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Novel Insights Into The Contribution Of Cellular Senescence To Cancer Therapy: Reversibility, Dormancy And Senolysis.

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NOVEL INSIGHTS INTO THE CONTRIBUTION OF CELLULAR SENESCENCE TO CANCER THERAPY: REVERSIBILITY, DORMANCY AND SENOLYSIS.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Dedication

To my younger brother Rashad, whose innocence and brilliance have been my inspiration to have hope, faