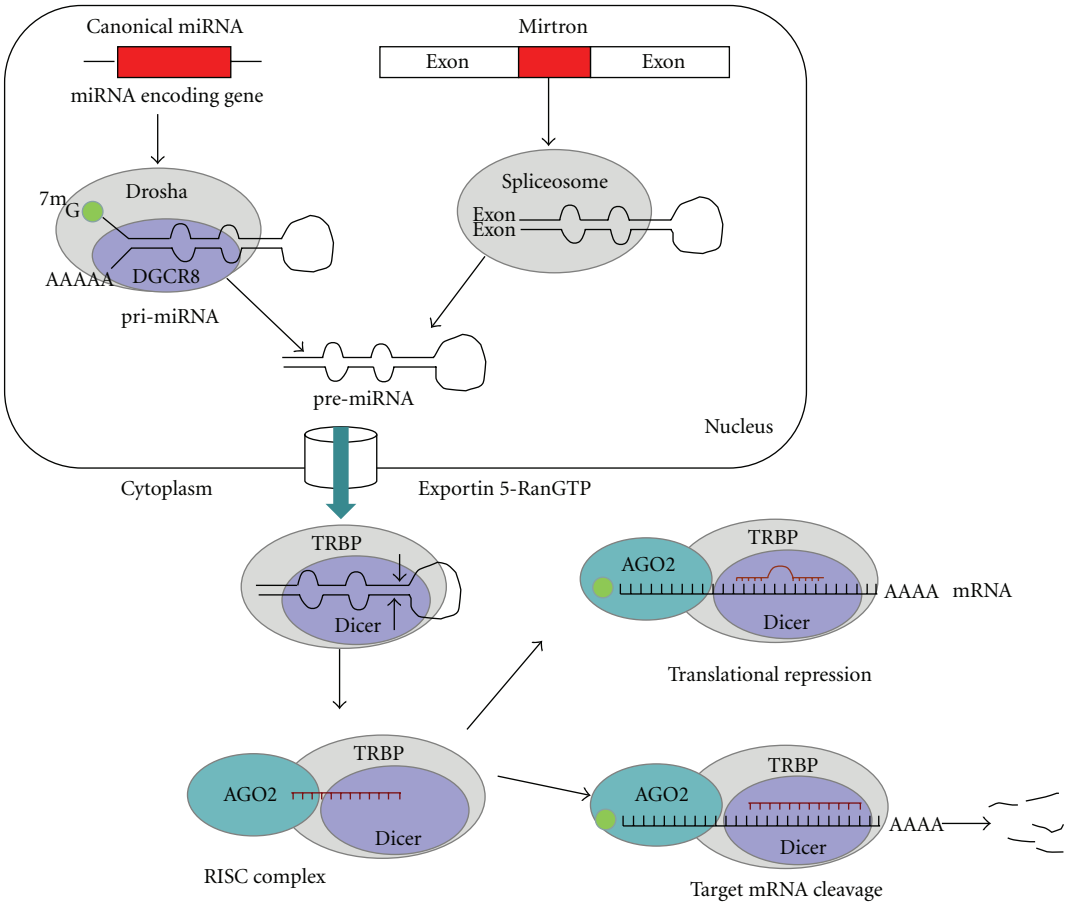

Figure 1: miRNA biogenesis. In the nucleus, canonical miRNAs are processed by the endoribonuclease, Drosha, in partnership with DGCR8. In contrast, mirtrons are processed by the spliceosome. The pre-miRNA is then exported from the nucleus into the cytoplasm by Exportin-5, and it is processed into a mature miRNA by Dicer. After RISC incorporation, mature miRNAs inhibit translation or promote degradation of their target mRNA transcripts depending on the degree of complementarity between the 3′-UTR of the target mRNA and the seed region of the miRNA.

pathways. p53 provokes growth arrest, in part by inducing the expression of $p21^{Cip1}$, which is a cyclin-dependent kinase (CDK) inhibitor that suppresses the phosphorylation and, hence, the inactivation of pRB [32, 33]. In addition, senescence signals that engage the $p16^{INK4A}$-pRB pathway generally do so by inducing the expression of $p16^{INK4A}$, which is another CDK inhibitor that prevents pRB phosphorylation and inactivation [32]. In this regard, the loss of tumor suppressors that function downstream of oncogenes, such as p53, impair senescence and allow progression to malignant stages providing a link between tumor suppression and the induction of senescence by $p16^{INK4A}$, $p19^{ARF}$, and p53 [30]. In general, oncogenes that elicit a senescence response often converge on the activation of p53 and/or pRB. However, RAF-induced senescence independent of both p53 and pRB has been reported in human cells [34].

Importantly, several groups have shown that benign tumors contain senescent cells and that these cells fully disappear in the corresponding malignant areas of the patients. Senescent cells are found in premalignant lesions in mice and humans, and they are absent in their corresponding malignant stages, which suggests a role for senescence as a barrier to tumor progression [35, 36]. In addition, senescent cells are relatively rare in young organisms, but their number increases with age. Consistent with a role in aging, senescent cells accumulate with age in many rodent, primate, and human tissues [37, 38]. Moreover, they are found at sites of age-related pathology including degenerative disorders, such as osteoarthritis and atherosclerosis [37], in addition to hyperproliferative lesions, such as benign prostatic hyperplasia [39].

Several miRNAs have been reported to be differentially expressed in senescent cells when compared to primary cells, providing a role for miRNAs in senescence (Figure 2, Table 1). Recently, it has been reported that miR-34a overexpression during senescence can be p53 dependent and p53 independent [40–44]. p53 activates transcription of a set of genes, which induces cell cycle arrest, senescence, or apoptosis. Moreover, p53 also regulates the expression of miR-34a because genes in the family of miR-34 genes contain p53-binding sites in their promoters, which are conserved among humans and rodents. In turn, miR-34a increases the activity of p53 by means of reducing expression of sirtuin 1 (SIRT1), which interacts with p53 and deacetylates the Lys382 residue

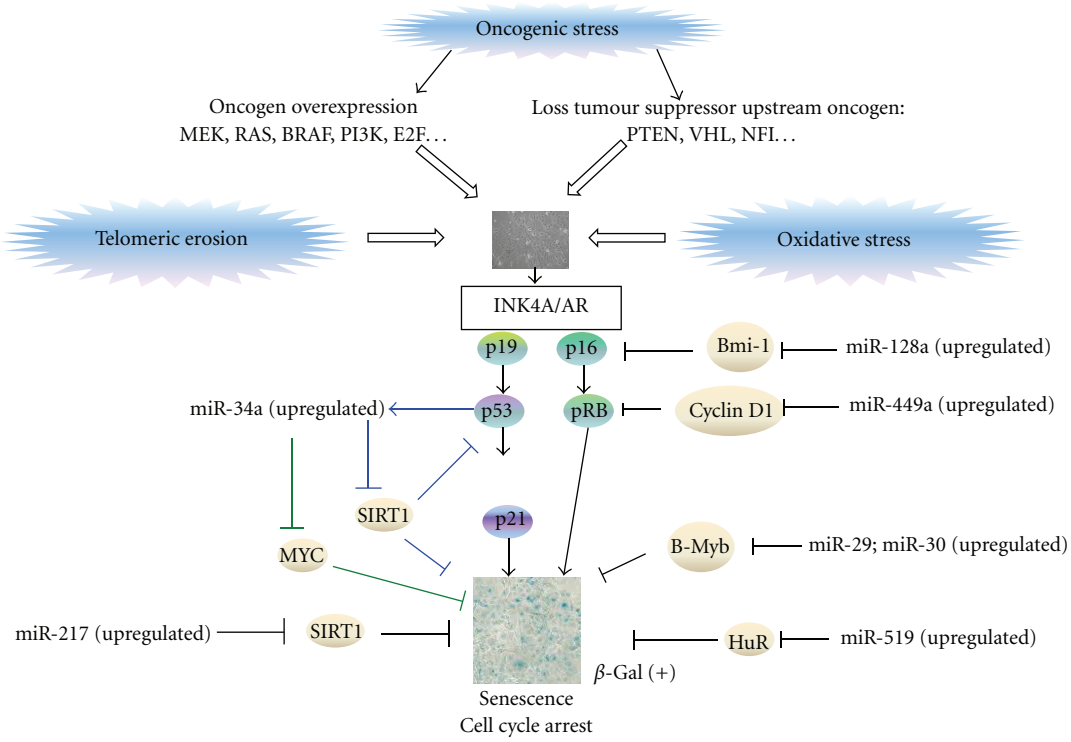

Figure 2: Role of miRNAs in senescence. Cellular senescence is triggered by several factors including telomeric erosion, oncogenic stress, oxidative stress, and miRNA modulation. The following miRNAs are key regulatory miRNAs modulating senescence: miR-128a, miR-449a, miR-29, miR-30, miR-519, miR-217, and miR-34a.

of p53 in a NAD^+-dependent manner, thereby decreasing p53-mediated transcriptional activation and reducing the expression levels of downstream proteins, such as $p21^{Cip1}$. Therefore, overexpression of miR-34a decreases SIRT1 expression, allowing an increase in p53 acetylation and p53 activity and thus mediating the senescence response [43, 45]. On the other hand, several studies have shown that miR-34a causes senescence in a p53-independent manner. miR-34a induces senescence and suppression of cell proliferation through downregulation of the E2F pathway in human colon cancer cells p53 (+/+) leading to the upregulation of the $p53/p21^{Cip1}$ pathway, but also in human colon cancer cells p53 (−/−) [42]. In addition, another study has reported a strong upregulation of miR-34a during B-RAF-induced senescence independent of p53. In this case, miR-34a is transcriptionally upregulated by ELK1, which is a member of the ETS oncogene family and has previously been implicated in cellular senescence. Moreover, miR-34a is upregulated after activation of the B-RAF oncogene. Finally, miR-34a provokes senescence through repression of v-myc myelocytomatosis viral oncogene homolog (Myc) [44]. Importantly, miR-34a, which is a tumor suppressor in the miR-34 family, is downregulated in pancreatic cancer cells, neuroblastomas, colon cancer cells, and lung cancer cells [40–42, 46].

The expression levels of miR-29 and miR-30 increase during cellular senescence in a Rb-dependent manner. Moreover, these microRNAs directly repress v-myb myeloblastosis viral oncogene homolog (avian)-like 2 (B-Myb) by binding to its 3′-UTR acting in conjunction with Rb-E2F complexes at the B-Myb promoter to mediate repression of B-Myb expression during Rb activation resulting in senescence [47]. miR-29 is downregulated in mantle cell lymphomas [48], and the overexpression of miR-29 is suppressed during tumorigenicity in lung cancer cells [49]. Moreover, miR-29 is upregulated in indolent human B-cell chronic lymphocytic leukemia (B-CLL) when compared to aggressive B-CLL and normal CD19+ B-cells, suggesting that miR-29 can function as an oncogene and contribute to the pathogenesis

Table 1: Novel miRNAs involved in senescence.

miRNA	miRNA function	Reference
hsa-miR-29/30	Oncomir/Tumour suppressor	[47–51]
hsa-miR-34a	Tumour suppressor	[40–44]
hsa-miR-519	Tumour suppressor	[52]
hsa-miR-449a	Tumour suppressor	[53, 54]
hsa-miR-128a	Tumour suppressor	[55–57]
hsa-miR-217	Tumour suppressor	[58, 59]
hsa-miR-372/373	Oncomir	[60–63]
hsa-miR-17-5p	Oncomir	[60, 64–69]
hsa-miR-130b	Oncomir	[60, 70]
hsa-miR-15b/25/141	Oncomir	[71–74]
mmu-miR-20a	Tumour suppressor	[75]
mmu-miR-290	Tumour suppressor	[76]

of indolent CLL. In contrast, miR-29 is downregulated in aggressive CLL when compared to indolent CLL, and miR-29 may function as a tumor suppressor in CLL by targeting T-cell leukemia/lymphoma 1 (TCL-1) [50]. In addition, miR-30 directly represses LIN28 (a lin-28 homolog of C. elegans) in embryonic stem cells and cancer cells. Importantly, LIN28 functions as an oncogene promoting malignant transformation and tumor progression [51]. Another miRNA involved in senescence is miR-449a. miR-449a induces senescence by suppressing Rb phosphorylation by directly repressing the upstream regulatory factors of Rb, such as cyclin D1 (CCND1) [53], histone deacetylase 1 (HDAC1) [53], cyclin-dependent kinase 6 (CDK6), and cell division cycle 25 homolog A (CDC25A) [54]. A recent study has shown that miR-449a is downregulated in prostate cancer, indicating that this miRNA regulates cell growth and viability, in part by repressing the expression of HDAC-1 [53].

The functional role of miR-128a in senescence is also evident. miR-128a directly targets the Bmi-1 oncogene (polycomb ring finger oncogene; BMI1), increasing $p16^{INK4A}$ expression and reactive oxygen species (ROS), which promote cellular senescence in medulloblastoma cell lines [55]. Recently, it has been reported that this tumor suppressive miRNA (miR-128a) is downregulated in medulloblastomas [55], glioblastomas [56], and acute myeloid leukemia [57], suggesting that this miRNA has an important role in these types of cancer.

miR-217, which is expressed in endothelial cells during aging, promotes premature senescence by inhibiting SIRT1 expression, thus increasing forkhead box O1 (FoxO1) expression and endothelial nitric oxide synthase (eNOS) acetylation [58]. In addition, miR-217 has been reported to be a novel tumor suppressive miRNA in pancreatic ductal adenocarcinoma due to decreases in tumor cell growth both *in vitro* and *in vivo* by targeting K-Ras [59].

miR-290 also acts as a physiological effector of senescence in murine cells including mouse embryonic fibroblasts (MEFs) [76], and miR-20a induces senescence in MEFs through the direct downregulation of the transcriptional regulator leukemia/lymphoma-related factor (LRF), leading to an induction of $p19^{ARF}$ [75]. In addition, miR-519 is another miRNA that induces senescence in cancer cell lines. miR-519 elicits these actions by repressing HuR expression [52]. On the other hand, there are miRNAs that are downregulated during senescence, such as miR-15b, miR-24, miR-25, and miR-141, which directly target mitogen-activated protein kinase kinase (MKK4) [71].

Importantly, the escape from OIS is a requirement for transformation into tumor cells. Therefore, high-throughput genomic and miRNA screens have been preformed to identify novel mediators of OIS in human mammary epithelial cells (HMECs), which contain OHT-inducible Ras^{G12V} [60]. Borgdorff and colleagues showed that 28 miRNAs prevented senescence upon Ras^{G12V} induction. These miRNAs are as follows: miR-17-5p, miR-20a-b, miR-93, miR-106a-b, miR-130b, miR-302a-d, miR-372, miR-373, miR-512-3p, miR-515-3p, miR-519c-e, miR-520a-g, miR-526b*, and miR-146a-b. These miRNAs bypass Ras^{G12V}-induced senescence by directly targeting the 3′-UTR of $p21^{Cip1}$. Moreover, miR-372, miR-373, miR-302, and miR-520 can also bypass Ras^{G12V}-induced senescence through the downregulation of LATS2 in addition to $p21^{Cip1}$ [60–63]. miR-372 and miR-373 also prevent Ras-induced senescence in human fibroblasts [61], suggesting that the immortalization mechanism of these miRNAs is universal. Importantly, these identified proliferative miRNAs have been demonstrated to be associated with cancer development. For example, miR-17-5p is overexpressed in pancreatic cancer [64], squamous cell carcinoma [65], breast cancer [66], hepatocellular cancer [67], renal cell carcinoma [68], and thyroid cancer [69], suggesting a potential oncogenic role of miR-17-5p. Furthermore, miR-130b promotes gastric cancer by downregulating the tumor suppressor, runt-related transcription factor 3 (RUNX3) [70]. In addition, miR-372 and miR-373 have been found to be upregulated in testicular germ cell tumors [61]. Finally, miR-302 is expressed specifically in embryonic stem cells and embryonic carcinoma cells [77, 78], suggesting a possible role of this miRNA in cancer stem cell biology.

4. Senescence Induction Based upon miRNA Modulation as a Therapeutic Approach

It is well known that the overexpression of several oncogenes (e.g., Ras^{G12V}) or tumor suppressor genes (e.g., ribosomal protein S6 kinase, 90 kDa, polypeptide 6; RSK4) [79] induces senescence. However, cancer cells can be induced to a senescent state with conventional anticancer treatments such as Doxorubicin [80, 81]. The use of senescence as a novel modality of cancer therapy has been considered in clinical trials with promising results [81].

Senescence may promote carcinogenesis in surrounding tissues [82] by secreting interleukins, chemokines, growth factors, and proteases, which stimulate malignant phenotypes in neighboring cells. In this regard, miR-146a and miR-146b have been demonstrated to negatively regulate the senescence-associated secretion of IL-6 and IL-8 by directly targeting IRAK1 and reducing NF-κB activity [83]. Therefore, these miRNAs may be promising tools to restore the protective potential against development of the senescence-associated secretory phenotype (SASP).

The rationale for using miRNAs as novel anticancer molecules is based on the following two major findings: (1) miRNA expression is deregulated in cancer when compared with normal tissues; (2) the cancer phenotype can be changed by targeting miRNA expression [13].

The therapeutic application of miRNAs involves two major strategies. For oncogenic miRNAs (oncomirs), which promote proliferation when overexpressed, the major therapeutic strategy is directed toward reducing oncomir expression. These therapies include anti-miRNA oligonucleotides, microRNA sponges, miRNA masking, and small molecule inhibitors. For TS-miRs, the therapeutic strategy is directed toward restoring the levels of TS-miRs by exogenous expression (Figure 3).

4.1. Anti-miRNA Oligonucleotides. The base pair interaction between miRNAs and mRNAs is essential for the function of

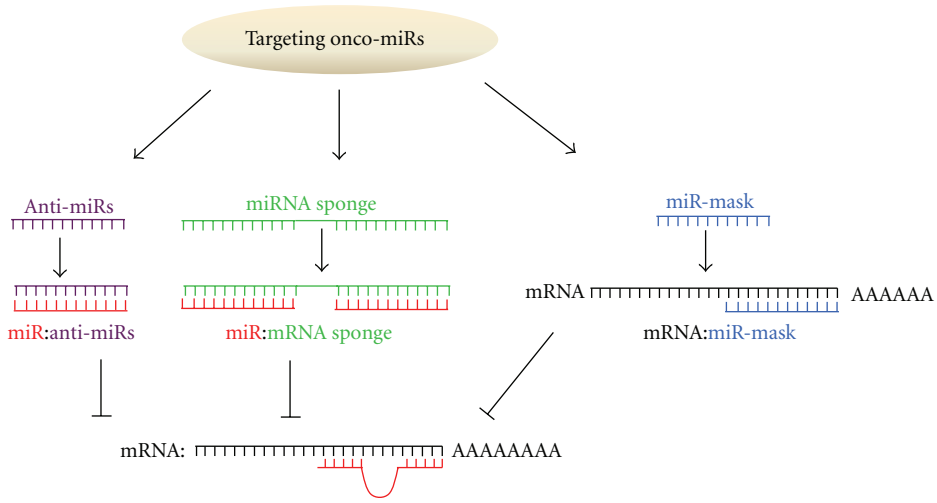

Figure 3: miRNA-based molecular cancer therapy for oncogenic miRNAs. The cancer therapies include anti-miRNA oligonucleotides (anti-miRs), miRNA sponges, and miRNA masking.

miRNAs. Therefore, a logical approach of silencing miRNAs is to use a nucleic acid that is antisense to the miRNA [84, 85]. These anti-miRNA oligonucleotides (AMOs) block the interactions between miRNAs and their target mRNAs by competition. Thus, the anti-miRNA oligonucleotides knockdown the oncogenic properties of the miRNA resulting in cancer suppression and decreased cancer progression. Studies targeting miR-21 represent one of the first examples of inhibiting cancer development by downregulating an oncogenic miRNA. miR-21 is overexpressed in most tumor types and acts by targeting many tumor suppressor genes related to proliferation, apoptosis, and invasion including the following genes: programmed cell death 4 (PDCD4) [86–88]; tropomyosin 1 (Tpm1) [89]; PTEN [90, 91]; ras homolog gene family, member B (RHOB) [92]; polymerase (DNA-directed), delta 4 (POLD4) [93]. Si and colleagues have knocked down miR-21 expression using an anti-miR-21 oligonucleotide transfected into MCF-7 breast cancer cells, and they demonstrated that the anti-miR-21 oligonucleotide suppressed both cell growth and tumor growth in a xenograft mouse model by increasing apoptosis and decreasing cell proliferation [94].

A modified AMO approach has recently been described in which multiple antisense units are engineered into a single unit that is able to simultaneously silence multiple miRNAs. For example, the multiple-target AMO targeting miR-21, miR-155, and miR- 17-5p has a greater inhibitory effect on cell growth in MCF-7 cells when compared to single-target AMOs or a combination of these single-target AMOs [95]. The multiple-target AMO approach may have a broad application in human tumors.

4.2. miRNA Sponges. miRNA sponges are transcripts that contain multiple tandem-binding sites to a miRNA of interest, therefore preventing the interaction between the miRNA and its endogenous targets. Ebert et al. (2007) [96] engineered such molecules by inserting a bulge between the miRNA-binding sites at the position normally cleaved by Argonaute 2, thereby enabling stable association of miRNA sponges with microribonucleoprotein complexes loaded with the corresponding miRNA. In addition, they specifically designed sponges with a complementary heptameric seed so that a single sponge can be used to effectively repress an entire miRNA seed family. These miRNA sponges can derepress miRNA targets as strongly as chemically modified AMOs *in vitro*. miRNA sponges have been applied to inhibit miRNA activity in *Drosophila* [97]. However, the efficacy of these stably expressed sponges in applications needs to be further evaluated.

4.3. miRNA Masking. A miRNA-mask is a gene-specific strategy developed by Xiao et al. (2007). miR-masks consist of single-stranded 2′-O-methyl-modified antisense oligonucleotides that are fully complementary to predicted miRNA binding sites in the 3′-UTR of the target mRNA. These modified oligonucleotides can form complementary duplex fragments with the target mRNA with higher affinity. In this study, Xiao and colleagues designed miR-masks complementary to HCN2 and HCN4 mRNA to prevent the repressive actions of miR-1 and miR-133 on protein expression of these genes [98]. The disadvantage of this gene-specific strategy is the limited scope (one target) for therapeutic purposes.

4.4. Restoring Tumor Suppressor miRNA Expression. For TS-miRs, which promote cancer when downregulated, small synthetic oligonucleotides that mimic endogenous mature miRNA molecules (designated miRNA mimics) restore expression of TS-miRs, thereby inducing cell death and blocking proliferation [99, 100]. The concept of miRNA replacement therapy is perhaps best exemplified by the let-7 miRNA family. Let-7 is underexpressed in nonsmall cell lung cancer relative to normal lung tissue, which inversely correlates with the expression of the Ras oncoprotein, suggesting that let-7 negatively regulates the Ras oncoprotein

[101]. In addition, let-7 expression is downregulated in other cancer types such as hepatocellular carcinoma, melanoma, and nasopharyngeal carcinoma. Let-7 acts by suppressing protein expression of several genes such as high mobility group AT-hook 2 (HMGA2), Myc, CCND1, and BCL2-like 1 (BCL-XL) [102–106]. Functional studies using cultured lung cancer cells and mouse models of lung cancer have shown that administration of let-7 mimics blocks the proliferation of cancer cells and reduces the growth of lung tumors, respectively [107–109]. Moreover, in hepatocellular carcinoma cells, the transfection of a let-7 family member, let-7g mimics, inhibits cell proliferation by downregulating the oncogene, c-Myc, and upregulating the tumor suppressor gene, $p16^{INK4A}$ [110]. miRNA replacement is also demonstrated by miR-34a [111], which is underexpressed in multiple cancer types. miR-34a, a key effector of the p53 signaling pathway, induces apoptosis, G2 arrest, and senescence in cancer cell lines by repressing the expression of direct targets, such as cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase 6 (CDK6), CCND1, SIRT1, cyclin E2 (CCNE2), E2F transcription factor 3 (E2F3), neuroblastoma-derived Myc (MYCN), and B-cell CLL/lymphoma 2 (BCL2) [42, 46, 112–114].

One of the major problems for the use of miRNAs as therapeutic molecules relates to the tissue-specific delivery and cellular uptake of sufficient amounts of synthetic oligonucleotides to achieve sustained target inhibition [115]. Consequently, strategies have been developed to deliver miRNA-based therapeutics, including viral and nonviral vector systems. Viral vector-systems have high gene transfer efficiency but have limitations due to their lack of tumor-targeting capability and to residual viral elements that can be immunogenic, cytopathic, or recombinogenic. However, adenovirus-associated vectors (AAVs) do not integrate into the genome and are efficiently eliminated with minimal toxicity as shown in Phase I and Phase II clinical trials [13]. Furthermore, systemic administration of mir-26a using an AAV in an animal model of hepatocellular carcinoma results in apoptosis induction and significant protection from disease progression without toxicity [116]. On the other hand, nonviral vector systems include cationic liposome-mediated, nanoparticle-mediated, and polymer-mediated gene transfer systems for *in vivo* human therapy [117–119].

5. Conclusions

miRNAs have an important role in tumor development, progression, chemosensitivity, and cellular senescence. A better understanding of the function of miRNAs is required for the development of novel therapies, such as restoring TS-miRs and targeting oncomirs with anti-miRNA technology.

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Research Article

Self-Renewal Signalling in Presenescent Tetraploid IMR90 Cells

Anda Huna,[1] Kristine Salmina,[1] Elina Jascenko,[2] Gunars Duburs,[2] Inna Inashkina,[1] and Jekaterina Erenpreisa[1]

[1] *Latvian Biomedical Research and Study Centre, Ratsupites 1, 1067 Riga, Latvia*
[2] *Latvian Institute of Organic Synthesis, Aizkraukles 21, 1006 Riga, Latvia*

Correspondence should be addressed to Jekaterina Erenpreisa, katrina@biomed.lu.lv

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Endopolyploidy and genomic instability are shared features of both stress-induced cellular senescence and malignant growth. Here, we examined these facets in the widely used normal human fibroblast model of senescence, IMR90. At the presenescence stage, a small (2–7%) proportion of cells overcome the 4n-G1 checkpoint, simultaneously inducing self-renewal (NANOG-positivity), the DNA damage response (DDR; γ-H2AX-positive foci), and senescence (p16inka4a- and p21CIP1-positivity) signalling, some cells reach octoploid DNA content and divide. All of these markers initially appear and partially colocalise in the perinucleolar compartment. Further, with development of senescence and accumulation of p16inka4a and p21CIP1, NANOG is downregulated in most cells. The cells increasingly arrest in the 4n-G1 fraction, completely halt divisions and ultimately degenerate. A positive link between DDR, self-renewal, and senescence signalling is initiated in the cells overcoming the tetraploidy barrier, indicating that cellular and molecular context of induced tetraploidy during this period of presenescence is favourable for carcinogenesis.

1. Introduction

Cellular senescence is a condition in which the cells remain alive but are unable to proliferate. Premature senescence can be triggered by certain stresses independently of the number of cell divisions or telomere length [1], possibly as a result of protracted DNA damage signalling [2]. Oncogene-induced senescence is thought to behave similarly, driven at the very early stages of tumour development where it serves as a barrier to cancer progression [3]. Subsequent progression to full-blown malignancy is favoured when tumour stem cells acquire further mutations that impair the senescence pathway, for example, mutations in TP53 or CDKN2a [4, 5].

During *in vitro* culture, human fibroblast cells undergo a presenescence phenomenon whereby they display evidence of chromosome instability (CIN) within an apparently highly heterogenous population with signs of chromosomal damage, and the appearance of polyploid interphase cells and their divisions [4, 6–12]. Whereas the frequency of diploid mitotic cells at presenescence is declining, the number of polyploid mitoses increases to a peak before a sharp fall as the cells change to the characteristic flat morphology indicative of replicative senescence [13, 14]. These data stimulated the hypothesis that telomeric loss at senescence may represent a "genetic time bomb" causally involved in both cell senescence and malignant transformation [13, 15].

In is clear that CIN associated with polyploidy at the presenescence stage may substantially increase the mutability and risk of malignant transformation [16–18]. Moreover, there are reports from normal cell cultures of revertant cells escaping senescence by acquiring mutations [19] and their ability to depolyploidise and restart mitoses [9–12, 17]. The features of CIN, including polyploidy, are also characteristic of malignant tumors where the degree of CIN is correlated with aggression [20]. Induced endopolyploidy is a typical response of tumour cells with deficient p53 function to the action of DNA or spindle-damaging agents [21–24]. For a decade, it has been generally accepted that sublethal genotoxic damage to cancer cells associated with anticancer clinical modalities accelerates cellular senescence [1, 25], with concomitant induction of polyploidy as a component.

However, we and others have recently shown that the induction of endopolyploidy followed by arrest and subsequent slippage from a spindle checkpoint is accompanied

Table 1: Antibodies: source and use.

Primary antibodies	*Secondary antibodies* (dilution, if not stated otherwise, 1 : 400)
Mouse monoclonal anti-hNANOG (N3038, Sigma) 1 : 75	Goat anti-mouse IgG-Alexa Fluor 488 (A31619, Invitrogen) or 594 (A31623, Invitrogen)
Rabbit polyclonal anti-hOCT4 (ab19857, Abcam) 1 : 200	Goat anti-rabbit-IgG-Alexa Fluor 594 (A31631, Invitrogen)
Goat polyclonal anti-hOCT3/4 (N-20) (sc-8630X, Santa Cruz) 1 : 400	Rabbit anti-goat IgG-Cy3 (C2821, Sigma) 1 : 500
Rabbit polyclonal anti-hP21 C-19 (sc-397, Santa Cruz) IF 1 : 50	Goat anti-rabbit-IgG- .Alexa Fluor 594 (A31631, Invitrogen)
Rabbit polyclonal anti-hP16 N-20 (sc-467, Santa Cruz) IF 1 : 50	Goat anti-rabbit-IgG- .Alexa Fluor 594 (A31631, Invitrogen)
Mouse monoclonal anti-hPML (sc-966, Santa Cruz) 1 : 150	Goat anti-mouseIgG-Alexa Fluor 488 (A31627, Invitrogen)
Rabbit polyclonal anti-hAURORA B (ab2254, Abcam) 1 : 300	Goat anti-rabbit-IgG- .Alexa Fluor 594 (A31631, Invitrogen)
Rabbit polyclonal anti-h-γH2AX (4411-PC-100, R&D Systems) 1 : 200	Goat anti-rabbit-IgG- .Alexa Fluor 594 (A31631, Invitrogen)

in p53-mutant tumour cells by the activation of meiotic proteins [24, 26, 27] and key self-renewal transcription factors (OCT4, NANOG, and SOX2) [28]. The majority of these polyploid cells senesce. However, a minor fraction retains divisional activities (thus counteracts or reverses senescence), accumulate self-renewal factors in their subnuclei, and subsequently undergo depolyploidisation to paradiploid descendants that provide clonogenic regrowth [28, 29].

Cycling tetraploidy, an illicit deviation from the normal cell cycle, is considered to serve as a crucial step from diploidy to cancer-related aneuploidy and from senescence to malignancy [17, 30–32]. Together, these data highlight the need to more closely investigate the role of endopolyploidy in the relationship between self-renewal and senescence. These investigations will greatly assist the current endeavours being made to induce reprogramming of somatic cells that are free from genomic damage and provide further information regarding the use of senescence-induction as a potential anticancer strategy [33, 34].

Therefore, we chose to examine these phenomena using a well-established model of cell senescence, involving *in vitro* cultured normal human fibroblast IMR90 cells. We show here that a small proportion of cells undergoing senescence are able to overcome the tetraploidy barrier and that these cells appear to simultaneously upregulate self-renewal and senescent factors.

2. Materials and Methods

2.1. Cell Culture. The wild-type p53 human embryo lung fibroblast cell line IMR90 was obtained from ATCC and also from Coriell collection kindly donated by Dr. A. Ivanov (Beatson Institute, Glasgow) after 21–23 population doublings (PDL). Cells were cultured in DMEM (Sigma) supplemented with 10% FBS (Sigma), without antibiotics, as monolayers in a humidified incubator in 5% CO_2/95% air atmosphere. The early passage cells were split 1 : 3 (~50 $\times 10^4$ of cells per flask (25 cm^2) twice weekly. Mid-passage cells were split 1 : 2 weekly, and late-passage cultures were split 1 : 2 once cultures attained confluence. Culture medium was changed two or three times between subculture. In this way, several subsequent passages were carried out until the cells failed to undergo >0.8 population doublings in a 7-day culture period. Under the given conditions of cultivation, the cells typically reached this state after 40–50 PDL.

2.2. Immunofluorescence (IF). Cells were trypsinized, pelleted, washed in warm PBS, resuspended in FBS and cytospun on to polylysine-coated slides. For detailed cytological studies, the cells were also grown on glass cover slips. Cells on coverslips were rinsed in PBS and FBS, then fixed in methanol at −20°C for 7 min (30 min for γ-H2AX staining) followed by 10 short rinses in cold acetone at −20°C. Slides were washed in TBS/0.01% Tween-20 (TBST) (0.05% Tween-20 for γ-H2AX staining) three times for 5 min each, after which they were blocked with 1% bovine serum albumin in TBS/0.05% Tween-20 for 15 min. Fifty microliters of the appropriate dilution of antibody was applied to each sample and the slides incubated overnight at 4°C. Samples were washed thrice for 5 min each time in TBST. The sources and dilutions of the primary and secondary antibodies are listed in Table 1. Poststaining was with DAPI (0.25 μg/mL). Cells were finally embedded in Prolong Gold (Invitrogen).

2.3. Microscopy. A fluorescence microscope (Leitz, Ergolux L03-10) equiped with a colour videocamera (Sony DX-S500) was used to examine cell preparations, record images, and perform image cytometry. For three-colour images and colocalisation studies, the BRG filter system (Leica) providing nonoverlapping excitation and transmission emission of blue, red, and green bands was used. In addition, confocal microscopy (Leica, DM 600) was used with the images scanned in the three different colour channels in sequence.

2.4. DNA Image Cytometry. Cells grown on coverslips were rinsed in PBS and serum. Alternatively trypsinised cells were washed in warm PBS and suspended in FBS and cytospun onto glass slides. Both preparations were then fixed in ethanol/acetone (1 : 1, v/v) overnight at 4°C and air dried. For stoichiometric DNA staining [35], slides were hydrolysed with 5 N HCl for 20 min and stained with 0.05% toluidine blue in McIlvain 50% buffer pH 4 for 10 min at room temperature, rinsed, dehydrated in warm butanol,

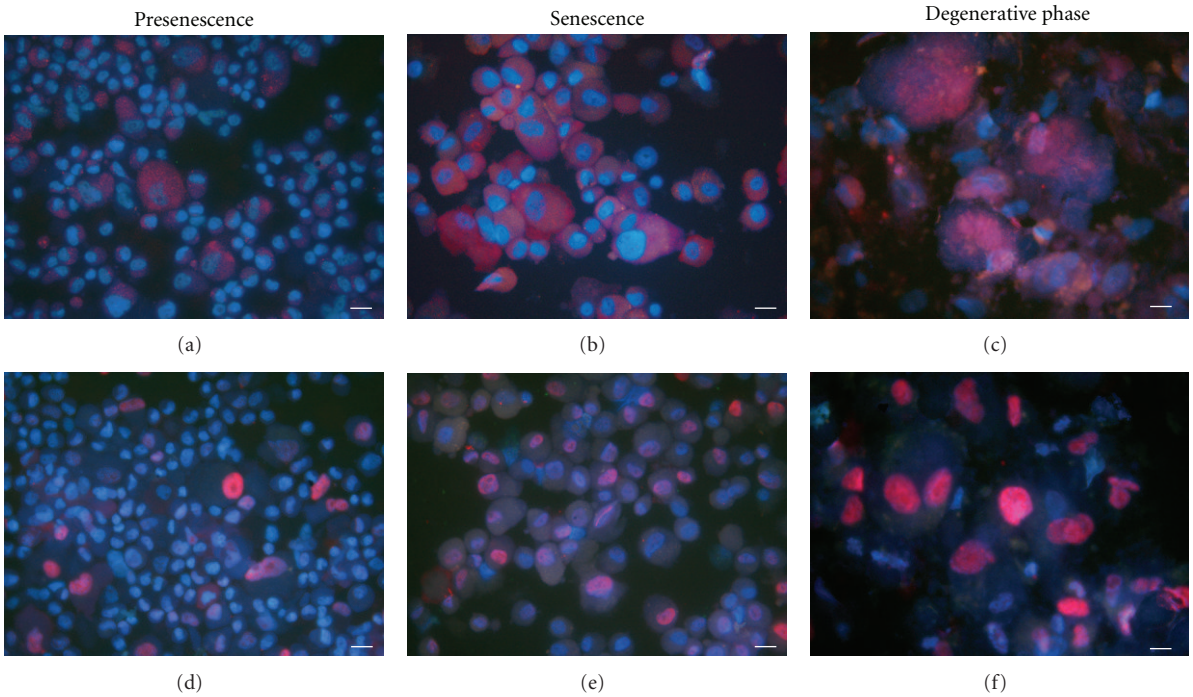


Figure 1: Phenotypic characteristics of IMR90 in the process of senescing showing cell enlargement and the accumulation of principal growth inhibitors involved in the regulation of senescence, p16 and p21, in three stages: ((a), (d)) presenescence, ((b), (e)) senescence, ((c), (f)) degeneration phase. ((a)–(c)) p16 (red) and DAPI (blue); ((d)–(f)) p21(red) and DAPI (blue). Scale bar = 20 μm.

and passed through xylene prior to embedding in DPX (Sigma-Aldrich). DNA content was measured as the integral optical density in 200 cell nuclei in the green channel of the calibrated video camera, using Image Pro Plus 4.1 software (Media Cybernetics; REO 2001, Latvia). In parallel, optical density and nuclear area were registered. The estimated sum of device and measurement error was <5%. The 2C DNA content was determined by the modal value of the first G1 peak. Mitotic indices were counted per 1,000–2,000 cells in the same samples.

2.5. RNA Extraction, RT-PCR, and DNA Sequencing of NANOG. These studies were performed on IMR90 cells in a logarithmic phase of growth as described before [28].

3. Results

3.1. Kinetics and Characteristics of Senescing Cells. Subcultivation of IMR90 cells invariably leads to diminishing growth after a number of passages. Under our experimental conditions, this was reached prematurely at passage 32–34 corresponding to 40–50 PDL. This was likely due to growing the cells in the air atmosphere shortening their lifespan [36]. During further passaging, full growth arrest was achieved, characterised by the mitotic index reaching zero and the cells attaining typical features of senescence such as cytoplasm enlargement and flattening and bi- and multinuclearity, as well as accumulation of senescence markers p16inka4a (p16) and p21inka4a (p21) as illustrated on Figures 1(a), 1(b), 1(d), and 1(e). Degenerative phase was characterised by nuclear swelling and cell lysis (Figures 1(c) and 1(f)).

3.2. DNA Cytometry Reveals a Minor Fraction of Cycling Tetraploid Cells. As the cells underwent this senescence phenomenon, they were analysed by DNA image cytometry in three independent experimental series. The following cytometric regularities were observed. In the stage of logarithmic growth (typified in Figure 2(a)), where the mitotic index was 4.5–3.0%, the cell population had a normal cell cycle, with typical DNA distribution of the major fractions between 2C and 4C. During presenescence, with the mitotic indices progressively lowering, the histogram of DNA distribution remained generally similar (exampled on Figure 2(b)), however the proportion of cells in the G1-2C phase increased and the S-phase decreased. At senescence, where no mitoses were observed, the proportion of interphase cells in the 4C-fraction was again increased (Figure 2(d)). This change was already noticeable at late presenescence, one-two passages earlier (Figure 2(c)). The average proportions of 4C cytometric fractions at the stages of logarithmic growth, presenescence and senescence, and corresponding proportions of the cells with p21-positive nuclei are presented on Figure 3.

In addition to the major DNA cytometric fractions, we also observed a small proportion (1–7%) of hypertetraploid cells, some of them reaching octoploidy. The number of hypertetraploid cells strongly inversely correlated with mitotic indices in each experimental series (Figure 4(a)). Furthermore, the number of octoploid cells was found

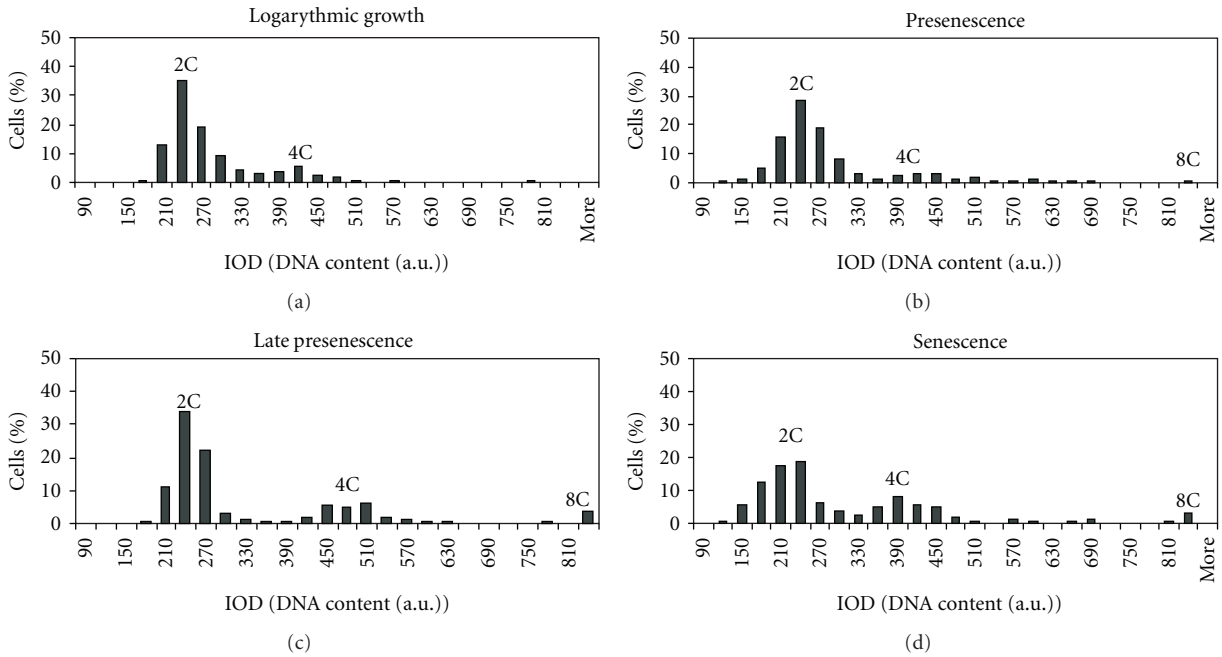


Figure 2: Histograms of IMR90 DNA content in several passages showing typical changes in the time course: (a) logarithmic growth, (b) presenescence, (c) late presenescence, and (d) senescence showing the accumulation of 4C cells and the increase of the proportion of hypertetraploid cells, some of which reach octoploidy.

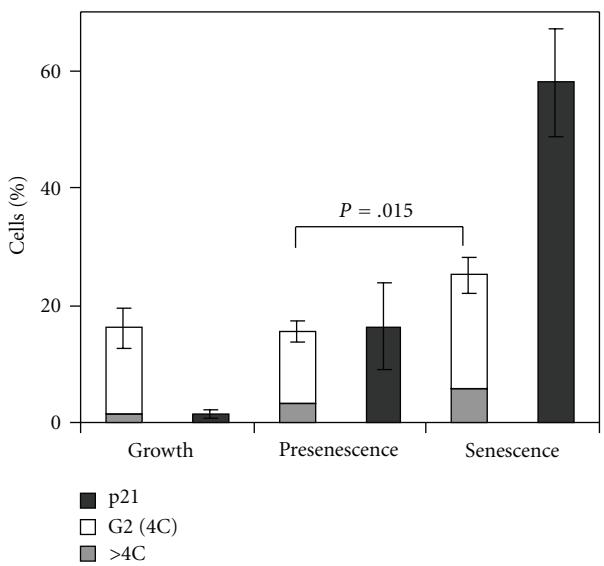


Figure 3: Quantitative data showing changes obtained from the DNA cytometry results, at the growth phase, presenescence, and senescence averaged from all experiments and the corresponding proportions of cells displaying nuclear positivity for p21. Significant increase of the 4C fraction accompany transition from presenescence to senescence ($P = .015$). Increase of the proportion of hypertetraploid cells, in parallel to considerable increase of the proportion of p21-positive cells, accompany the whole process.

increasing with hypertetraploidy (Figure 4(b) and can be seen from Figure 2). These data indicate that the process of (accelerated) senescence encourages the cells to leave the normal cell cycle favouring their cycling as tetraploids.

3.3. Self-Renewal Markers Appear at Presenescence in Tetraploid Cells Simultaneously with Senescence and DDR Markers. We were further interested to see the association of these polyploid features with DDR, self-renewal, and senescence markers. At presenescence, we found that the embryonal transcription factors of pluripotency and self-renewal OCT4 (mostly cytoplasmic) and NANOG (nuclear) were activated in parallel with the initial activation of senescence factors in the same cells. Notably, this occurred in the cells with larger nuclei and larger (often polygonal) p16-positive cytoplasm, suggesting that these were hypertetraploid cells which had initiated the process towards senescence (illustrated in Figure 5). In an effort to better characterise the position of these particular cells in the cell cycle, we undertook two kinds of analysis.

First, we stained samples stoichiometrically with Toluidine blue for DNA and recorded the integral optical density (DNA content), nuclear area, and subsequently optical density (OD, concentration of DNA) of 200 cells in each sample using Image Pro Plus software. Importantly, this imaging method is interactive and excludes any cell aggregates, which are either excluded or separated for single cells before the measurement by the operator. Using this approach, the concentration of DNA remained constant in all samples (the data are not shown), with dispersion of OD around the average within only 2-3%, up to the degeneration phase. The positive correlation between the DNA content per nucleus and its area counted for each sample is high ($r = 0.57$–0.76);

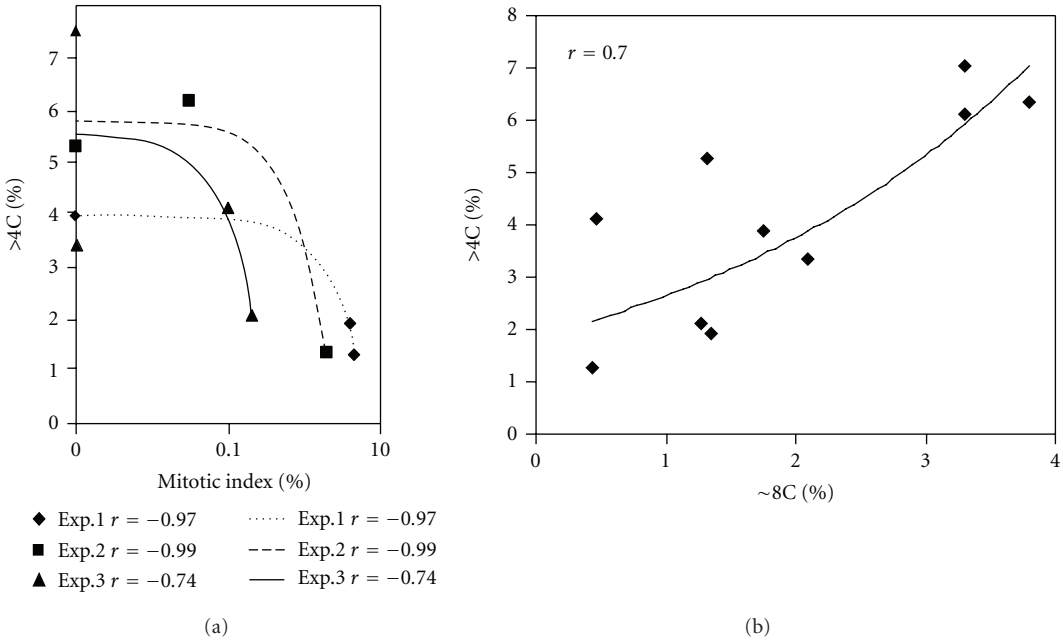


Figure 4: Tetraploidy increases in the course of IMR90 senescence. (a) The number of hypertetraploid cells increases with senescence and strongly inversely correlates with mitotic indices in each experimental series; (b) the number of cells with octoploid DNA content increases with hypertetraploidy indicating to increase of tetraploid cycling (the united data from three experiments).

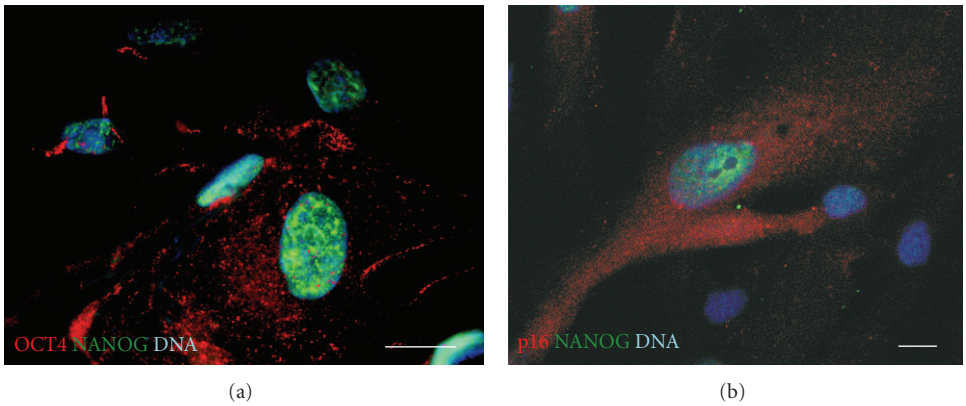


Figure 5: Examples of pre-senescent IMR90 fibroblasts grown on the coverslip, which differ from the surrounding cells by enlarged nuclei, cytoplasm volume, and the tendency of flattening. These cells express: (a) enhanced OCT4 (mostly in cytoplasm) and nuclear NANOG and (b) paranucleolarly localized NANOG, which is combined with enhanced expression of the senescence regulator, p16. Both, self-renewal and senescence regulators are undetectable or at the background level in the neighbouring fibroblasts possessing small nuclei. Scale bars = 10 μm. On (a) OCT4 was stained with the antibody for both A and B isoforms (Abcam polyclonal antibody, see Table 1).

an example is shown in Figure 6(a). This confirms the accepted observation that nuclear area is proportional to DNA content; hence, its concentration remains constant [37, 38]. In Figure 6(a), sampled from presenescence phase, it is seen that the nuclear size of the 4C nuclei cluster is roughly twice as large as that of the 2C nuclei cluster, while the nuclear size of the cells with hypertetraploid DNA content exceeds that of the average 4C nuclei. It follows that the cells with a nuclear area visibly larger than that for G1 and G2 cells should contain the hypertetraploid DNA content.

Second, we stained samples for NANOG and one of the main senescence regulators (p16 or p21) in combination with DAPI. Using this approach, we identified cells possessing visibly larger nuclei as compared with G1- and G2-sized nuclei of surrounding fibroblasts which simultaneously expressed markers of both self-renewal (NANOG) and senescence. We subsequently applied DNA measurement of the integral nuclear fluorescence (INF) of these and neighbouring cell nuclei as exemplified on Figures 7(a) and 7(b), through 16 optical fields (total 242 cells); a selection of seven microscopic fields is also presented in supplemental

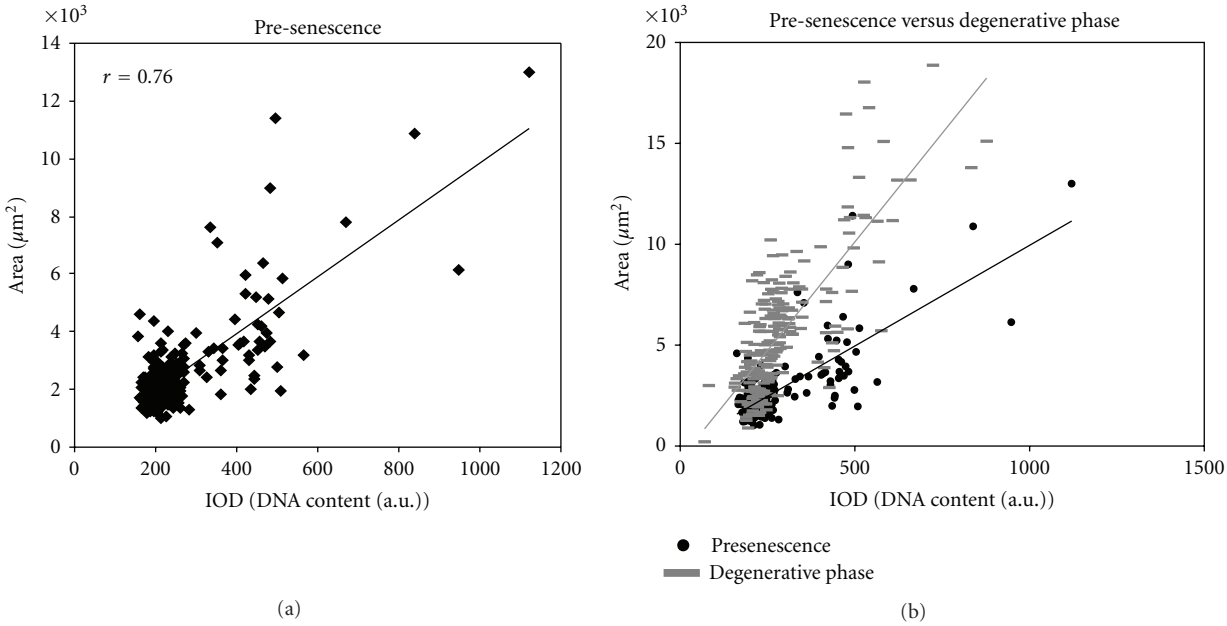


Figure 6: The relationship between the nuclear area and the DNA content (IOD) per each cell nucleus in: (a) a typical sample of presenescence phase and (b) the comparison of the latter and a sample in the degeneration phase. It is seen that the nuclear size is increasing roughly proportionally to the DNA content in the presenescence phase, while in the degeneration phase, the nuclear size is much larger indicating to nuclear swelling.

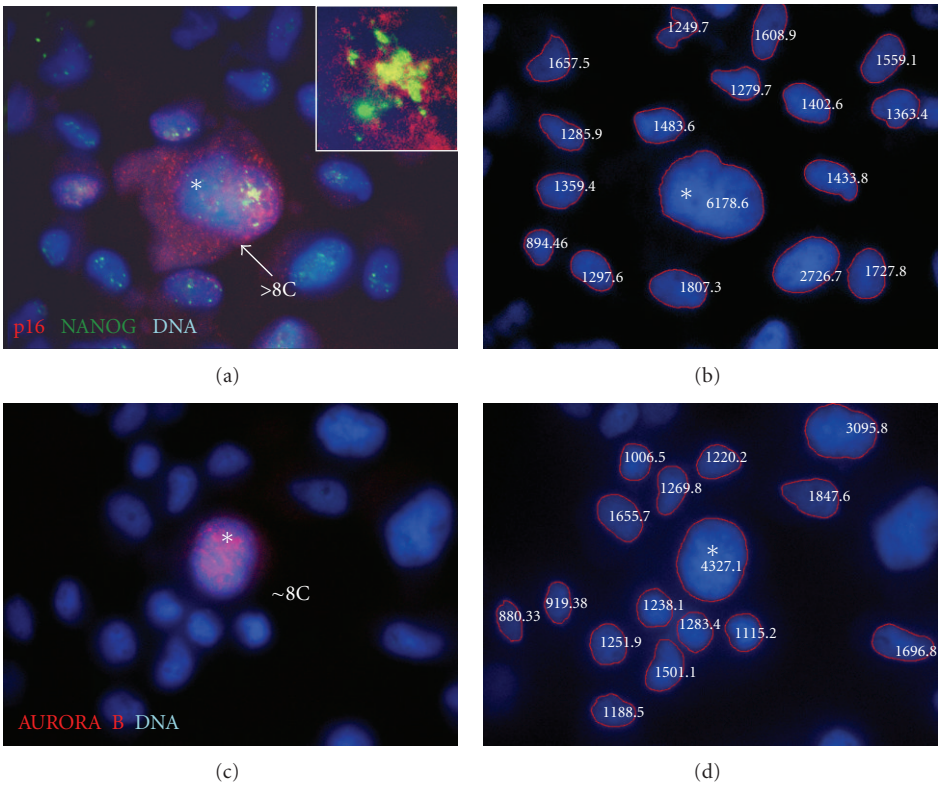


Figure 7: Examples of DNA content measurements by integral fluorescence in the DAPI channel using Image Pro Plus software in microscopic fields including cells with enlarged nuclei and stained for: ((a), (b)) NANOG, p16, and DNA by DAPI, ((c), (d)) Aurora B-kinase and DNA by DAPI. The selected hyperoctoploid cell (*) shown in (a) has an enlarged p16-positive cytoplasm and some amount of both NANOG and p16 in the nuclear region, and this region is magnified in the insertion; the paraoctoploid cell in (c) is positive for Aurora B-kinase.

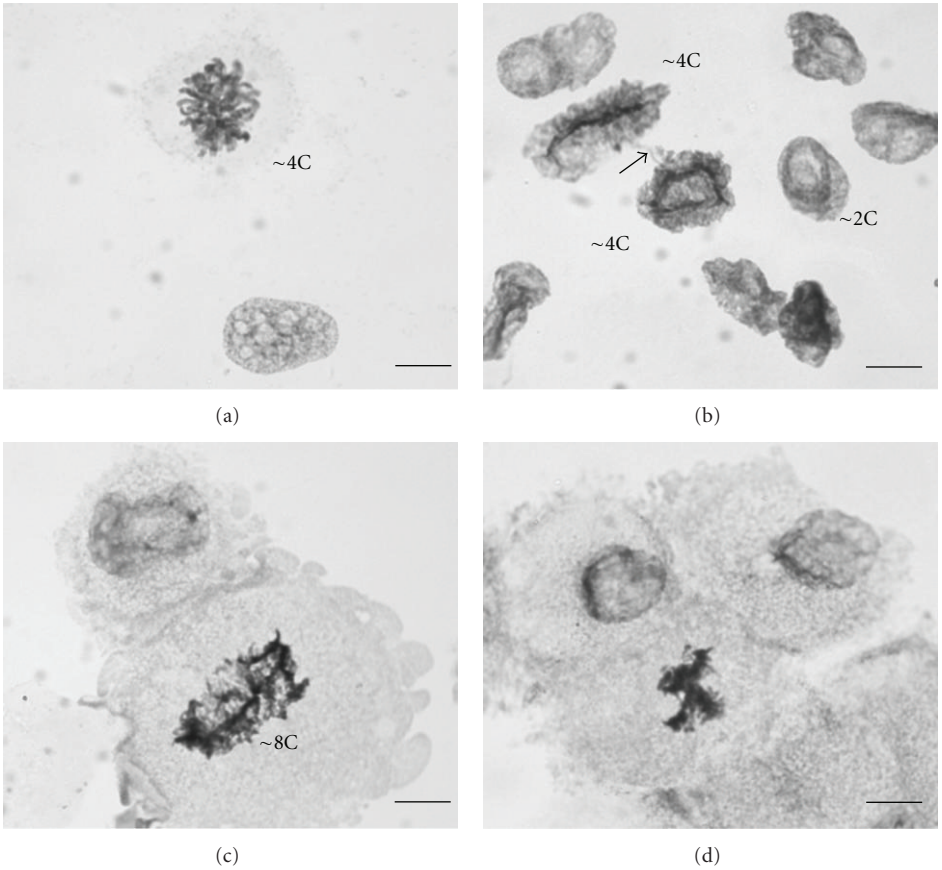


Figure 8: Mitoses of IMR90: (a) normal mitosis of a diploid cell in the growth stage, ((b)–(d)) mitoses in the presenescence stage. (b) Anaphase of the tetraploid cell, chromosome bridge (arrowhead) indicates to CIN. (c) Metaphase of the large tetraploid cell (~8C). (d) An attempt of the tetrapolar mitosis indicating to CIN. ((a),(b)) stoichiometric staining for DNA with Toluidine blue pH 4.0 after extraction of RNA. ((c),(d)) Similar staining after partial extraction of RNA. Scale bar = 10 μm.

Figures 1–6 in Supplementary Material available online at doi: 10.4061/2011/103253. The standard error of such nuclear measurements comparing the INF recorded on the smallest cell nuclei (2C-G1) was around 20%. The selected cells which expressed markers of self-renewal and senescence contained tetraploid, hypertetraploid, or octoploid amounts of DNA more frequently than surrounding cells.

We were interested subsequently if these cells retained proliferation capacity. Using immunostaining for Aurora B-kinase as a marker of cell division potential for endopolyploid cells [39], we often found that cells with enlarged nuclei containing the tetraploid or octoploid amounts of DNA (as also detected in DAPI channel), were Aurora B-positive (exemplified on Figures 7(c) and 7(d)). At presenescence, we also found mitoses of tetraploid cells. Although commonly aberrant or pycnotic, some proceed to anaphase with signs of CIN such as chromosome bridges or multipolar mitoses (Figure 8).

The enlarged nuclei were also often labelled by the DDR marker γ-H2AX coupled to the NANOG staining (Figures 9(a) and 9(f)). Thus, at the presenescence stage, the cells with enlarged nuclei possessing enlarged p16-positive cytoplasm, simultaneously expressing small amounts of self-renewal, senescence, and DDR markers in their nuclei were found to be tetraploid, had overcome the 4n-G1 barrier, were cycling to octoploidy, and possessed division potential.

3.4. Self-Renewal, DDR, and Senescence Markers Are Initially Localised in the Perinucleolar Compartment of Tetraploid Cells. Interestingly, manifestations of all three kinds of response (γ-H2AX, NANOG, and p16/p21) were seen to be partialy colocalised as foci detected by BRG three-band optical filter (with nonoverlapping excitation and emission bands); and confirmed by confocal microscopy (supplemental Figure 7). These concentrated in the perinucleolar compartment either to one side of the large central nucleolus or surrounding it (Figures 9(a)–9(c)). Initial signs of senescence-associated heterochromatin foci (SAHF) also appear to emerge from the perinucleolar chromatin in such cells (Figure 9(d)). The initially observed PML-bodies, partially colocalised with γ-H2AX -positive speckles, were also found in this area (Figure 9(e)). The IMR90 cells at the logarithmic stage of growth had very low background IF staining for NANOG confirmed by RT-PCR and DNA sequencing (data not shown). The amount of NANOG usually seen by IF in

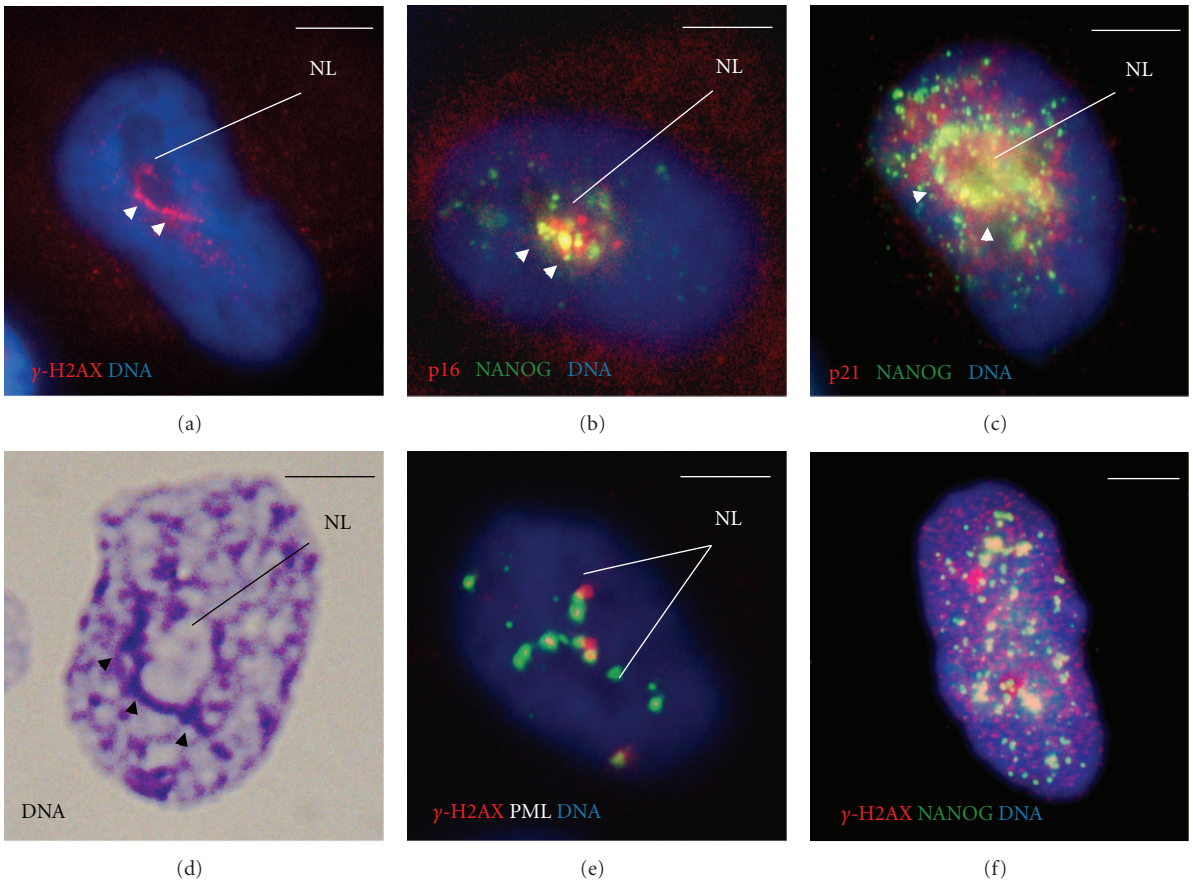


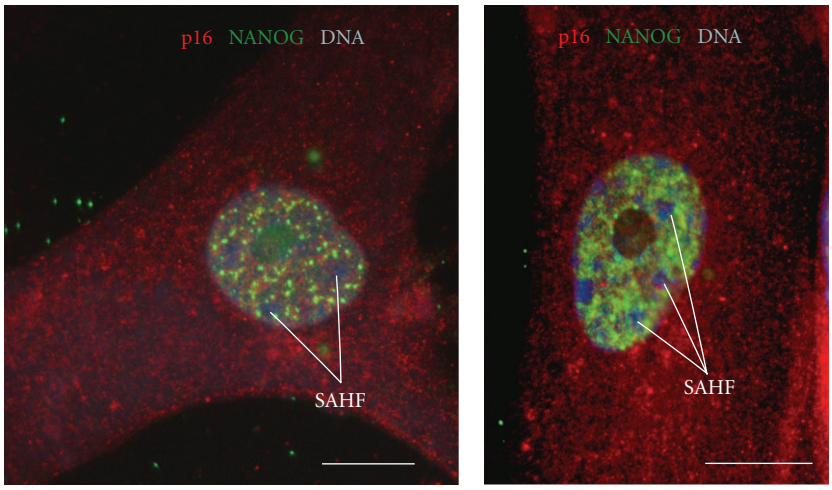
Figure 9: Characteristic cytological nuclear IF features of the tetraploid cells in the presenescence phase showing synergism of several labels located near the nucleoli. (a) Initial DDR in the perinucleolar chromatin indicated by the γ-H2AX-positive label (arrowheads), NL-nucleolus. (b) The accumulation of p16 and NANOG-positive, partly colocalising granules in the perinucleolar region (arrowheads). (c) The accumulation of p21 and NANOG—partial colocalisation around the central nucleolus (arrowheads). (d) The emergence of initial gentle SAHFs revealed by DNA-specific metachromatic staining with Toluidine-blue in the perinucleolar chromatin (arrowheads). (e) PML bodies in the vicinity of the central lobulated nucleolus, where they partly colocalise with γ-H2AX-foci. (f) The tetraploid cell from the mid-presenescence with the network of γ-H2AX and some amount of NANOG, partially colocalised. Images were obtained using BRG three-colour optical filters system. Scale bars =10 μm.

the enlarged cell nuclei at early presenescence exceeded the background of surrounding cells with normal nuclei by ~2–4-fold (supplemental Figure 7).

We compared the frequency of this coexisting self-renewal and senescence nuclear landmarks (mostly perinucleolar) in the cells with enlarged nuclei with that in the surrounding fibroblasts possessing normal 2C–4C-sized cell nuclei in one of early presenescence passages (Figure 11). From these counts, it can be seen that initial DDR and simultaneous senescence and self-renewal signalling were found in 84–96% of the cells with enlarged nuclei at far greater frequency (many-fold) than in the cells of the normal cell cycle. It should be stressed that it was practically impossible to find at this stage the cells where the nuclear enlargement and initial expression of DDR, NANOG, and senescence landmarks were dissociated. As such, also this approach confirmed that the expression of key markers of both senescence and pluripotency become selectively and simultaneously activated in tetraploid cells.

3.5. Through Intermediate Stage, NANOG Is Gradually Lost from Most Tetraploid Cell Nuclei, Correlating with an Accumulation of Senescence Markers during the Time Course of Senescence. Development of the tetraploid cells in subsequent passages of the culture as it progresses towards terminal senescence is characterised by the shaping of clear SAHFs, further increase of p16 in the enlarging cytoplasm, while smaller was this increase in the nuclei, and much stronger positivity of nuclear p21. p21 begins to extend from the perinucleolar region into the nucleoplasm (Figure 9(c)). Like p21, γ-H2AX also forms an elaborate network mostly in polyploid cells (Figure 9(f)). In these cells, NANOG can still be found, albeit in a more disseminated form, sometimes colocalised with regions of the p21 (Figure 9(c)) and γ-H2AX network (Figure 9(f)).

Figure 9(f) shows some intermediate state, when the both markers are still expressed. However, in mid-presenescence passages, γ-H2AX vanishes from the cells with the stronger expression of NANOG, and contrary to that, 2-3% of cells



(a) (b)

Figure 10: Relatively rare flattening giant fibroblasts displaying at presenescence a considerable accumulation of NANOG filling the whole nucleus in parallel to clear DAPI-positive SAHFs; in addition, the cells contain p16-positive material in the cytoplasm. Images were obtained using BRG three-colour optical filters system. Scale bars = 10 μm.

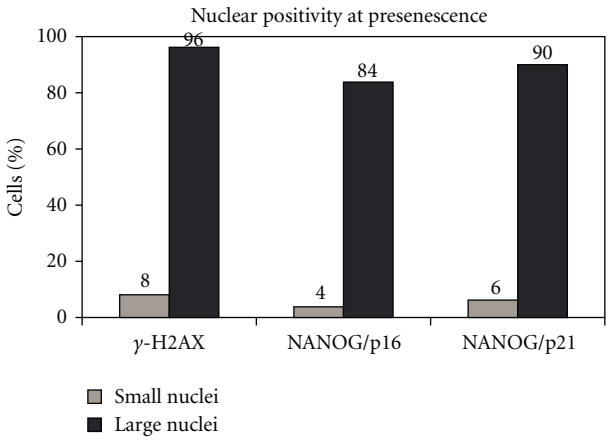


Figure 11: Proportions of the fibroblasts with enlarged (hypertetraploidy) nuclei and with conventional small nuclei in the early presenescence stage, estimated in one of the samples by the labelling frequency for γ-H2AX (n = 400 cells), NANOG/p16 positive (n = 300 cells), and NANOG/p21-positive nuclear granules (n = 300 cells). All nuclear labels were mostly present in this phase in the perinucleolar region. Evaluation reveals a many-fold prevailing signalling for DDR, self-renewal, and senescence regulators occurring simultaneously in the enlarged hypertetraploidy cells, which left normal cell cycle.

which develop very clear thick γ-H2AX foci do not contain NANOG (not shown). The cells with large nuclei and stronger expression of NANOG are more notable in the "midpassages" of the presenescence (2–5%), a few may accumulate it in their nuclei in the considerable amounts (Figure 10). However, the main tendency towards further senescing, contrary to the early phase of presenescence, when the initial expression of self-renewal and senescence markers appears positively linked, at more extended passages *the expression of NANOG in the giant nuclei generally decreases proportionally to the accumulation of cytoplasmic p16 and of the enhanced nuclear expression of p21*, as illustrated on Figure 12.

In the very last passages, approaching terminal senescence with the mitotic index tending to zero (<0.1%), this trend is further extended as illustrated in Figures 1(b), 1(c), 1(e), and 1(f), where the giant polyploid cells with large cytoplasm display considerable accumulation of p21 and p16. They are void of the NANOG expression or contain it at the background level. However, rare exceptions of the NANOG-positive cells with the only background expression of senescence factors were encountered (Figures 12(d)–12(e), arrowed). Further, the senescent cells appear to enter the terminal degeneration phase. Figures 1(c) and 1(f) show the appearance of cells at the terminal degeneration phase of the senescent culture, sampled from one typical experiment. These cells are characterised by the accumulation of p16 and p21 in the cell nuclei, absence of NANOG, lysis of the cytoplasm (Figures 1(c) and 1(f)), and by swelling of the nuclei as testified by DNA image cytometry presented in Figure 6(b).

4. Discussion

Here, we have documented the cellular behaviour of IMR90 cells as they undergo senescence after protracted *in vitro* culture. A notable observation in this process is the appearance of tetraploid cells. Clearly, tetraploid cells appearing in senescing cultures represent a deviation from normal cell cycle regulation. Such cells and their aberrant proliferative activities were reported from the very early studies of *in vitro* senescing cultures of normal fibroblasts occurring before terminal arrest of proliferation [6–8], and confirmed more recently [10]. Our results are entirely in accord with them.

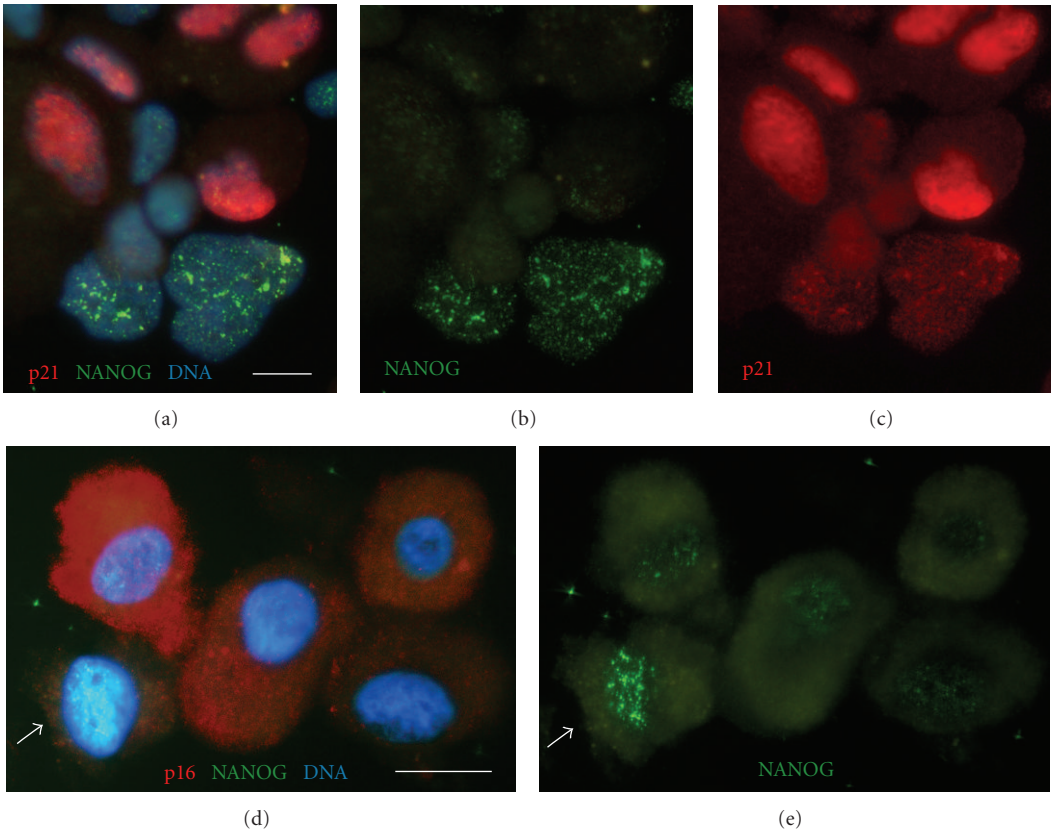


Figure 12: The antagonism between expression of NANOG and senescence regulators at the late presenescence and senescence phases: ((a)–(c)) late presenescence (MI = 0.1%): in the group of ten cells it is seen that nuclear staining of NANOG is found only in the cells with enlarged nuclei, where NANOG staining intensity is inverse to the staining for p21; ((d), (e)) senescence (MI = 0)—a rare NANOG-positive cell was encountered (arrowed), which has the weakest staining for p16 in its cytoplasm among five cells of the group. ((a), (d)) were imaged through the BRG filter; ((b), (e))—through blue, and (c)—through green filter. Scale bars = 10 μm.

These deviations, indicative of chromosomal instability during presenescence, were shown to be related to telomeric dysfunction [13–15, 40].

It is difficult to judge to what extent the induction of polyploidy is associated with the stress due to *in vitro* cultivation. Previously, more stressful conditions of passage were shown to have a slight increase in polyploidy induction, although the same results could have been obtained, if more laboriously, from unstressed cells [10]. On the whole, our present data and that generated previously show that in the senescing culture, there is a tendency for a small proportion of normal human fibroblasts to increasingly form tetraploid cells, to cycle to octoploidy, and then divide. Cycling tetraploidy is generally considered as a dangerous step towards carcinogenesis as it brings CIN and can result in aneuploidy [30, 32]. It is known that functional p53/p21CIP1 should prevent tetraploidy at the 4n-G1/S checkpoint [41, 42], the existence of which was also disputed [16]. Our DNA cytometric data showed that at presenescence, this barrier is leaky and is linked to accumulation of 4C cells during the senescence process as the cells approach full growth arrest. The same increase in the 4C cell fraction in senescing IMR90 cells, then interpreted as arrest at the 4n-G1 checkpoint, was previously reported by Sherwood and colleagues [43] following analysis by DNA flow cytometry and karyotyping and the same was found in an accelerated senescence model of Ras-oncogene transfected IMR90 cells [44].

In addition, we found here that illegitimate cycling of tetraploid cells at the early presenescence phase was associated with activation of the self-renewal response manifested by expression of the embryonal transcription factor NANOG. In our preliminary studies, we have also observed that the OCT4B splicing form (POU5F1_B) was activated; however, as its function is unknown, we concentrated here mostly on studies of NANOG expression.

The homeodomain gene, NANOG, is a key intrinsic determinant of self-renewal and pluripotency in embryonic stem cells. Beside its transactivation function, NANOG was also reported to directly propagate the G1-S transition by ativating cdk6 [45]. As the senescence regulator, p16 prevents endogenous Cdk6 and Cdk4 from associating with its catalytic unit cyclin D1 [46], NANOG should counteract this activity of p16. Moreover, normal embryonal stem cells in response to damage were shown to upregulate self-renewal transcription factors (Oct4 and Nanog) readily undergoing mitotic slippage and reversible tetraploidy [47]. Therefore, it remains possible that NANOG expression may force

the normal fibroblasts (or their stem cells) in stressed senescing cultures to bypass this tetraploidy limiting control.

Our IF observations showed directly that NANOG is expressed in tetraploid cells of pre-senescing cultures unless the cells accumulate considerable amounts of senescence regulators (which occurs increasing as the cultures approach terminal senescence); in particular, clear antagonism was found at late passages between NANOG and accumulation of nuclear p21. These observations are entirely in line with the suggestion that p16 represents a second barrier to senescence, which in the absence of the main barrier p53/p21 may be reversible [48]. Our observations on more accumulation of NANOG in some tetraploid cells, which lose the DDR signalling in the intermediate presenescence stage, may be cautiously interpreted as tendency to revert senescence. It follows that at the presenescence phase, the amount of the p53/p21 growth inhibitor appears insufficient to downregulate NANOG, which may to some extent neutralise p16 and that, therefore, more time (or activation of more p53 activators including for example, the p38 pathway [16, 49]) is needed for p53 to become an efficient enforcer of senescence barrier. This explanation of our observations is in line with the data that activated p53 suppresses the *NANOG* gene promoter [50].

Recently, Davoli and colleagues [40] showed that simultaneous elimination of telomerase and p53 causes chronic DDR resulting in the prolonged G2 arrest and tetraploidisation through licensing DNA rereplication origins. This data also well fit our observations as senescing is associated with telomere dysfunction (however, induction of self-renewal may counteract it by activating telomerase; this aspect needs further research), while found delay of p21 accumulation means relatively retarded activation of p53.

Although IMR90 are normal, nonmalignant cells containing wild-type p53, our observations importantly show for the first time that these normal cells can temporarily activate the self-renewal factor NANOG and enter "a window" when senescence regulators are as yet insufficiently active to irreversibly neutralise its activities. In line with this suggestion, upregulation of several embryonal pluripotency and self-renewal factors have also been reported by Riekstina and coauthors [51] in putative stem cells obtained from explanted adult human mesenchymal tissues during their first adaptive passages of *in vitro* cultivation. The step into tetraploidy is associated with DDR and genome instability, known to greatly increase the probability of chromosomal and genetic mutations and escape of revertants [30]. The same concern was formulated by Romanov et al. [19] and Walen [17], who showed that in presenescence the tetraploid cells display depolyploidisation activities and can ultimately escape senescence with a mutated genotype.

Although the IMR90 model is one of replicative or accelerated senescence *in vitro*, it has clear relevance to pathologic conditions *in vivo* where adult stem cells may be involved such as chronic inflammation and/or trauma.

However, a puzzle remains for the seemingly simultaneous initial induction of the opposing responses of DDR, self-renewal, and senescence in the early pre-senescent tetraploid cells. The site of convergence may be the RAS-RAF-MEK-ERK pathway priming both the mitogenic and accelerated senescence pathways [52]. It was shown that moderate activation of the wild-type *Ras* is mitogenic, while overexpression causes p38-MAPK-dependent senescence [49]. This trigger leads, in particular, to positive versus negative regulation of Cyclin D1 [53], the catalytic unit of cdk6 activated by Nanog [45]. In the negative loop, suppression of Nanog by MEK-ERK was shown through chemical inhibition of MEK [54]. Clearly, the impact of the MEK/ERK pathway on NANOG and its role in the signalling of senescence require further attention.

The data, somewhat supportive for our observations, were reported by Banito and colleagues [55] who showed upregulation of cellular senescence by transduction in IMR90 cells of the four pluripotency-inducing oncogenes.

Interestingly, all three kinds of the initial response, self-renewal, DDR and senescence, were found spatially confined to the perinucleolar compartment, and their partial or full colocalisation suggests a cross-talk between these pathways. Moreover, it is likely that the SAHFs which represent regions of epigenetically changed chromatin also start to form from the same region of the perinucleolar chromatin and are associated with the emergence of the DNA double-strand breaks. The question is why these various aspects are appearing in the nucleolus.

The nucleolus is involved in the regulation of senescence in several ways. Nucleostemnin, a nucleolar protein specifically involved in regulating the cell cycle in stem and tumour cells, can sequestrate MDM2 and MDM4 and, thus, favour accumulation of p53, the main player in senescence induction [56, 57]. Similarly nucleolar Arf will activate p53 in the senescence response [58]. In addition, relocation of hTERT to the nucleolus is associated with initiation of senescence [59]. Finally, PML binds MDM2 and sequesters it into the nucleolus [60], thus protecting p53 from proteosome-mediated degradation.

However, the most important aspect may be that, in eukaryotic systems, rDNA contains fragile sites that are extremely sensitive to replication-induced stress [61, 62]. This replication stress may be related to the p53 independent license of rereplication origins fired at prolonged G2 arrest [40]. The level of the DNA Polymerase I in IMR90 tetraploid cells may be insufficient at this time-point, causing stalling of replication forks and converting the underreplicated sites into rDNA strandbreaks [63]. This idea is compatible with cancer development from its earliest stages being associated with DNA replication stress, leading to DNA strandbreaks and subsequently to DDR [3, 64, 65].

In relation to p53-function-deficient tumours, our data [28, 29] also show that endopolyploid cells induced after genomic insult undertake sustainable activation of the pluripotency and self-renewal genes, and undergo a stage of competition between self-renewal and senescence with an improved chance for self-renewal to succeed. A proportion of these p53-mutated polyploid cells is capable of accumulating considerable amounts of self-renewal factors and subsequently depolyploidise into mitotic paradiploid descendants.

5. Conclusion

Our findings on senescing normal human IMR90 fibroblasts clearly provide us with insight into the risks of cancer development. Since it is assumed that a cancer clone develops from a single adult stem cell which receives a mutation(s), including those compromising the senescence barrier, our observations suggest that this may be favoured in the normal stressed tissue due to the unique cellular and molecular setting of the presenescence stage. It is hypothesised that telomere dysfunction causing DDR and temporary activation of self-renewal on a background of insufficient activity of senescence inducers may allow putative adult stem cells to overcome the G1 tetraploidy limit controlled by p53 leading to their replicative stress and aberrant divisions. This would favour acquisition of CIN, aneuploidy, and tumorigenic mutations, thereby driving tumorigenesis.

Conflict of Interests

The authors declare no conflict of interests.

Authors Contribution

A. Huna performed DNA image cytometry, participated in design and analysis of experiments and editing of the manuscript; K. Salmina performed immunofluorescence stainings, participated in design and analysis of experiments and editing of the MS; E. Jascenko carried out cell cultures, participated in design and analysis of experiments; G. Duburs participated in design and analysis of experiments; I. Inashkina performed RT-PCR with sequence analysis of NANOG expression, participated in design and analysis of experiments and editing of the MS; J. Erenpreisa designed experiments, performed microscopy and analysis of results and prepared the draft and editing of the manuscript, A. Huna and K. Salmina made an equal contribution.

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Review Article

Regulation of Senescence in Cancer and Aging

Yahui Kong, Hang Cui, Charusheila Ramkumar, and Hong Zhang

Department of Cell Biology, University of Massachusetts Medical School, 55 Lake Avenue North, S7-125, Worcester, MA 01655, USA

Correspondence should be addressed to Hong Zhang, hong.zhang@umassmed.edu

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Senescence is regarded as a physiological response of cells to stress, including telomere dysfunction, aberrant oncogenic activation, DNA damage, and oxidative stress. This stress response has an antagonistically pleiotropic effect to organisms: beneficial as a tumor suppressor, but detrimental by contributing to aging. The emergence of senescence as an effective tumor suppression mechanism is highlighted by recent demonstration that senescence prevents proliferation of cells at risk of neoplastic transformation. Consequently, induction of senescence is recognized as a potential treatment of cancer. Substantial evidence also suggests that senescence plays an important role in aging, particularly in aging of stem cells. In this paper, we will discuss the molecular regulation of senescence its role in cancer and aging. The potential utility of senescence in cancer therapeutics will also be discussed.

1. Introduction

Senescence was first described as a state of irreversible growth arrest that normal human fibroblasts enter at the end of their replicative lifespan [1]. This phenomenon has been observed in a variety of somatic cells derived from many species, which is in contrast to the infinite replicative capacity displayed by germline, cancer, and certain stem cells [2]. Senescent cells are irreversibly arrested in G1/G0 phase of the cell cycle and lose the ability to respond to growth factors [3, 4]. They show sustained metabolic activity for long periods of time [5] and become resistant to apoptosis [6, 7]. In addition, senescent cells undergo distinctive changes in morphology to a flat and enlarged cell shape [8] and are often accompanied by the induction of acidic senescence-associated β-galactosidase (SA-β-gal) activity [9]. At the molecular level, alterations in gene expression specific to senescent cells have been identified [10–14], including those constituting senescence-associated secretome, which can trigger profound changes in the surrounding cells and microenvironment [15–17]. The changes of gene expression in senescent cells can be partially explained by alterations in chromatin structure [13], including the formation of senescence-associated heterochromatic foci (SAHF), which is associated with trimethylated lysine 9 of histone H3, heterochromatin protein 1, and high-mobility group A protein [18–20]. The formation of SAHF requires the recruitment of pRb to E2F-responsive promoters and is responsible for the stable repression of E2F target genes, possibly contributing to the irreversibility of senescence [18].

2. Telomere-Dependent Replicative Senescence and Telomere-Independent Premature Senescence

The onset of replicative senescence is determined by the number of times that a population of cells can divide, suggesting that a mitotic clock recording cell divisions governs this cellular process [21, 22]. The discovery that telomeres get progressively shortened with each cell division provides a plausible explanation for the nature of this mitotic clock [23–26]. Because of the inability of DNA polymerases to replicate DNA at the very ends of linear chromosomes, telomeres become progressively shortened during successive cell divisions [23, 27, 28]. Telomerase, which is responsible for *de novo* synthesis of telomeric repeats and maintenance of telomere length [29], is expressed in germline, stem and cancer cells, but undetectable in majority of normal somatic cells [30, 31]. In the absence of telomerase, progressive telomere shortening is thought to be the major cause of replicative senescence. Supporting this notion, enforced expression of the telomerase catalytic subunit (TERT) has been shown to prevent telomere shortening and extend

the lifespan of human somatic cells [32–34]. Conversely, inhibition of telomerase in immortal cells has been found to limit their replicative lifespan [35, 36]. Critically shortened telomeres lose the protection of telomere-binding proteins, leading to telomere "uncapping" [26]. Recent studies have revealed that DNA damage foci containing multiple DNA damage-response proteins, such as 53BP1, γH2AX, MDC1 and MRE11, are found at telomeres in senescent cells, suggesting that uncapped telomeres are recognized as DNA breaks and thus trigger a DNA damage response [37–39].

In addition to telomere attrition, senescence can be activated by many types of stress, including aberrant activation of certain oncogenes [40–42], damage to chromatin structure [43–45], oxidative stress [46–48], DNA damage [49, 50], and inadequate culture conditions [48, 51, 52]. Collectively, they are referred to as stress-induced premature senescence. Among these senescence-inducing stimuli, oxidative stress has been shown to accelerate telomere shortening [47, 53], possibly by inducing telomeric single-strand breaks [54]. However, stress-induced premature senescence, unlike replicative senescence, is largely independent of the telomere length or the number of cell divisions [55–57].

The final outcome of both replicative senescence and stress-induced premature senescence is remarkably similar in that they share common changes in cell cycle regulation and morphological properties [40, 41, 46, 49, 50, 58, 59]. Although gene expression pattern can vary depending on the specific types of tissues and cells or the specific stimuli to trigger the senescence response, senescent cells display a unique pattern of gene expression that differs from proliferating cells or quiescent cells. In addition to the cell cycle regulatory genes, the expression of DNA damage checkpoint genes, inflammation and stress-associated genes, genes encoding extracellular matrix proteins and extracellular matrix-degrading enzymes, and cytoskeletal genes and metabolic genes is generally altered during replicative and premature senescence. Recent studies suggest that DNA damage could be a common cause for different forms of senescence induced by various stimuli [11, 12, 14, 60–63]. Senescence is now considered as a general stress response in normal cells to various types of cellular damage [64].

3. Molecular Regulation of Senescence

Despite the commonality shared by senescence induced by various stimuli, regulation of senescence varies significantly among cells derived from different species, or even different types of cells from the same species [65]. For example, telomere shortening is the major cause of senescence in human fibroblasts [34], whereas mouse fibroblasts undergo senescence that is independent of telomere shortening and probably mediated by oxidative stress [48, 52]. Diverse senescence-inducing stimuli can trigger the senescence response through multiple genetic pathways. However, these pathways seem to converge on p53 and pRb, and inactivation of both the p53 and pRb pathways is often required to prevent the activation of senescence [66–70].

In senescent cells, p53 is phosphorylated and its transactivation activity is elevated, although its mRNA and protein levels are largely unchanged [38, 71–74]. DNA damage response elicited by telomere dysfunction leads to activation of ATM/ATR and Chk1/Chk2, which in turn phosphorylate and stabilize p53 [37–39, 75, 76]. In addition, p53 is activated and plays an important role in stress-induced premature senescence [40, 50, 77–79]. This p53 activation is mediated by $p14^{ARF}$ (or $p19^{ARF}$ in mouse) encoded by the *INK4a/Arf* locus. ARF stabilizes p53 by sequestering Mdm2, an E3 ubiquitin ligase targeting p53 for degradation [80]. The ARF-p53 axis plays an important role during senescence in mouse cells. Inactivation of p53 or ARF in mouse embryo fibroblasts (MEFs) is sufficient to prevent senescence [81–83].

One of the p53 targets is $p21^{(CIP1/WAF1)}$ (p21), whose increased expression transactivated by p53 is responsible for cell cycle arrest [84]. The expression of p21 is up-regulated during replicative senescence [85–89]. This p21 up-regulation is dependent on signal(s) initiated by telomere shortening, as expression of TERT blocks this up-regulation [89–91]. Overexpression of p21 is able to induce a senescence-like growth arrest in some cells [92, 93], while deletion of p21 can postpone senescent arrest [94, 95]. Collectively, these studies suggest that p53 regulates senescence at least in part by inducing p21. As a cyclin-dependent kinase inhibitor, up-regulation of p21 in senescent cells leads to inhibition of pRb phosphorylation, which controls cell cycle progression [18, 84]. There are instances that inactivation of either p53 or pRb can significantly delay the onset of senescence, supporting a linear p53-pRb pathway [68, 96]. In many other instances, both p53 and pRb need to be inhibited to prevent replicative senescence, suggesting two independent pathways [66–69].

In parallel to p21, $p16^{INK4a}$ (p16) is another cyclin-dependent kinase inhibitor that leads to pRb hypophosphorylation [84]. The expression of p16 is increased during replicative senescence [88, 97–99], but whether increased p16 expression is regulated by telomere shortening is controversial. As telomere shortening is the major cause of replicative senescence in human fibroblasts [34], and inactivation of both the p53 and pRb pathways is required to prevent replicative senescence [67], it is reasonable to expect that dysfunctional telomeres may signal into p16-pRb axis. There is indeed an example showing that telomere dysfunction induces p16 expression [69]. However, the dynamics of p16 and p21 elevation in senescent cells are different. The increased expression of p16 occurs after senescence has already been established in culture [88, 97, 98, 100], in contrast to the rapid increase of p21 expression in cells approaching replicative senescence [89]. Within a senescent population of human cells, some cells express p16, while others express p21 [38, 96, 100]. DNA damage foci at telomeres are found only in cells expressing p21, but not in p16 positive cells [38], suggesting that p16 elevation is independent of telomere shortening. Consistent with this notion, p16 induction during senescence, unlike p21, is not prevented by ectopic expression of TERT [53].

The expression of p16 is readily increased during premature senescence induced by a variety of stress [40–42, 49, 51, 101]. It is not entirely clear how p16 expression is regulated by various senescence signals [102–104]. Under certain circumstances, p16 is coordinately regulated with Arf, which is also encoded by the *INK4a/Arf* locus. For example, polycomb complex proteins have been shown to repress the *INK4a/Arf* locus [100, 105–108]. Decreased expression of polycomb complex proteins relieves the repression of the *INK4a/Arf* locus and is responsible, at least in part, for the elevation of p16 and Arf in senescent cells [100, 106, 107]. The expression of p16 and Arf can also be regulated independently. Id1, whose expression is decreased in senescent cells [109], has been shown to specifically suppress p16 expression by forming heterodimers with transcriptional factors Ets1/2 or E47 and inhibiting their ability to transactivate p16 [110–112]. Down-regulation of Id1 in human and mouse fibroblasts has been shown to induce p16 expression and senescence [110, 112], while ectopic expression of Id1 delays senescence in human fibroblasts, mammary epithelial cells, keratinocytes, and endothelial cells [98, 113–115], suggesting an important role for Id1 in regulating p16 and senescence.

The expression of p16 varies significantly among different human cell lines [100], and this variable expression seems to hold the key to as whether p53 and pRb function in a linear manner or in parallel. In cells with low or no p16 expression, p53 and pRb may function in a linear pathway, whereas p53 and pRb work in parallel in cells with significant p16 expression. In mouse embryo fibroblasts (MEFs), inactivation of p53 or ARF, but not p16, is sufficient to prevent senescence [81–83, 116], indicating that p53-Arf axis is the major regulator of senescence pathway in mouse cells. Human mammary epithelial cells quickly encounter a growth arrest state that is not associated with telomere shortening but mediated by p16 up-regulation [33, 101]. A subset of cells with p16 inactivation emerge from the arrest population and continue to divide until reaching a second growth arrest state that is associated with telomere shortening [33, 51, 101]. Depending on cell types, culture conditions, and the extent of stress, inactivation of either p53-p21-pRb or p16-pRb pathway individually, or both pathways together, is required to prevent senescence.

4. Senescence As a Barrier to Tumorigenesis

Tumorigenesis is a multistep process, in which a normal cell acquires mutations in a number of cancer-causing genes [117]. By restricting cell proliferation and thereby impeding the accumulation of mutations, senescence acts as an important tumor suppression mechanism. Furthermore, senescence induced by aberrant activation of oncogenes, oxidative stress, or DNA damage prevents cells at risk of malignant transformation from proliferating [55, 59, 118, 119]. Senescence represents a physiologic response that cells must overcome in order to divide indefinitely and develop into tumors. Consistent with the notion that senescence is a tumor suppression mechanism, well-established tumor suppressors, including p53, pRb, p16, Arf, and p21, are regulators of senescence [102, 118, 120].

In contrast to normal somatic cells, cells derived from tumors divide indefinitely in culture. The ability to escape senescence (i.e., immortality) is a necessary step for cells to become transformed and one of the hallmarks of cancer cells [120]. 80%–90% of human cancer cells acquire unlimited proliferative potential through reactivation of telomerase [30, 31], while the rest maintain telomere length by a recombination-mediated process termed alternative lengthening of telomeres [121, 122]. These observations in human cancer strongly suggest a connection between telomere checkpoint and tumor suppression. Supporting this connection, inhibition of telomerase activity in cancer cells limits their growth by triggering telomere shortening and cell death [35, 36]. Conversely, ectopic expression of telomerase in normal human cells leads to immortalization and enhances the ability of these cells to be neoplastically transformed [33, 34, 123]. Furthermore, transgenic mice overexpressing TERT show increased propensity to tumorigenesis [124–128].

Genetic studies in mice deficient in telomerase provide further support for telomere shortening as a tumor suppression mechanism. Mice deficient in the telomerase RNA component ($mTERC^{-/-}$) gradually lose telomeres over several generations [129], and tumorigenesis is significantly reduced in late generations of $mTERC^{-/-}$ mice with telomere attrition [130–140]. Decreased tumorigenesis is also observed in late generation of mice with a null mutation in telomere catalytic subunit ($mTERT^{-/-}$), and p53 mutation enables tumor progression in these mice [141]. More importantly, two recent studies provide evidence that senescence induced by telomere shortening is responsible for tumor suppression [142, 143]. When apoptosis is blocked by the expression of Bcl-2 or a specific p53 mutant (R172P), shortened telomeres reduce tumorigenesis in $mTERC^{-/-}$ mice. Suppression of tumor development requires p53-dependent activation of senescence [142, 143], demonstrating that senescence induced by telomere shortening is an effective tumor suppression mechanism *in vivo*.

The discovery that oncogenic Ras protein can induce a senescent arrest after causing an initial hyperproliferation in normal cells suggests that induction of senescence is an intrinsic cellular response that prevents cells at risk from proliferating [40]. In mouse tumor models with oncogenic Ras, senescent cells are found in premalignant lesions in lung [61], spleen [144], breast [145], and pancreas [146]. The observation of senescent cells has been extended to many premalignant lesions or benign tissues induced by different oncogene activation or tumor suppressor inactivation in mouse [147–155] and human [148, 156–159]. Importantly, senescent cells are absent in malignant tumors [61, 144, 145, 147–150, 152, 156, 158, 160], suggesting that oncogene-induced senescence is a powerful tumor suppression mechanism by restricting proliferation of cells with oncogenic mutations and this senescence block must be evaded for malignancy to progress. Consistently, deletion of senescence regulators such as p53, Arf, p16, p27, SUV39H1 or PRAK abrogates senescence and causes progression of tumors to

the malignant stage [144–146, 148–150, 152, 153, 160]. These observations point to a causal link between loss of senescence and malignant transformation.

5. Senescence in Anticancer Therapy

In theory, senescence offers an attractive therapeutic option if it can be induced in tumor cells. Because of the uncertainty in reactivating in cells, a response that otherwise has been overcome, senescence remains as an underappreciated therapeutic approach [161, 162]. Surprisingly, many cancer cells retain the ability to senesce either spontaneously or in response to external stress stimuli, even though most cancer cells have overcome the senescent arrest during tumorigenesis. As tumors often develop resistance to apoptosis induced by anticancer treatment, induction of senescence in tumor cells serves as an alternative approach in cancer therapy, and could be especially effective in treatment of cancer cells in which apoptotic pathways are disabled [163].

Telomerase is an attractive target for inducing senescence in cancer cells. As telomerase is critical for the maintenance of telomere length [29], inhibition of telomerase in cancer cells leads to shortening of telomeres, which is a major cause of senescence activation [24, 33, 34]. Since 80–90% of human cancers acquire unlimited proliferative potential through activation of telomerase [30, 31], the strategy of inhibiting telomerase in cancer therapeutics targets a broad range of malignancies. In addition, this approach offers desired specificity in targeting cancer cells, as telomerase is expressed in most cancer cells, but undetectable in the majority of normal cells including adult stem cells [164, 165]. The emerging cancer therapeutics targeting telomerase include small molecule or oligonucleotide inhibitors of telomerase enzymatic activity, antitelomerase immunotherapy, inhibitors of telomerase expression and telomere-disrupting agents [166–168]. The strengths and weaknesses of these different approaches are discussed in an excellent review [166]. Although apoptosis is induced by inhibition of telomerase in some studies, induction of senescence as a result of telomerase inhibition is clearly demonstrated to be responsible for tumor suppression [169–173]. The effectiveness of these approaches has been demonstrated in many studies [174–177], and several clinical trials targeting telomerase for cancer therapeutics are now ongoing [166].

Senescence induced by oncogene activation or inactivation of tumor suppressor genes must be evaded for tumors to progress to full malignancy, which is often associated with inhibition of crucial senescence regulators. Reactivation of senescence response offers a great opportunity in cancer therapeutics. Considering the critical role of p53 in senescence regulation and common occurrence of p53 mutations in cancer cells, p53 is an attractive target for reactivation of senescence in cancer cells. Various approaches have been developed to target p53 in order to restore normal p53 function in cancer cells, including pharmacological depletion of mutant p53 protein [178, 179], restoring normal function in mutant p53 [180, 181], and reactivation of p53 [182–189]. In most of these reports, apoptosis is the overwhelming response that is responsible for tumor suppression. Senescence as a tumor suppression mechanism after restoring p53 expression has been demonstrated in two recent elegant studies [190, 191]. In a mouse model of hepatocellular carcinoma, reactivation of p53 in these tumors results in rapid activation of senescence and subsequent immune cell infiltration which leads to clearance of tumor cells [191]. In a separate study, restoration of p53 in p53-deficient mouse models of lymphoma, and osteosarcoma leads to tumor regression. Apoptosis is selectively induced by p53 in lymphomas, while senescence induced by p53 in osteosarcomas is responsible for tumor regression [190], suggesting that tissue type and/or genetic context play a critical role in determining the cellular response in p53-mediated tumor regression. Taken together, restoration of p53 function in tumors offers an effective way to restrict tumor growth by inducing senescence or apoptosis. As p16 and p21 have been shown to induce senescence efficiently [92], these senescence regulators together with Arf and pRb may provide additional targets for the effective activation of senescence in cancer therapeutics.

In addition to restoration of tumor suppressor genes, oncogene inactivation offers another possible intervention to induce senescence in cancer cells. Suppression of *c-Myc* oncogene induces senescence and leads to tumor regression in diverse tumor types including hepatocellular carcinoma, lymphoma and osteosarcoma [192]. Senescence induced by Myc inhibition depends on critical senescence regulators such as p53, p16 or pRb. Inactivation of these senescence regulators impairs senescence induction and tumor regression [192]. Inhibition of Myc as therapeutic intervention is further illustrated in lung carcinoma mouse model initiated by oncogenic Ras. Inhibition of Myc triggers rapid tumor regression associated with apoptosis and senescence induction [193]. These studies indicate that senescence response not only is functional in cancer cells, but also can be reactivated to cause tumor regression. Furthermore, these studies suggest that therapeutic intervention aimed at molecules required to support tumor growth may also lead to senescence induction and ultimately tumor regression.

The finding that senescence can be induced by DNA damage [49, 50] suggests that chemotherapeutic drugs, which cause DNA damage, may activate senescence in tumor cells and therefore determine the drug response in cancer treatment [194]. Chemotherapeutic drugs induce senescence in various types of tumor cells in culture [195–199]. In a Myc-driven mouse lymphoma model, chemotherapeutic drug cyclophosphamide induces p53- and p16-dependent senescence in lymphomas, leading to better prognosis following chemotherapy [163]. In human breast cancer, a high percentage of tumors after chemotherapy show positive staining for senescence markers, and induction of senescence in these tumors is associated with p53 and p16. Induction of senescence is not observed in tumors before chemotherapy [199], suggesting that senescence observed in tumors is induced by chemotherapy. Taken together, these studies show that senescence induction can positively influence the outcome of cancer treatment. Senescence-inducing drugs may be effective

alone or in combination with classic therapeutic approaches to reduce tumor growth and toxicity to normal cells.

6. Senescence and Aging

Aging is characterized by progressive deterioration of physiological function in all tissues and organs after a period of development. This biological process is associated with increased susceptibility to major chronic diseases and ultimately mortality. Since the discovery of senescence in cultured cells, it is recognized that cellular senescence and organismal aging may be closely related because of their shared ability to limit lifespan [21]. It is hypothesized that constant tissue regeneration results in accumulation of senescent cells in somatic tissues, which limits tissue renewal, perturbs normal tissue homeostasis and ultimately leads to aging [59, 118, 200]. Cells with characteristics of senescence have been reported to increase with advancing age in mice, primates and humans [9, 201–206]. In addition, accumulation of senescent cells is linked with age-associated pathological conditions, such as osteoarthritis [207], atherosclerosis [208–211], dementia [212], liver cirrhosis [203], and respiratory disease [213, 214]. The initial support for the senescence theory of aging comes from the observation of an inverse correlation between the *in vitro* lifespan of cells and the age of donors from which they are derived [215–219], although this correlation has been disputed [220]. Subsequent support comes from studies of cells derived from progeroid patients, such as Werner syndrome, which achieve fewer cell divisions before entering senescence than cells derived from normal individuals of same age [221]. Direct evidence supporting senescence as one of the aging mechanisms, however, is still missing. It remains to be determined whether accumulation of senescent cells is responsible for aging or age-related diseases.

Recent studies suggest that telomere checkpoint plays an important role in the aging process. It is evident that telomere shortening occurs in aged human tissues [222–235], at sites of age-related pathological conditions [203, 236–243], or associated with stress and obesity [244, 245]. Although it remains to be demonstrated whether telomere shortening leads to the accumulation of senescent cells *in vivo*, and more importantly makes a substantial contribution to aging, studies of human premature aging syndromes support a link between telomere attrition and aging. Patients of dyskeratosis congenita and aplastic anemia have mutations in telomerase RNA or catalytic subunit [246–248], and are characterized by accelerated telomere shortening [239, 246]. Further evidence for a role of telomere attrition in aging comes from genetic studies of mice deficient in telomerase. While mice with a null mutation in telomerase RNA ($mTERC^{-/-}$) are apparently normal in early generations, these mice in later generations gradually lose telomeres [129] and show accelerated aging phenotypes [140, 249]. Similarly, premature aging phenotypes are observed in $mTERC^{-/-}$ mice on a CAST/EiJ background, which have shorter and more homogenous telomere length than C576BL/6 strain. Even with the presence of telomerase, shortened telomeres in $mTERC^{+/-}$ mice on CAST/EiJ background are associated with premature aging [250]. A recent study shows that telomerase reactivation can reverse much of the premature aging phenotypes in telomerase-deficient mice [251], indicating that telomere attrition plays a critical role in aging. Furthermore, mutations in *WRN* or *BLM* in the telomere dysfunctional background in mouse cause premature aging phenotypes that are characteristics of Werner or Bloom syndrome in human. Such premature aging phenotypes are absent in mice with *WRN* or *BLM* mutation but with long telomeres [252, 253]. These studies clearly establish a link between telomere attrition and aging. Whether this link is mediated through senescence triggered by telomere shortening is currently unknown.

Premature aging phenotypes in late generation $mTERC^{-/-}$ mice are associated with reduced renewal capacity in highly regenerative tissues such as skin, intestine, bone marrow and reproductive organs [140, 249–251], suggesting that stem cells may be affected by telomere shortening. Tissue-specific or adult stem cells, which are capable of self-renewal and differentiation, are essential for the normal homeostatic maintenance and regenerative repair of tissues throughout the lifetime of an organism. The self-renewal ability of stem cells is known to decline with advancing age, eventually leading to the accumulation of unrepaired, damaged tissues in old organisms [59, 254–256]. By limiting cell proliferation, senescence in stem cells is hypothesized to contribute to aging by reducing the renewal capacity of these cells [21, 59, 118]. Not all stem cells express high level of telomerase. For example, human mesenchymal stem cells have no detectable telomerase activity [257], and hematopoietic stem cells from human and mouse have low level of telomerase activity [258–260]. Telomere attrition has been observed in these stem cells [257, 260–263]. It is possible that senescence induced by telomere attrition may occur in stem cells over the lifespan of an organism and would result in the reduction of the renewal capacity of stem cells. However, it remains to be determined whether stem cells undergo senescence during aging.

Several senescence regulators have been found to play a critical role in aging. The expression of p16 increases with advancing age in humans and rodents [264–270]. Increase of p16 in aged rodents is attenuated in several tissues (adrenal, heart, kidney, ovary, and testis) by caloric restriction [264], which potently slows aging. Moreover, age-related increase of p16 is found to be associated with a decline in the renewal capacity of stem cells in brain, pancreas, and hematopoietic system, and these stem cells derived from aged mice lacking p16 have increased regenerative potential [271–273]. In addition, p53 and p21 have also been implicated to impact aging. It has been shown that p21 is required to maintain quiescence of hematopoietic stem cells (HSCs). In the absence of p21, increased cell cycling leads to stem cell exhaustion, which is responsible for impaired self-renewal of HSCs [274]. Interestingly, deletion of p21 in late generation $mTERC^{-/-}$ mice improves stem cells function and rescues much of the premature aging phenotypes associated with telomere attrition [275]. HSCs from p53-deficient mice have increased stem cell population and enhanced renewal capacity [276, 277]. Suppression of

stem cells function by p53 is also observed in neural stem cells [278]. Furthermore, mice with excessive p53 activity maintain cancer protection, but age prematurely including impairment of HSCs [279–282], which is at least in part due to increased sensitivity to senescence-inducing stimuli [280]. Interestingly, concomitant increase of normal p53 and $p19^{Arf}$ leads to increased longevity in mice [283], although elevation of p53 alone is not sufficient to increase longevity [284, 285]. Collectively, these recent studies support an emerging link between senescence regulation and aging, and show the potential importance of senescence regulation in stem cells aging.

Senescence is regarded as an antagonistic pleiotropy: beneficial as a tumor suppressor, but detrimental to organisms by contributing to aging. Great progress has been made in our understanding of senescence regulation in cancer and aging. Challenges remain as how to effectively utilize senescence as a potent treatment for cancer. The exact function of senescence-associated secretome in cancer and aging is of great interest and needs to be investigated. Investigation of telomere shortening and senescence in stem cells during physiological aging is much needed for our understanding of the role of senescence in aging, which leads to the intriguing question as whether inhibition of senescence may slow aging.

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Review Article

Sirtuins, Bioageing, and Cancer

D. McGuinness,[1] D. H. McGuinness,[1] J. A. McCaul,[2] and P. G. Shiels[1]

[1] *College of Medical, Veterinary and Life Sciences, Institute of Cancer Sciences, University of Glasgow, Glasgow G11 6NT, Scotland, UK*
[2] *Maxillofacial and Head and Neck Unit, Bradford Institute for Health Research, Bradford Teaching Hospitals NHS Foundation Trust, Bradford BD5 0NA, UK*

Correspondence should be addressed to P. G. Shiels, ps6g@clinmed.gla.ac.uk

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The Sirtuins are a family of orthologues of yeast Sir2 found in a wide range of organisms from bacteria to man. They display a high degree of conservation between species, in both sequence and function, indicative of their key biochemical roles. Sirtuins are heavily implicated in cell cycle, cell division, transcription regulation, and metabolism, which places the various family members at critical junctures in cellular metabolism. Typically, Sirtuins have been implicated in the preservation of genomic stability and in the prolongation of lifespan though many of their target interactions remain unknown. Sirtuins play key roles in tumourigenesis, as some have tumour-suppressor functions and others influence tumours through their control of the metabolic state of the cell. Their links to ageing have also highlighted involvement in various age-related and degenerative diseases. Here, we discuss the current understanding of the role of Sirtuins in age-related diseases while taking a closer look at their roles and functions in maintaining genomic stability and their influence on telomerase and telomere function.

1. Sirtuins

Sirtuins are a highly conserved family of proteins found in all organisms from yeast to mammals. All are orthologues of the yeast protein, silent information regulator 2 (Sir2) [1] and their primary targets are acetylated lysines of various peptides and proteins, including histones. Along with sequence homology, they also share functional similarities although the functions performed in mammals are more complex than in yeast, as reflected in the number of distinct orthologous forms. These play key roles in cellular stress and ageing, and as such, their function has been linked to diseases associated with ageing, including Alzheimer's [2], Parkinson's Disease [3], cancer [4], type II diabetes [5], and atherosclerosis [6].

Every member of the family contains a highly conserved core domain consisting of a NAD^+-binding site and a catalytic domain [7]. Sirtuin function is tied to cellular energy production through nicotinamide adenine dinucleotide-(NAD^+-) dependent deacetylation reactions, as well as o-ADP ribosylation, in response to changes in the cellular NAD^+/NADPH ratio. Sirtuins appear to be involved in the extension of life span and health promotion in several species including yeast, nematodes and flies [8]. Pertinent to this is the observation that Sirtuins can be activated through caloric restriction, stress, or by pharmacological agents [9]. Sirtuins have a pivotal role in the expansion of lifespan in lower organisms via caloric restriction [10–15]. This phenomenon is also believed to occur in higher mammals, and ongoing studies in monkey models have demonstrated promising results in proving this connection [16]. Additionally, some small-scale studies with centigenarians have demonstrated that allelic variants of some Sirtuin genes are linked to longevity in humans [17–19]. Despite this, the involvement of Sirtuins in enhanced human health and lifespan is still the subject of great debate. There is, however, increasing corroborative evidence of their links to cancer processes, genomic instability and other diseases of ageing.

Central to such associations is the observation that Sirtuin activity is directly correlated with the metabolic state of the cell [20]. Sirtuins act as substrate-specific type III protein lysine deacetylases, in contrast to the classic deacetylases, which facilitates a link between cell metabolism and

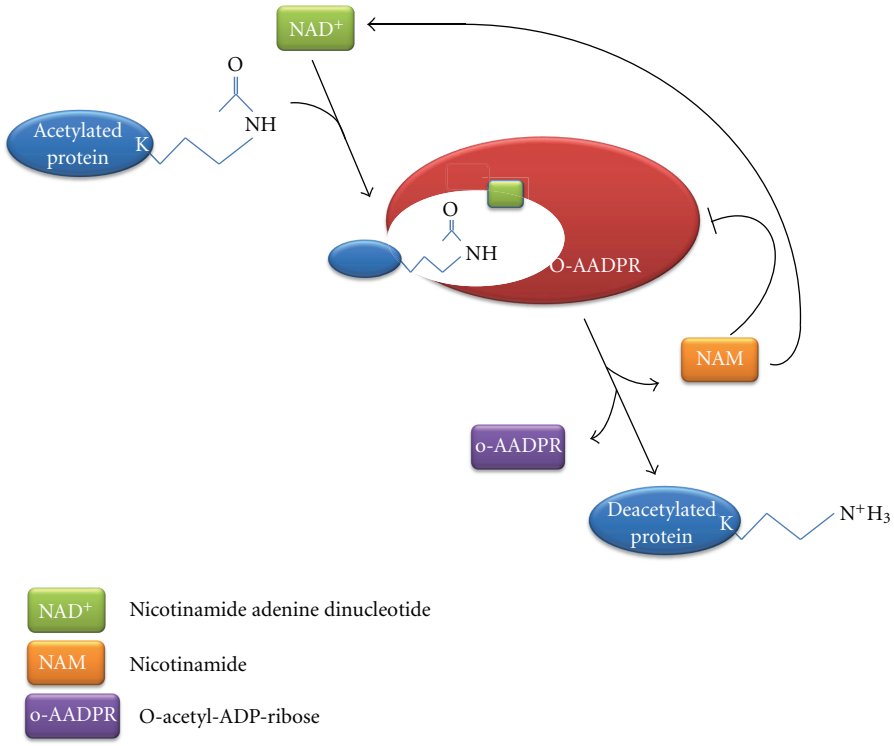

Figure 1: Protein deacetylation by Sirtuins. Sirtuins deacetylate lysine (K) residues of target proteins using cofactor—Nicotinamide-adenine-dinucleotide (NAD^+) and releasing Nicotinamide (NAM). 2′-O-acetyl-ADP ribose is generated as a result of transfer of the acetyl group of K onto ADP-ribose residue. Deacetylation is inhibited by NAM, which can also reverse the reaction to reproduce NAD^+.

control of transcription. Briefly, the deacetylation involves a unique enzymatic NAD^+-dependent reaction which begins with amide cleavage from NAD^+ leading to the formation of Nicotinamide (NAM) and a covalent ADP-ribose peptide-imidate intermediate (ADPR). This intermediate is transformed to O-acetyl-ADP-ribose and the deacetylated protein is released from the complex (Figure 1, [21]). Due to the reliance of Sirtuin deacetylation activity on NAD^+, it is hardly surprising that evidence suggesting NAD^+ and NAD^+ generating pathways are directly involved in the regulation of Sirtuin activity is mounting rapidly. This is supported by the observation that the Nicotinamide (NAM) product site can be occupied in the presence of substrates and reaction intermediates [22, 23]. Bound NAM is able to inhibit the enzymatic activities of Sirtuins and can, in some cases, reverse the reaction, thus regenerating NAD^+ and the acetylated substrate. Sirtuins, together with other NAD^+ consumers (ADP-ribosyltransferases and cAMP ribose synthetase), have also been implicated in the salvage/elimination of NAM, thus playing a vital role in the homeostatic maintenance of NAD^+ metabolism [7, 24].

In humans, seven Sirtuins have been identified (Sirt1–7) [25, 26], all with unique characteristics, functions, and localisations (Table 1).

Sirt1, Sirt6, and Sirt7 are localised mainly in the nucleus, whereas Sirtuins 3–5 are found mainly in the mitochondria [27]. Conversely, Sirt2 has a predominantly cytoplasmic localisation [28, 29].

The Sirtuins contain nuclear localisation signals (NLSs) as well as nuclear export signals (NESs) and their intracellular localisation is determined by cell/tissue type and physiological conditions. Sirt6 and Sirt7 contain a single NLS, while Sirt1 contains 2 NLS and 2 NES domains [29, 30]. The presence of an N-terminal mitochondrial targeting sequence ensures Sirt3–5 localisation within the mitochondrial matrix [31, 32], whereupon the signal sequence is cleaved, activating the enzymatic function of the proteins. Interestingly, it has been recently suggested that under specific conditions (stress), Sirt3 can translocate from mitochondria to the nucleus [33, 34].

Sirtuins are actively involved in the regulation of gene expression, principally due to their histone deacetylase activity and the consequential ability to influence the activity of a wide range of transcription factors. It has been shown that all Sirtuins, with the exception of Sirt4 and Sirt7, have histone deacetylase activity (HDAC). Sirt1 can affect core histones (H1, H2, H3, and H4), but it preferentially deacetylates H3 (K9, K14 and K56 residues), H4 (K16) and H1 (K26) [35–37]. The specific deacetylation of lysine residues at H3K9/H4K16 and H1K26 by Sirt1 has been linked to gene silencing and chromatin remodelling. Additionally, histone deacetylation can facilitate the methylation of histones, for example, di/tri-methylation of H3 on the K9 residue and H4 on the K20 residue. These modifications have been linked to global transcriptional repression and are characteristic for facultative heterochromatin [38]. This reaction can be

Table 1: The mammalian Sirtuins.

	Enzymatic activity	Localisation	Substrates/targets	Function
SIRT1	Deacetylase	Nuclear/cytoplamic	p53, FOXO, NFκB, MyoD, Ku70, LXR, PPARγ, p300, Tat, PCAF, ERα, AR, SMAD7, PCAF, p73, Sox9, HES1, PGC1α, HEY2, NcoR/SMRT, E2F1, RelA/p65	Glucose metabolism, fatty-acid and cholesterol metabolism, differentiation, insulin secretion, and neuroprotection
SIRT2	Deacetylase	Nuclear/cytoplamic	α-tubulin, FOXO	Cell-cycle control, tubulin deacetylation
SIRT3	Deacetylase	Mitochondrial	AceCS2, GDH complex1	ATP production, regulation of mitochondrial proteins deacetylation, and fatty-acid oxidation
SIRT4	ADP-ribosylotransferase		GDH, IDE, ANT	Insulin secretion
SIRT5	Deacetylase		CPS1	Urea cycle
SIRT6	Deacetylase ADP-ribosylotransferase	Nuclear	NFκB, Hif1α, helicase, DNA polymeraseβ	Telomeres and telomeric functions, DNA repair
SIRT7	Deacetylase	Nuclear	RNA polymerase type I, E1A, SMAD6	RNA polymerase I transcription

further enhanced by Sirtuins; for example, H3K9 methylation is enhanced by Sirt1. Sirt1 binds to the histone methyltransferase Suv39H1 (suppressor of variegation 3–9 homolog 1) and facilitates binding of this protein to chromatin. Sirt1 is then activated by deacetylation of Suv39H1 [36, 39]. There are additional regulation mechanisms involved in the chromatin silencing mediated by Sirt1-Suv39H1 complex, it has recently been shown that deleted in breast cancer 1 (DBC1), not only inhibits both Sirt1 and Suv39H1 activity, but also disrupts the interaction between these two molecules leading to the increased methylation of H3K9 [40]. The other interesting aspect of Sirt1 involvement in the epigenetic regulation of gene expression is its association with aberrant expression of the methylated genes that can be facilitated by its interaction with Dnmt3b [41] or Dmnt1 [42]. Overall, the ability of Sirt1 to remodel chromatin, together with the ability of this enzyme to deacetylate and/or interact with a broad range of transcription factors (i.e, p300, NFκB, FOXO, E2F1, and Smad7 (Table 1)) suggest that Sirt1 may be a major player in the regulation of organism homeostasis, stress responses, endocrine signalling, and cell metabolism.

Acetylated lysine residues on Histone 4 (H4K16) and Histone H3 (H3K9) are targets for Sirt2 deacetylation when the nuclear envelope disassembles during the mitotic process [43]. This makes Sirt2 a regulator of the cell cycle, involved in the promotion of chromatin condensation. Similarly, Sirt3 deacetylates H4K16 and H3K9 *in vitro* although the importance of this process under *in vivo* conditions remains somewhat controversial [34].

Finally, Sirt6 deacetylates H3K9 at telomeres, indicating that this particular molecule may be a modulator of cellular senescence and ageing induced chromosomal abnormalities [44].

Sirtuins are also involved in the regulation of RNA Polymerase II transcribed genes although their involvement in the formation of the transcription initiation complex has not been proven to date [45]. Interestingly, Sirtuins affect transcription of ribosomal RNA also. Sirt1 and Sirt7 have opposing effects on rRNA transcription; Sirt1 deacetylates TATA box-binding protein associated factor (TAF_I68) leading to the inhibition of Polymerase I [46], while Sirt7 directly binds to polymerase I and induces enzyme activity [47].

A search for Sirtuin-binding sites revealed many putative targets. However, all of these targets follow on a common theme in Sirtuin function, namely, cellular stress responses [48]. These incorporate cell death responses, senescence, stress-related transcription regulation, cell-cycle control, cell metabolism, genomic stability and formation, and maintenance and control of telomeric function (Table 1). These activities for Sirtuins reinforce the link between key features of cellular bioage and disease, centred on telomere stability and cellular lifespan. Extrapolating Sirtuin activity to longevity, already established in lower organisms is thus intuitive for higher animals though it remains unproven. The increasing evidence for Sirtuin involvement in age related diseases is a key link to their function in the control of the cell lifespan and genomic stability [49–51]. This involvement in age-related disease further supports the link between Sirtuin function and longevity, possibly making Sirtuins the key to unlocking the causes and treatments for many age related diseases.

2. Sirtuins and Genomic Instability

The involvement of Sirtuin function in disease is typified by Sirt1, which is rapidly emerging as a tumour keystone,

providing both tumour suppressor and tumour promoter functionality [52]. Sirt1 has been shown to be overexpressed in several cancers, including prostate [53], acute myeloid leukaemia [54], colon cancer [55], and some nonmelanoma skin cancers [56]. Sirt1 has also been observed to be repressed in many other cancers, including glioblastoma, bladder, ovarian, and prostate cancers [57]. This duality of purpose indicates the pivotal role this Sirtuin exerts in the cell. Overexpression of Sirt1 can lead to deacetylation of p53 [58, 59] and reduction of many tumour suppressor genes, thus promoting genomic instability by reducing the cell's ability to respond to DNA damage and stress. Conversely, it can also deacetylate B-catenin causing the oncogenic form of this protein to translocate to the cytoplasm, thus reducing the growth of tumours [60]. More recently, Oberdoerffer et al. have demonstrated that redistribution of Sirt1 in a mouse model of genomic instability results in improved survival rates and transcription profiles similar to those found in the ageing process, particularly involved in repairing DNA breaks and other forms of genomic instability [61]. Further investigation into Sirt1 involvement in genomic instability has been hampered by an inability to produce a viable null model, as Sirt1 knockout mice die during the mid-gestation stage although it was determined that these mice showed histone modifications and impaired DNA-damage repair. Additionally, Sirt1 and p53 heterozygotes showed an increase in tumour formation in multiple tissues, a phenotype that could be at least partially rescued by activation of Sirt1 using Resveratrol [57]. It is still undetermined whether this Sirtuin acts as a tumour keystone with suppressor or promoter functions.

Sirt2, which acts as a G2 checkpoint mitotic regulator, appears to have a similar dichotomous role in both the formation and prevention of gliomas [62]. Increased expression of Sirt2 has been linked to a prolonged cell-cycle, with severe delays in cell cycle progression, suggesting a tumour suppressor role [63]. Furthermore, its role as a mitotic checkpoint protein helps prevent chromosome instability and the development of hyperploidy [64]. Downregulation of Sirt2 has been shown to interfere with cell cycle progression and in some cases can induce cell-cycle arrest [65], while overexpression has been shown to cause a prolongation of the mitotic phase of the cell cycle [63] resulting in multinucleated cells [28, 65]. The induction of multiploidy phenotypes indicates that Sirt2 plays a role in chromosomal stability by controlling the cell division associated separation of recently replicated chromosomes. Sirt2 directly deacetylates α-tubulin, providing it with a further mechanism for the control over mitosis and its ability to ensure single ploidy cells [64], thus ensuring genomic stability during mitosis.

Sirt3 may play a role in mitochondrial redox regulation [27] though data on its role is equivocal. Two independent studies have demonstrated that Sirt3 null mice have no associated phenotype, with normal development and fertility [66, 67]. Conversely, another Sirt3 null model, using mouse embryonic fibroblasts, has demonstrated abnormal mitochondrial function, including an increase in stress-induced ROS and genomic instability [68]. In this model, expression of a single oncogene (c-myc or ras) was sufficient for neoplastic transformation of the cells, which could be reversed by introducing superoxide dismutase to counteract the increase in ROS. It has now been shown, by the same group, that this effect is dependent upon deacetylation of mnSOD [69]. Mice with Sirt3 knockouts also developed oestrogen receptor $(ER)^+$ and Progesterone receptor $(PR)^+$ mammary tumours, suggesting that Sirt3 is a mitochondrially localised tumour suppressor. However, there is still some debate over the localisation of Sirt3, with the majority of studies claiming that Sirt3 is exclusively mitochondrial [70, 71]. Notably, two distinct forms (long and short) of Sirt3 have been reported [72–74] with the short version lacking a mitochondrial localisation signal peptide, indicating it may be localised elsewhere. This may account for the equivocal reports that Sirt3 can be localised to the nucleus. There are also reports that suggest Sirt3 translocates to the nucleus upon overexpression of Sirt5 or oxidative challenge to the cell [33, 34]. These reports did not investigate whether this was accomplished by translocation of Sirt3 from mitochondria to the nucleus, *de novo* synthesis, or expression of the short version of Sirt3.

Sirt4 shows no discernable NAD^+-dependent deacetylase activity *in vitro* [75, 76], confirmed by a lack of mitochondrial protein acetylation variation in a null mouse model [67]. Like Sirt3 null mice, Sirt4 null mice demonstrate an overtly normal phenotype [50]. Sirt4 is associated with insulin secretion by pancreatic β-cells, which may link it to type II diabetes, an age-related disorder. There is no direct evidence to date, however, that Sirt4 has any direct affect on genomic stability, through either over- or underexpression. Recently it has been suggested that Sirt3 and Sirt4 activities are antiapoptotic in response to DNA damage when extremely low levels of NAD^+ are present [77].

Sirt5 localises to the mitochondrial matrix, where its N-terminus is cleaved. Sirt5 appears to operate exclusively in the mitochondria and one of its major targets is carbamoyl phosphate synthase 1 (CPS1) [78], which is responsible for converting ammonia to urea. It also regulates the entry of ammonia into the urea cycle. Therefore, it would appear that the major function of Sirt5 *in vivo* is to enhance the body's reaction to the breakdown of amino acids during calorie restriction via CPS1 and Cytochrome C [79, 80]. Very little else is known about the function of Sirt5 other than that it has been demonstrated that Sirt5 plays a role in the localisation of Sirt3 [34]. Sirt3 is ordinarily present in the mitochondria; however, overexpression of Sirt5 causes Sirt3 to localise to the nucleus this phenomenon has also been shown as part of the cell stress response [33]. Whether this is due to increased expression of Sirt3 Short has yet to be established. This indicates that Sirt5 may contribute, in part, to the cellular response to stress, or that it is produced as a result of the stress response. Given the dependence of Sirtuins on NAD^+ for their action, it is feasible that Sirt5 is part of the sensing apparatus to initiate the stress response and would then activate it's deacetylation functions to affect other transcription factors, thus initiating the cell wide stress reaction, which may include sending Sirt3 to the nucleus. Therefore, Sirt5 may exert an influence over genomic stability via the action of Sirt3.

The role of Sirt6 has been established as being a key component of base excision repair (BER), as part of

intra-cellular DNA-damage responses. Sirt6 directly stabilises DNA-dependant protein kinase at the site of dsDNA breaks, allowing the formation of the DNA repair complex and the initiation of repairs [81]. Sirt6 also associates directly with chromatin, demonstrated by its association with chromatin enriched cellular fractions [82]. Sirt6 has also been shown to localise to the promoter regions of NF-κB activated proteins, whereupon it deacetylates the associated H3 histone at Lysine 9, thereby silencing the recently activated genes [65]. Sirt6 deficiency is associated with shortened lifespan and accelerated ageing phenotypes. In fact, mice with Sirt6 knockouts have been shown to have a progeroid phenotype, with extreme hypoglycaemia and are unable to survive beyond 4 weeks. The lethal hypoglycaemia observed in Sirt6 deficient mice is a direct result of its H3K9 deacetylase function which controls the expression of glycolytic genes [83]. Furthermore, knockout mice demonstrate a very high level of genomic instability and hypersensitivity to DNA damage [82, 84], confirming Sirt6's key role in DNA damage repair and also demonstrating its close relationship with the original Sir protein in yeast, Sir2. It was also noted that the increased sensitivity to DNA damage did not appear to be a function of impaired cell-cycle checkpoints, nor the dsDNA break repair mechanism. Deletion of Sirt6 results in chromosomal abnormalities including breaks and fusions, as well as a breakdown in BER, a phenotype that can be rescued by introduction of a fragment of Polymerase β (Polb), which has been determined as a target for Sirt6 [85]. The deacetylation of histones by Sirt6 is likely to have a stabilising effect on the genome, for example, H3K56 [86] although a direct link has yet to be established.

Sirt7 directly interacts with RNA polymerase I (Pol I) and histones, giving a direct link between this Sirtuin and genomic stability [87]. This link is demonstrated by increasing Sirt7 levels directly increasing Pol I function and inhibition of Sirt7 leading to decreased Pol I activity [47]. Complete depletion of Sirt7 results in cell death, after a complete halt to cell proliferation; it is believed that this direct linkage allows Sirt7 to regulate Pol I function with regard to NAD^+ levels, tying it to cell metabolism and energy levels in keeping with the original postulate of Shiels and Davies (2003) [48]. They argue that cellular responses to stress and damage centre on how much damage has been accrued, how much energy the cell needs to effect any repair, and how much fuel it must burn to achieve this. If the damage is too great; the cell will effect death, however, if the damage is not critical, then cellular energy metabolism is regulated to allow repair, and ribosome biogenesis is modulated to facilitate this [48, 88].

All cells have an in built mitotic clock associated with telomeres [89], this clock is continually reset in germ line cells by telomerase, and it also appears to be modified, turned off, or reset in cancer to allow tumours to grow unabated. It has been well established that ageing is associated with the degradation of telomeres, which ultimately leads to cell senescence and apoptosis when the cell has reached the end of its useful life [48]. The system of telomeric instability associating with age is an essential checkpoint in the control of life and disease, in particular cancer. Sirtuins are rapidly emerging as the key link between ageing, disease, metabolism and cellular stress.

3. Sirtuins and the Regulation of Cellular Stress Responses

The intricate role Sirtuins play in the control of the cell metabolism is mediated through their dependence on NAD^+; this control inextricably links their function with the metabolic status of the cell. It also provides a sensing platform for the response to cellular stress.

p53 tumour suppressor is involved in the regulation of apoptosis and its reactivity is tightly regulated. Under physiological condition, this molecule is maintained at very low levels in the cells, but its expression is rapidly increased in response to stress in order to fulfil its regulatory functions [90]. It has been documented that p53 activity can be modulated by SIRT1 in particular; overexpression of SIRT1 not only abrogated p53 dependent apoptosis in response to oxidative stress, DNA damage, and ionizing radiation, but also sensitised cells to apoptosis induced by these factors [58, 59]. Various studies have demonstrated that SIRT1 plays a critical role in the regulation of both p53 dependent and p53 independent apoptosis in response to oxidative stress. This regulation occurs via the deacetylation of p53 which leads to its retention in the cytoplasm and enhances passage of p53 into the mitochondria [91, 92]. The ability to modulate p53 acetylation establishes SIRT1 in the inhibition of cell senescence in response to oxidative stress. In this case, SIRT1 is recruited to the PML bodies and p53, where it blocks p53-dependent transactivation; this phenomenon has been observed in human endothelial cells, where Downregulation of SIRT1 led to increased acetylation of p53 and development of a premature senescence phenotype [93, 94]. In contrast to SIRT1, SIRT2 overexpression promotes neurodegeneration and affects the ability of cells to recover after cellular stress, mainly due to Downregulation of 14-3-3ζ [95, 96].

Another mechanism by which Sirt1 can regulate the cellular response to stress is the ability of Sirt1 to regulate members of the FOXO (Forkhead box class O) transcription factor family. Sirtuin 1 deacetylates 3 members of FOXO family, Foxo1, Foxo3a, and Foxo4 [97, 98]. Sirt1 regulation of Foxo3a function in mammalian cells reduces apoptosis in response to cellular stress, but also increases the expression of genes involved in DNA repair and cell-cycle check points [97]. SIRT1 activates Foxo1 and Foxo4 which are involved in the promotion of cell-cycle arrest by induction of $p27^{Kip1}$ and in enhancing cellular defences against oxidative stress through the regulation of manganese superoxide dismutase, catalase, and GADD45 (growth arrest and DNA damage inducible α) [98, 99]. It has been demonstrated that Sirt2 under oxidative stress deacetylates Foxo3a, and thus enhances the expression of Foxo-regulated genes and reduces ROS levels in cells [100]. Similarly to Sirt1, Sirt7 depletion in mice leads to a specific phenotype, characterised by p53 hyperacetylation and lack of resistance to the oxidative or genotoxic stress [101].

4. Sirtuins, Telomeres, and Telomerase

TNFα has been shown to induce telomerase activity in lymphocytes [102], this proinflammatory cytokine is controlled by NF-κB which in turn is influenced by Sirt1. Therefore, Sirt1 has direct influence over TNFα activation of telomerase activity. Whether this activation can be achieved in cells other than lymphocytes or whether it can contribute to the immortalisation of tumour cells has yet to be elucidated. Inhibition of Sirt1 has also been associated with increased telomerase activity in human cells [103].

Sirt2 is predominantly cytoplasmic and is unlikely to play any role in telomere biology. Sirt3–5 are mitochondrial and to date have no information linking them to telomeric sites, telomerase, or mitotic division. However, Sirt6 is absolutely essential for dsDNA repair, playing an active role in the recruitment of other factors to the site of dsDNA breaks [81]. Sirt6 also appears to be extremely important in the maintenance of telomeres and telomeric function. Recent studies have demonstrated that reduction or removal of Sirt6 results in telomere dysfunction and end-to-end chromosomal fusions. This absence of Sirt6 is similar in symptoms to Werner's syndrome, which is a disease characterised by premature ageing. It is an extremely rare, autosomal recessive disorder caused by a mutation in the *WRN* gene encoding DNA helicase [44]. This results in genomic instability and telomeric attrition, the process by which this occurs is unknown. It is believed that Sirt6 is essential for proper telomere maintenance and function. Sirt6-deficient cells have been shown to have an increased susceptibility to genotoxic DNA damage resulting in the accumulation of chromosomal abnormalities resulting in genomic instability. Sirt6-deficient mice exhibit an accelerated ageing phenotype; however, the researchers were unable to determine any cellular lifespan change [82]. In another study using Sirt6 null mice it was demonstrated that these mice have a progeroid like syndrome, profound hypoglycaemia, and premature death at around the 4-week stage [50]. This appears to indicate that Sirt6 does, in fact, have a major impact on organismal lifespan control.

Very little is known about Sirt7, and although it is localised to the nucleolus, there has been no evidence presented that suggests any involvement with telomere function, formation, or stability.

5. The Association of Sirtuins with Diseases of Ageing

Sirt1 is heavily implicated in several diseases associated with ageing, as well as with ageing itself. This Sirtuin has been shown to protect axons from damage in animal models of the Wallerian degenerative disease (Parkinson's disease) [104]. Furthermore, the use of resveratrol (a Sirt1 activator) in models of Huntington's disease shows that Sirt1 is able to reduce cell death by inhibition of NF-κB signalling [105]. Alzheimer's disease has also been linked to Sirt1 function and calorie restriction in monkeys [106]. A recent study has demonstrated that Sirt1 overexpression in the brain of mice directly reduces β-Amyloid production and the formation of plaques [107]. Another study demonstrated that induction of Sirt1 function also reduced macular degeneration by protecting retinal ganglial cells [108]. Furthermore, it has been shown that Sirt1 has a direct influence on the pancreatic β cell production of insulin. Along with Sirt3, altered expression of these Sirtuins has been implicated in the development of Type II Diabetes [8]; however, no links with Type I diabetes have yet been established. This activity is believed to occur through acetyl coenzyme A synthetase (AceCS) upon which both Sirt1 and 3 act to produce acetate. The production of acetate has been shown to be disrupted in diabetes as well as in ageing. Sirt4 has also been shown to be downregulated in pancreatic b cells in response to calorie restriction implicating it in diabetes although no links have yet been demonstrated.

Although Sirt2 is associated mainly with the brain, there have been no links made between this Sirtuin and neurodegenerative diseases. The limited amount of information available on Sirt5 makes it very difficult to make any connections between this Sirtuin and diseases of ageing; however, its heavy involvement in the mitochondria leads to speculation that it may be related to metabolic disorders.

Sirt3 has been linked to overall longevity in humans, although the studies conducted were small scale. The first study linked a polymorphism in Sirt3 to increased longevity in males [19], and the authors also determined that the chromosomal location of Sirt3 is also home to four other proteins associated with longevity (tyrosine hydroxylase, proinsulin, IGF2, and HRAS1). A subsequent study confirmed this observation but went further to suggest that decreased levels of Sirt3 was detrimental to longevity in males [17]. Furthermore, Lescai et al., (2009) [109] linked a Sirt3 SNP to longevity in centenarians from Italy, France, and Germany. Recently, Sirt3 has been directly linked to age-related hearing loss [110].

Sirt6 is heavily associated with DNA damage, telomeres, and cancer. Another link to degenerative disease exists with the association between Sirt6 and WRN which is implicated in premature ageing like Werner Syndrome [111]. Furthermore, Sirt6 actively represses genes associated with age-related cellular senescence and it is, therefore, highly likely that more associations will be discovered and that Sirt6 will become a key player and target in the research and treatment of cancer and other age-related diseases. There is also a suggestion that it may play a key role in the maintenance of organ integrity particularly associated with ageing [8]. Another key mediator in age-related diseases is inflammation, which in this context is generally induced by age-related increases in NF-κB activity. This activity is directly opposed by both Sirt1 and Sirt6, where Sirt1 acts directly on the RelA subunit causing deacetylation and reducing its action. Sirt6 is also sequestered to NF-κB activated targets and shuts them down at the transcription level. Thus, both Sirtuins may be active in age-related inflammatory disorders. Although no direct causal links between these Sirtuins and inflammatory disorders have been made, the level of circumstantial evidence suggest that formal demonstration may be a matter of time and research.

Sirt1 and Sirt7 are associated with age-related cardiovascular disease through their interactions with p53, Fox01 and nitric oxide synthetase (NOS). Sirt1 has also been shown to improve the regeneration of vascular endothelia and smooth muscle cells [112].

6. Sirtuins and Cancer

Cancer is now established as a disease of ageing. Consequently it was inevitable that Sirtuins would play a vital role in tumourigenesis. The roles played by Sirtuins at key points in the cell are also highly indicative of their roles in modulating the aberrant survival and replication of tumour cells. The most obvious involvement for Sirtuins in cancer comes from Sirt1 and Sirt7 mediation of p53-function, which is well established as a focal point in many cancers. Sirt1 (and Sirt7) deacetylates p53 reducing its influence over cell cycle control during stress and in response to DNA damage. Thus overexpression of Sirt1 deactivates p53 and disrupts p53 dependent pathways and this results in a large reduction in the cell's ability to respond to stress and DNA damage [58, 59]. This has lead to many researchers describing Sirt1 as a tumour promoter, a suggestion that has now been supported by several studies. These identify increased levels of Sirt1 associated with various cancers including prostate [53], AML [54], primary colon [55] and several nonmalignant skin cancers [56]. Overexpression studies resulted in lowered production and or action of several key tumour suppressors including FOXO family members [113], p73 [114], RB [115], and several others [116–119]. However, the story for Sirt1 is not so simple. Several studies have reported decreased levels in cancer, for example glioma, bladder, prostate, and ovarian cancers [57]. Several studies have reinforced this connection demonstrating a reduced level of Sirt1 associated with tumourigenesis [118, 120–122]. In fact, Sirt1 acts as a tumour keystone, where its level and action maintain a fine and delicate balance between suppression and promotion of oncogenesis. Based on the available evidence, it is plausible that Sirt1 acts as a suppressor and then a promoter (or vice versa) depending on the stage and situation of tumourigenesis.

Control of cell-cycle progression by Sirt2 has been shown to be essential in the prevention of tumours, as it is suppressed in gliomas [62]. Sirt3 is the only mitochondrial Sirtuin to have a demonstrated role in tumourigenesis to date and its reduction in several cancers leads to an increase in ROS which results in enhanced tumour growth [68]. Interestingly, Sirt5 overexpression has been implicated in a study of pancreatic cancer [123].

The role of Sirt6 in controlling NF-κB function and DNA damage repair also indicate a key role in tumourigenesis although very little information is available on specific correlations with cancer to date some studies have been conducted which demonstrate a link through interaction with GCIP in colon tumours [124]. Our group has previously demonstrated that Sirtuins 3–7 are elevated in some forms of breast cancer [49] and mRNA levels of Sirt7 have been inversely correlated with the ability to undergo tumourigenesis in mouse cell lines [125]. Sirtuin influence and types of cancer where associations have been shown are summarised in Table 2.

Table 2: Cancers associated with Sirtuins and their proposed mechanism of involvement.

	Association with cancer
Sirt1	Acute myeloid leukemia, colon, nonmalignant skin, bladder, prostate ovarian cancers, and glioma—mediates p53 function
Sirt2	Glioma—control of cell cycle progression
Sirt3	Breast cancer—decrease in levels is associated with a general increase in tumour growth due to increase in ROI
Sirt4	Breast cancer—metabolic
Sirt5	Pancreatic, breast cancers—metabolic
Sirt6	Colon, breast cancers—mediates NFκB and GCIP function
Sirt7	Breast cancer—mediates p53 function

An interesting link between Sirtuin levels and circadian rhythm has also been reported [126]. This is noteworthy given the understood disruption of circadian rhythm in cancer [127]. This opens the possibility of the use of chronotherapy using Sirtuin regulators at specific times to target tumours [126].

It is obvious that the Sirtuins, in line with the function of Sir2 in yeast, play critical roles in the maintenance of the genome in all organisms. These vital roles have led to speculation that these molecules are heavily involved in two key areas, tumourigenesis and ageing. Further, evidence for these proteins in such crucial roles is accumulating at an accelerating rate. As this area of molecular science consolidates and advances, the Sirtuin family of proteins are gaining significance in human biology and disease. This group show strong potential to become valuable predictive and prognostic markers for disease and as therapeutic targets for the management of a variety of cancer types and other age-related diseases.

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Review Article

Cellular Senescence as a Target in Cancer Control

Mar Vergel,[1] Juan J. Marin,[2] Purificacion Estevez,[1] and Amancio Carnero[1,3]

[1] *Instituto de Biomedicina de Sevilla, Hospital Universitario virgen del Rocio, 41013 Sevilla, Spain*
[2] *Department of Preventive Medicine and Public Heath, University of Seville, 41012 Seville, Spain*
[3] *Consejo Superior de Investigaciones Cientificas, Avenida Manuel Siurot s/n, 41013 Sevilla, Spain*

Correspondence should be addressed to Amancio Carnero, acarnero@ibis-sevilla.es

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Somatic cells show a spontaneous decline in growth rate in continuous culture. This is not related to elapsed time but to an increasing number of population doublings, eventually terminating in a quiescent but viable state termed *replicative senescence*. These cells are commonly multinucleated and do not respond to mitogens or apoptotic stimuli. Cells displaying characteristics of senescent cells can also be observed in response to other stimuli, such as oncogenic stress, DNA damage, or cytotoxic drugs and have been reported to be found *in vivo*. Most tumors show unlimited replicative potential, leading to the hypothesis that cellular senescence is a natural antitumor program. Recent findings suggest that cellular senescence is a natural mechanism to prevent undesired oncogenic stress in somatic cells that has been lost in malignant tumors. Given that the ultimate goal of cancer research is to find the definitive cure for as many tumor types as possible, exploration of cellular senescence to drive towards antitumor therapies may decisively influence the outcome of new drugs. In the present paper, we will review the potential of cellular senescence to be used as target for anticancer therapy.

1. The Biology of Senescence

Over 40 years ago, Hayflick [1] established that human diploid fibroblasts show a spontaneous decline in growth rate in continuous culture related not to elapsed time but to an increasing number of population doublings, eventually terminating in a quiescent but viable state now known as *replicative senescence*. These cells show a flat, enlarged morphology with low pH β-gal activity, are commonly multinucleated, and are irresponsive to mitogens or apoptotic stimuli. Similar behaviour has since then been observed in a wide variety of normal cells, and it is now widely accepted [2] that normal human somatic cells have an intrinsically limited proliferative lifespan, even under ideal growth conditions. Moreover, the senescent phenotype is associated with a typical gene-expression profile [3–5]. Cells displaying characteristics of senescent cells, however, can be observed in response to other stimuli, such as oncogenic stress, DNA damage, or cytotoxic drugs [6].

Cells displaying senescent characteristics have not only been observed in cell culture but also in their maternal tissue environment. A number of reports have related reduced cellular lifespan with metabolic disease, stress sensitivity, progeria syndromes, and impaired healing, indicating that entry into cellular senescence may contribute to human disease. Indeed, it has been suggested that cellular senescence is in part responsible for the pathogenesis of a number of human diseases, such as atherosclerosis, osteoarthritis, muscular degeneration, ulcer formation, Alzheimer's dementia, diabetes, and immune exhaustion.

Most cancers contain cell populations that have escaped the normal limitations on proliferative potential. This capability, known as *immortality*, contrasts with the limited lifespan of normal somatic cells. It has therefore been proposed that cellular senescence is a major tumor suppressor mechanism that must be overcome during tumorigenesis [2].

The kinetics of replicative senescence do not show an abrupt arrest of the whole population, but a gradual decline in the proportion of dividing cells [7], the exact timing of which varies between both cell types and sister clones [8]. This behaviour is best explained as the result of (i) an intrinsic control mechanism linked to elapsed cell divisions—*the senescence clock*—which progressively desensitises the cell-cycle machinery to growth factor stimulation, together with

(ii) a stochastic component probably having the same (still unknown) basis as that observed in immortal cells under conditions of growth factor restriction. Stem cells can give rise to differentiated progeny and are capable of autorenewal. In some renewing tissues, stem cells undergo more than 1000 divisions in a lifetime with no morphological signs of senescence [8]. This indicates that at a certain point of lineage differentiation, cells activate the senescence clock that ultimately induces cell senescence through a series of effectors.

More recently, the finite number of divisions—referred to as the "*Hayflick limit*"—was attributed to the progressive shortening of chromosomal ends. *Telomere shortening* is considered to be the most probable molecular mechanism explaining the existence of such a senescence clock controlling replicative senescence [9, 10]. Eukaryotic cells cannot replicate the very ends of their chromosomes, the telomeres, resulting in shortening their lengths with every cell division until they reach a critical threshold, at which point cells stop replicating [11]. However, enforced replication despite short telomeres ends in high chromosomal instability and apoptosis, a process known as *crisis*. Many other mechanisms, however, have been also proposed (Table 1).

Senescent cells display molecular markers characteristics of cells bearing double-strand breaks. These markers include nuclear foci of phosphorylated histone H2AX and the localization at double-strand break sites of DNA-repair and DNA-damage checkpoint factors, such as 53BP1, MDC1, and NBS1 [12, 13]. Senescent cells also contain activated forms of the DNA-damage checkpoint kinases Chk1 and Chk2. These and other results suggest that telomere shortening initiates senescence trough a DNA-damage response. This will explain why other DNA-damaging stresses, such as culture shock, might initiate senescence without telomere involvement. The initiation of senescence triggers the generation and accumulation of distinct heterochromatic structures known as senescence associated heterochromatic foci (SAHF). The formation of SAHF coincides with the recruitment of heterochromatic proteins and the pRB tumor suppressor to E2F-responsive promoters. SAHF accumulation is associated with stable repression of E2F target genes and does not occur in reversibly arrested cells. SAHF formation and promoter repression depend on the integrity of the pRb pathway [14]. These results provide an explanation for the stability of the senescent state.

Consistent with a role in aging, senescent cells accumulate with age in many rodent and human tissues [15]. Moreover, they are found at sites of age-related pathology, including degenerative disorders such as osteoarthritis and atherosclerosis [15] and hyperproliferative lesions such as benign prostatic hyperplasia [16] and melanocytic naevi [17]. A limited number of cell culture and mouse xenograft studies support the idea that senescent cells secrete factors that can disrupt tissue structure and function and promote cancer progression [18–20]. Recent studies on the senescence-associated secretory phenotype (SASP) of human and mouse fibroblasts show that it is conserved across cell types and species and that specific secreted factors are strong candidates for stimulating malignant phenotypes in neighboring cells [21–23].

The idea that a biological process such as cellular senescence can be both beneficial (tumor suppressive) and deleterious (protumorigenic) is consistent with a major evolutionary theory of aging termed antagonistic pleiotropy [23]. The SASP may be the major reason for the deleterious side of the senescence response [24].

In addition to telomere dysfunction, cellular senescence can be elicited by other types of stress, including oncogene activation [25]. This phenomenon is not observed for oncogenic RAS exclusively; many—but not all—of its effectors, including activated mutants of RAF, MEK, and BRAF, were shown to cause senescence as well [26–29]. Some oncogenes, such as RAS, CDC6, cyclin E, and STAT5 which induce senescence also trigger a DNA-damage response (DDR), which is associated with DNA hyperreplication and appears to be causally involved in oncogene-induced senescence (OIS), *in vitro* [30–33]. During most of the last decade, OIS has been studied predominantly in cell culture systems, triggering a long debate as to whether or not OIS corresponds to a physiologically relevant phenomenon *in vivo*. In favour of OIS representing an *in vitro* phenomenon only is that artificial conditions, such as the use of bovine serum and plastic dishes, as well as the presence of supraphysiologic O2, generate a stress signal that at the very least contributes to triggering a cellular senescence response [34, 35]. However, conversely, senescence bypass screens have identified several genuine human oncogenes, including TBX2, BCL6, KLF4, hDRIL, BRF1, and PPP1CA [36]. Furthermore, virtually all human cancers lack functional p53/pRB pathways, two key senescence-signalling routes [37], and often carry mutations in sets of genes, which are known to collaborate *in vitro* in bypassing the senescence response.

2. Effector Pathways

Cellular senescence pathways are believed to have multiple layers of regulation, with additional redundancy built into these layers [38]. On the basis of the complementation studies, there are at least four senescence genes or pathways. There are, however, many more chromosomes that can induce senescence than there are senescence complementation groups. Furthermore, there are some immortal cell lines that have been assigned to multiple complementation groups [39]. This indicates that in any one immortal cell line, there are probably multiple senescence genes/pathways that are abrogated [40]. Many of the functional studies, where a putative senescence gene is overexpressed in cells, indicate that although multiple genes/pathways may be abrogated in a particular cell line, as little as one gene/pathway is required for repair and subsequent reversion to senescence.

Pathways known to regulate cellular senescence/immortalisation, including the p16INK4a/pRB pathway, the p19ARF/p53/p21CIP1/WAF1 pathway, and the PTEN/p27KIP1 pathway, are reviewed in [36, 41–44]. Other genes that have been shown to induce a senescence-like phenotype include PPP1A [45], SAHH [46, 47], Csn2, Arase and BRF1 [48], PGM [49], IGFBP3 and IGFBPrP1 [50], PAI-1 [51, 52], MKK3 [53], MKK6 [53, 54], Smurf2 [55], and HIC-5 [56]. All these genes have shown to be related to human

Table 1: Cellular clock driving senescence hypothesis.

Cellular clock	Cause	Molecular readout
Error-catastrophe theories		
Somatic mutation accumulation	Metabolism/oxygen free radicals	Altered protein function, DNA damage
Mitochondrial DNA mutation	Oxygen free radicals	Altered mitochondrial function
Posttranslational modification of proteins	Oxidation, glycosylation, acetylation, methylation, and so forth	Altered function of proteins
Altered proteolysis	Errors in proteolysis machinery	Accumulation non functional proteins
Deterministic theories		
Telomere shortening	no replication of the telomere ends	DNA damage, exposure ends of telomeres, Liberation regulatory proteins, and so forth
Changes in heterochromatin domains		changes in transcription
Changes in DNA methylation		changes in transcription
Codon restriction	Switching codon preferences in early development, restrict availability later In life	Altered protein synthesis
Terminal differentiation	Senescence is a form of terminal differentiation genetically controlled	

Several hypotheses for cellular clocks driving senescence have been proposed. Most of them lay into error-catastrophe theories, suggesting that senescence is a byproduct of cell living, and deterministic theories, suggesting a genetic program for cellular senescence. Some of the most representative theories are collected in this table.

tumorigenesis. However, all these genes and their pathways, as indicated earlier, can act in sequential steps conforming a well-regulated process.

Two major effector pathways have been directly related to senescence: the p14ARF/p53/p21 pathway and the INK4/CDK/pRb pathway [57] (Figure 1). The absence of p53 function induced by dominant negative mutants, specific p53 antisense mRNA, oligonucleotides, or viral oncoproteins (such as SV40 T antigen or HPV16 E6) is sufficient to substantially extend the lifespan of several cell types in culture [58]. Consistent with this, senescence is associated with a switch-on of the transactivation function of p53 in culture [59]. Coincident with telomere shortening, DNA-damage checkpoint activation, and associated genomic instability, p53 is also activated *in vivo*[60]. Deletion of p53 attenuated the cellular and organismal effects of telomere dysfunction, establishing a key role for p53 in the shortening response [60].

Other p53 regulatory proteins are also involved in senescence (Figure 1). MDM2 protein has p53 ubiquitin ligase activity and forms an autoregulatory loop with p53 [61]. Overexpression of MDM2 targets p53 for degradation and induces functional-p53 loss [62]. The product of another gene upregulated in senescence—p14ARF—can release p53 from inhibition by MDM2 and cause growth arrest in young fibroblasts [62]. Seeding mouse embryonic fibroblasts (MEFs) into culture induces the synthesis of ARF protein, which continues to accumulate until the cells enter senescence [63]. MEFs derived from ARF-disrupted mice [63] or wild-type fibroblasts expressing an efficient ARF antisense construct [64] are also efficiently immortalised. Concomitant with this observation, overexpression of MDM2 in naïve MEFs produces efficient immortalisation [64].

Activation of p53 induces the upregulation of the cyclin-dependent kinase (CDK) inhibitor p21WAF1, which has a direct inhibitory action on the cell-cycle machinery [37] and correlates well with the declining growth rate in senescing cultures. In mouse embryo fibroblasts, however, the absence of p21WAF1 does not overcome senescence [65, 66]. This suggests that at least one additional downstream effector is needed for p53-induced growth arrest in senescence. In contrast, a different behaviour is observed in human cells, where elimination of p21 by a double round of homologous recombination is sufficient to bypass senescence [67]. Other p53 effectors might be also involved, such as 14-3-3 and GADD45, which inhibit G2/M transition or downregulation of Myc [68] (Leal and Carnero, Unpublished results).

The retinoblastoma tumor suppressor pathway, pRb, has also been related to senescence (Figure 1). Overexpression of pRb, as well as some of the regulators of the pRb pathway such as CDK inhibitors, leads to growth arrest mimicking the senescent phenotype [26]. Moreover, inactivation of pRb by viral oncoproteins such as E7, SV40 large T antigen, and E1A leads to extension of lifespan [69–71]. Other members of the pocket protein family comprising pRb, p130, and p107 may also be involved. In MEFs, p130 levels decrease with population doublings and MEFs from triple pRb, p130, and p107 knockout mice are immortal [72]. Nevertheless, since a certain degree of complementation has been observed among the pocket protein family [72], it is difficult to assess the role of each protein in replicative senescence.

Given that p16INK4a functions to inhibit the inactivation of pRb by CDKs [73], a loss of functional p16INK4a may be expected to have similar consequences with the loss of functional pRb. Several types of human cells accumulate p16INK4a protein as they approach senescence [74]. Senescent fibroblasts may contain p16INK4a levels at least 40-fold

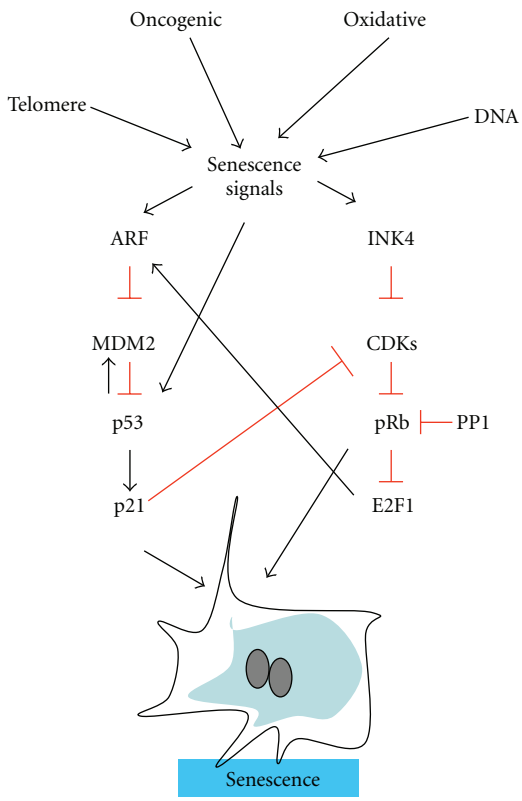

Figure 1: Scheme representing the senescence effector pathways crosstalk.

greater than early passage cells. The deletion of p16INK4a is common in immortalised tumor cell lines [75], and several nontumorigenic *in vitro* immortalised cell lines also lack functional p16INK4a protein. Expression of p16INK4a-specific antisense in naïve MEFs increases the probability of immortalisation of these cells [64]. In accordance with this observation, mice cells which are made nullizygous for p16INK4a by targeted deletion undergo immortalisation more readily than normal control cells [76, 77] although they show normal senescence kinetics. Knockout mice for p16INK4a proteins develop normally to adulthood and are fertile, indicating that the individual INK4 proteins are not essential for development. p16INK4a deficiency, however, results in a low susceptibility to spontaneous tumor development and increased tumor susceptibility under specific carcinogenic protocols [76, 77]. A crosstalk among the different pathways involved in senescence has been found. This crosstalk might ensure the correct functioning of the senescence program. Moreover, genes such as *myc* that are involved in all the pathways are able to bypass senescence in human primary cells. Myc can bypass CDK4/6 inhibition by activating CDK2-cyclinA/E complexes and inducing the Cdk-activating phosphatase Cdc25A [78]. Moreover, *myc* induces degradation of p27, thus influencing the inhibitory effects of PTEN. Finally, expression of myc induces telomerase activity by activating the transcription of the catalytic subunit [79]. The overall result is a single step immortalisation of human cells induced by myc gene amplification [80].

Over all steps, DNA methylation regulates expression of senescence genes, with the capability of controlling the process [44]. In human cancers, the silencing of tumour suppressor genes through aberrant DNA methylation of the CpG island(s) in promoters in these genes is a common epigenetic change [81]. There are an assortment of pathways from which genes have been shown to be hypermethylated in cancer cells, including DNA repair, cell-cycle control, invasion, and metastasis. The tumour suppressor genes BRCA1, p16INK4a, p15INK4b, p14ARF, p73, and APC are among those silenced by hypermethylation although the frequency of aberrant methylation is somewhat tumour-type specific. Recently, we found S-adenosylhomocysteine hydrolase (SAHH) [46], which has also been previously identified in an independent short hairpin RNA (shRNA) screening [82], the inactivation of which confers resistance to both p53- and p16(INK4)-induced proliferation arrest and senescence. SAHH catalyzes the hydrolysis of S-adenosylhomocysteine to adenosine and homocysteine. In eukaryotes, this is the major route for disposal of S-adenosylhomocysteine formed as a common product of each of the many S-adenosylmethionine-dependent methyltransferases, therefore, regulating the methylation processes. Interestingly, SAHH inactivation inhibits p53 transcriptional activity and impairs DNA-damage-induced transcription of p21(Cip1). SAHH messenger RNA (mRNA) was lost in 50% of tumour tissues from 206 patients with different kinds of tumours in comparison with normal tissue counterparts. Moreover, SAHH protein was also affected in some colon cancers [46, 47].

3. Clinical Implications

The implication of senescence as a barrier to tumorigenesis first comes from the realisation that a limited number of duplications necessarily reduces the possibility of tumor growth. However, the proliferative lifespan before reaching the Hayflick limit could be sufficient to generate a tumor mass greater than that required for lethality. This argument fails to take into account the existence of ongoing cell death and differentiation within a tumor and the occurrence of clonal selection driven by different senescence barriers or barriers unrelated to senescence. Finally, a clinically significant cancer can be composed of entirely mortal, presenescent cells if the cell of origin has a sufficient proliferative lifespan, and the tumor develops with few successive clonal expansion steps and/or with a low cell death rate. Even with these examples, however, senescence may of course still be a significant barrier to the recurrence of tumors from the small number of residual cells remaining after therapy.

As mentioned, several studies *in vivo* show that oncogene-induced senescence provides a bona-fide barrier to tumorigenesis. Michaloglou and coworkers [83] have shown that an oncogenic BRaf can induce senescence in fibroblasts and melanocytes and that human nevi display markers of senescence. Therefore, sustained exposure of melanocytes to aberrant mitotic stimuli provokes senescence after an initial proliferation burst. Collado and coworkers [84] identified senescent cells *in vivo* after generating new

senescence biomarkers from array studies. Using conditional Kras-val12 mice strains, they observed senescence markers to be predominant in premalignant lesions of the lung and pancreas, but not in those that have progressed to full-blown cancers. Direct evidence that hyperproliferative signals can trigger a program of permanent arrest *in vivo* have been provided in a transgenic model conditionally expressing E2F3 in the pituitary gland [85]. E2F3 induced hyperplasias that failed to progress because the cells became insensitive to further mitogenic signals. This insensitivity correlated with the appearance of senescence markers and a terminally arrested cellular state. Disruption of PTEN in mice also produces hyperplastic conditions analogous to prostatic intraepithelial neoplasia (a precancerous lesion in men). These lesions display senescence markers [86]. Loss of p53 prevents senescence in response to PTEN ablation and cooperates to produce invasive prostate carcinomas. These results are consistent with the notion that senescence actively limits malignant conversion.

In human fibroblasts in culture, the senescence program involves chromatin reorganisation involving H3 methylation at the Lys9 residue concomitant with the recruitment of heterochromatin proteins to some proliferation-related genes. Braig and coworkers [87] found that disruption of Suv39h1 methyltransferase, which methylates the Lys9 residue of H3, blocked ras-induced senescence and accelerated ras-induced lymphomagenesis in mice. Interestingly, Suv39h1-expressing tumors responded through senescence to chemotherapy; however, Suv39h1-null tumors did not show any senescent response but still maintained the apoptotic response. Treating ras transgenic mice with DNA-methyltransferase or histone deacetylase inhibitors, which mimic the effects of Suv39h1 disruption, accelerated ras-induced tumorigenesis.

The concept of cancer being a disease whereby cells have lost the ability to senesce leads to a critical evaluation of the benefits that can be achieved for cancer diagnosis, and therapy through the knowledge surrounding molecular pathways (both genetic and epigenetic in origin) that induce senescence. Until just a few years ago, it was accepted that tumor cells were no longer capable of senescence. Today, however, it is accepted that neoplastic cells can be forced to undergo senescence by genetic manipulations and by epigenetic factors, including anticancer drugs, radiation, and differentiating agents [26, 88]. However, although not fully studied *in vivo*, it has been shown that senescent cells might increase the oncogenic potential of tumor cells. Therefore, it will be necessary to understand the contribution of senescent stromal cells to tumors, before applying drug-induced senescence program to tumors.

Immortalising defects are recessive and can be blocked by imposing the process of senescence [89]. The first approach to inducing senescence to tumor cells was through somatic cell fusion. These studies identified four senescence-determining complementation groups. In recent years, it has been found that different tumoral cell lines show cellular growth arrest along with senescence markers after the genetic expression of tumor suppressor genes commonly involved in senescence, such as p53, p21, p16, pRb, or p21 [90]. Similarly, the restoration of cellular levels of p53 in a cell line conditionally immortalised by p53 antisense expression induces growth arrest with a senescent phenotype [91]. Adenovirus vectors carrying CKIs (p16INK4a, p15INK4b, p21cip1, and p27kip1) as vehicles for delivery and expression are a powerful approach to examining therapeutic applications both *in vitro* and *in vivo*, with promising results [92]. When a 16-amino acid transmembrane carrier segment derived from the *Drosophila* antenappedia protein was linked to the third ankyrin repeat of the p16INK4a protein and inserted into cells, Rb-dependent G_1 arrest was observed. In a breast-derived cell line, the chimera containing the antennapedia peptide and the carboxyl-terminal residue of p21waf1 had higher specificity for CDK4/cyclin D than for CDK2/cyclin E and arrested the cells in G_1 phase [93].

These observations indicate that tumor cells maintain at least some of the components of the cellular senescence program, including terminal growth arrest. It is now clear that depending upon the cell proliferation kinetics of the tissue of origin, tumor development can be initiated by genetic events, causing either a block in terminal differentiation or/and inappropriate activation of growth stimulatory signaling pathways. The net result in both cases is the generation of a cellular clone capable of infinite expansion if it is not constrained by physical barriers or lack of blood supply. Schmitt and collaborators [94] convincingly showed that in a lymphoid mouse tumor model, an intact senescence pathway appears to be pivotal to the efficacy of cyclophosphamide, and its disruption makes tumor cells highly refractory to the drug. On the other hand, as mentioned, Suv39h1-expressing tumors responded to chemotherapy by inducing senescence. However, Suv39h1-null tumors did not show any senescent response but still maintained the apoptotic response. Suv39h1-null tumors with altered apoptotic response do not respond to therapy.

These results suggest that drug efficacy and tumor formation are not fully independent processes. Until recently, tumor formation and the development of drug resistance were thought to be independent processes. Mutations in factors that regulate tumor-suppressive fail-safe mechanisms, such as apoptosis and senescence, allow transformation. Chemotherapeutic compounds activate a separate set of effector pathways that eliminate malignant clones. Mutations in factors that are involved in these separate pathways inhibit the effect of chemotherapy to induce the effector programs to eliminate the tumors. Consequently, defects in antineoplastic fail-safe programs, even if required to allow for tumor formation, do not interfere with the effector program initiated by therapeutic agents. Nevertheless, preclinical data have provided evidence that key regulators, such as p53, participate in tumor prevention and drug action and that tumor mutations acquired during tumor development also confer chemoresistance [95]. Therefore, the "joint model" [96] proposes a functional overlap between the fail-safe and therapeutic effector programs, such that some of the mutations that allowed transformation can also confer chemoresistance by disabling drug effector programs.

The *in vitro* observation that DNA-damaging agents not only promote apoptosis but also induce cellular senescence [97, 98] indicates that genes that control senescence might

also determine treatment outcome. Using a MYC-driven mouse lymphoma model, p53 and p16INK4A were recently shown to control drug-induced senescence *in vivo* [94]. Drug-treated lymphomas with apoptotic defects were forced into senescence, and tumors that resumed growth frequently displayed defects in either p53 or p16INK4A. Importantly, drug-induced senescence was shown to contribute to long-term host survival after cancer therapy, as mice bearing lymphomas that were unable to enter senescence in response to therapy had shorter survival times. Notably, drug-inducible senescence is not a phenomenon that is restricted to a mouse lymphoma model, as tissue specimens taken from human breast tumors after chemotherapy also displayed typical features of cellular senescence [98].

Depending on the initiating oncogene, transformation relies on fail-safe defects that disrupt either apoptosis or senescence. There are a number of reports that drug-inducible senescence could become detectable only after apoptosis has been disabled [99]. It is conceivable that senescence occurs with much slower kinetics, serving as a "backup" fail-safe program in case the first-line response is corrupted. This is supported by sequential disruption of apoptosis- and senescence-controlling genes during tumor formation and subsequent therapy reported in human cancers [100, 101].

4. Senescence-Based Therapy

Different chemical agents can induce cellular senescence epigenetically. Treatment of primary cells with H_2O_2 or butyrate provokes early senescence [102]. Similar results were obtained after treatment with high doses of radiation and other damaging agents [102]. Interestingly, the treatment of different tumor cell lines with different chemotherapeutic agents, radiation, or differentiating agents induces irreversible growth arrest, with enzymatic and morphologic changes resembling those occurring during replicative senescence. Moderate doses of doxorubicine induced a senescent phenotype in 11 out of 14 tumor cell lines analysed, independently of p53 status [103]. A similar effect has been observed in lines from human tumors treated with cisplatin [104], hydroxyurea [105], and bromodeoxyuridine [106]. In mammary carcinoma cell lines treated *in vitro* and *in vivo* with differentiating agents, terminal proliferative arrest with minimal toxicity for normal cells has been observed [107].

The propensity of tumor cells to undergo senescence in response to different kinds of damage induced by commonly used chemotherapeutic treatments was compared on cell lines from different tumor origins [66]. Under equitoxic doses, the strongest induction of a senescent phenotype was observed with DNA-interacting agents (doxorubicin, aphidicolin, and cisplatin), and the weakest effect was observed with microtubule-targeting drugs (Taxol and vincristine). A medium response was observed with ionising radiation, cytarabine, and etoposide. Induction of senescence by the drugs was dose-dependent and correlated with the growth arrest observed in the cultures [102, 105–107]. The drug-induced senescent phenotype in tumor cells was not associated with telomere shortening and was not prevented by the expression of telomerase [108].

Drug-induced senescent phenotypes have been confirmed *in vivo* ([94] and references therein). A study from Poele et al. [98] revealed the correlation between chemotherapeutic treatment in clinical cancer and the senescence response. In frozen samples from breast tumors treated by neoadjuvant chemotherapy (cyclophosphamide, doxorubicin, and 5-fluoracyl), senescent markers were detected in 41% of samples from treated tumors. Normal tissue was negative, suggesting that the chemotherapy-induced senescence was a specific response of tumor cells. Interestingly, senescence response was associated with wild-type p53 and the increased expression of p16. Similarly, in treatment-induced senescence, murine Eμ-myc lymphoma response required wild-type p53 and p16 [94].

The Chk2 kinase is a tumor suppressor and key component of the DNA damage checkpoint response that encompasses cell-cycle arrest, apoptosis, and DNA repair. It has also been shown to have a role in replicative senescence resulting from dysfunctional telomeres. Some of these functions are at least partially exerted through activation of the p53 transcription factor. High-level expression of Chk2 in cells with wild-type p53 led to arrested proliferation with senescent features [109]. These were accompanied by p21 induction, consistent with p53 activation. However, Chk2-dependent senescence and p21 transcriptional induction also occurred in p53-defective cells. Small interfering RNA-mediated knockdown of p21 in p53-defective cells expressing Chk2 resulted in a decrease in senescent cells. DNA-damage response is also induced by cytokines, such as interferons. Sustained treatment with interferon triggers a p53-dependent senescence program. Interferon-treated cells accumulated gamma-H2AX foci and phosphorylated forms of ATM and CHK2. The DNA-damage-signalling pathway was activated by an increase in reactive oxygen species (ROS) induced by interferon and was inhibited by the antioxidant N-acetyl cysteine. RNA interference against ATM inhibited p53 activity and senescence in response to betainterferon [110]. It seems that p53 activation is the primary response to DNA damage, but its absence does not preclude a response with a senescent phenotype.

Comparable to p53, which functions as a fail-safe mediator of DNA-damage response, the p16 inhibitor has been implicated in both response to DNA-damage and control of stress-induced senescence. Although the molecular mechanism used by p16 to control not only temporary but permanent cell-cycle arrest is unclear, p16 responds to DNA-damage in a delayed manner and appears to be indispensable for the maintenance of cellular senescence [94, 98]. A synthetic inhibitor of CDK4, possibly mimicking the role of p16, produced a DNA-damage-independent form of senescence in cells lacking p16 expression and inhibited the growth of tumors in mice. Use of siRNAs to inactivate the papilomavirus oncoproteins E6 and E7, which deregulate p53 and pRb, restored cellular senescence in cervical cancer cells. Introduction of E2 protein, a negative regulator of E6 and E7, induced senescence in almost all cervical carcinoma cells tested. The effect of E2 was not accompanied by

telomere shortening, nor was it prevented by telomerase expression. Induction of senescence by E2 was associated with p53 stabilisation and strong induction of p21, and it was prevented by using p21 antisenses [111].

Many observations indicate that p53, p21, and p16, which regulate cellular senescence, play an important role in treatment-induced senescence of tumor cells. Since these genes are commonly lost in human tumors, we can expect that most human tumors do not respond by undergoing senescence. However, this is not the case. Chemotherapeutic drugs induced senescence in p53- and p16-defective tumor cell lines [107]. *in vivo*, 20% of tumors undergoing senescence after treatment showed p53 mutations [98]. We have been able to induce senescence with several chemotherapeutic drugs in p53-null cells independently of p16 (Moneo and Carnero, unpublished). We have found that the induced senescence correlated with p53-independent p21 induction. Moreover, knockout of p53 or p21 in HCT116 cells decreased but did not abolish cellular senescence. Hence, p16, p53, and p21 might acts as positive regulators but are not absolutely required for this response. Other related tumor suppressors, such as p63 or p73, could be involved, and their role in drug-induced senescence should be explored.

Treatment with 6-anilino-5,8-quinoline quinone, a previously described inhibitor of guanylate cyclase, induced cellular senescence [112]. Microarray analysis revealed that this compound induced the Cdk inhibitor p21WAF1 in a p53-independent manner. Furthermore, p21, though not p53, was required for inhibition of proliferation by the drug. The lack of p53 involvement suggests that this compound acts independently of DNA-damage induction. Growth inhibition was also observed in malignant melanoma and breast cancer cell lines. Functional inactivation of the retinoblastoma tumor-suppressor protein converted 6-anilino-5,8quinolinequinone-induced growth arrest into apoptosis. Tumor cell senescence was also found to be induced by TGFb and by differentiating agents including retinoids. The induction of senescence has been analyzed in more detail with derivatives of vitamin A, which regulate cell growth and differentiation through their effects on gene expression [113].

A prominent feature of immortal cells is a resistance to oxidative stress. By contrast, primary cells undergo senescence when grown for extended periods in tissue culture or exposed to agents that increase production of reactive oxygen species. It has been also found that enhanced glycolysis enables primary mouse cells to avoid senescence by protecting them from oxidative damage, and that immortal ES cells have intrinsically high levels of glycolysis [49]. siRNA downregulation of PGM, an enzyme regulating glycolytic flux, triggers senescent phenotype recovery in tumor cells. Therefore, regulation of glycolysis and/or ROS production might be interesting approaches to the induction of senescence in tumors.

5. Telomerase Inhibitors

Restoration of the limited replicative potential in tumors as an anticancer therapy has been widely examined through the targeting of telomerase activity. Early studies indicated that telomerase activity is absent in somatic tissues and present in most cancers [114]. It was, therefore, reasonable to suggest that inhibition of telomerase activity, with a consequent shortening of telomeres and arrest of cell growth, might be an effective treatment of cancer.

Several different approaches to telomerase inhibition have been adopted to prevent the multiplication of neoplastic cells in culture. These have included treatment of the cells with the alkaloid berberine, transfection with an antisense vector for the human telomerase RNA component, introduction of a catalytically inactive, dominant-negative mutant of human telomerase reverse transcriptase, and low-level expression of a mutant-template telomerase RNA. All of the treatments inhibit the multiplication of neoplastic cells in culture, and those tested also inhibit tumor formation in mice. It should, however, be noted that the transfection of neoplastic cells with telomerase-inhibitory vectors was accomplished either in culture before their inoculation into mice or (in the case of the antisense RNA) through daily injections into the growing tumors for 7–14 days. No attempt was made to assess the long-term systemic injection of vectors into mice carrying the tumors, leaving the matter of effects on normal cell function yet to be investigated. Telomere shortening has been observed in the treated tumor cells and correlates with inhibition of their proliferation [115]. The expression of threshold levels of mutant-template telomerase RNA decreases cell viability despite the retention of endogenous wild-type telomerase RNA, wild-type telomerase activity, and unaltered stable telomere lengths.

One reported advantage of telomerase inhibition as a cancer chemotherapy was that it was not expected to induce cancer in normal cells, as telomerase activity is closely associated with advanced tumors [114]. Knockout of the gene for the RNA component of telomerase in mice does not, however, prevent either tumor formation or neoplastic transformation of cells cultured from such mice [116, 117]. The incidence of spontaneous malignancies is even higher than that of normal mice [117]. A similarly increased risk of cancer is found in individuals with the inherited syndrome dyskeratosis congenita (DKC) that is caused by a mutation in one of the components of telomerase, such that individuals with DKC are deficient for telomerase activity [118]. This increased incidence of cancer is presumably a result of end-to-end fusion of chromosomes destabilized by inadequate capping [119]. There is, therefore, the distinct possibility that systemically introduced inhibition of telomerase in cancer chemotherapy would increase the frequency of chromosome aberration and the risk of secondary cancers in normal tissue, particularly when p53 mutations already exist [120].

The situation became more complicated when it was found that telomerase activity is present in stem cells and dividing transit cells of renewing tissues, and even when cell division is induced in tissues conventionally regarded as quiescent. Thus, it seems likely that all tissues with cells able to divide have either ongoing or potential telomerase activity with a capacity for telomere maintenance during cell division.

Treatment of cancer by telomerase inhibition is still considered potentially valid for several reasons that might mitigate side effects on normal tissues [121]. One reason is that telomeres are longer in normal tissues than in most cancers, and treatment of tumors can be designed to end before telomere depletion in normal tissues [120]. However, further studies with this approach must be carried out to protect renewing tissues, such as intestine, epidermis, and hematopoietic tissue, in which stem cells and transit cells are constantly dividing at a high rate.

It is expected that telomerase inhibitors will be developed that have far fewer side effects than many of the cancer chemotherapeutic agents that are currently available. Individuals with DKC show features that include abnormalities of the skin and nails and eventual failure of proliferation in the bone marrow, which indicates that telomerase is required for normal proliferative capacity in these somatic tissues. Despite this telomerase deficiency, onset of pancytopaenia in these individuals does not occur until a median age of 10 years, which indicates that it might be relatively safe to administer telomerase inhibitors continuously for several years.

Telomerase inhibitors will not be useful, however, for the minority of tumors that use ALT. In addition, in telomerase-positive tumors it can be predicted that effective telomerase inhibitors will exert an extremely strong selection pressure for the emergence of resistant cells that use the ALT mechanism. Activation of ALT was not observed in cell-culture experiments in which telomerase-positive cell lines were treated with small-molecule inhibitors of telomerase or dominant-negative TERT mutants [122], indicating that it is not a high-frequency event. This might be a problem, however, in clinically significant tumors containing as many as 10^{12} cells. Development of ALT inhibitors may, therefore, be necessary. For tumors that use both telomere maintenance mechanisms, treatment might need to be initiated with a combination of telomerase and ALT inhibitors. Both telomerase and ALT must access the telomere, but how this might be achieved is at present unknown. A further possibility could be to identify molecular targets for simultaneous inhibition of both telomere maintenance mechanisms, since proteins involved in telomerase-based and ALT-mediated events may overlap.

6. Concluding Remarks

The concept of senescence as a barrier to tumorigenesis, either by natural replicative limits or as stress-induced senescence leads to a critical evaluation of the benefits that can be achieved for cancer diagnosis and therapy. It is accepted that neoplastic cells can be forced to undergo senescence by genetic manipulations and by epigenetic factors, including anticancer drugs, radiation, and differentiating agents. These senescent features can be imposed even in the absence of the two functional effector pathways, p53 and pRb. This lead to speculate the possible benefits of inducing an unspecific senescence program to stop tumor growth. This might be of value added to surgery or radiation; however, possible escape from a yet uncontrolled senescent phenotype and the unknown effect *in vivo* of senescent stromal cells might hamper these efforts. A more controlled induction of senescence through the knowledge of pathways involved and targeting specific targets might rend a less profitable but more valued effort. The use of tools such as oncolytic viruses driven by telomerase promoters might also work better than direct inhibition of the protein. However, it is too early and more research is needed in the basic understanding of the molecular mechanisms driving the senescence processes before embarking patients in such therapy.

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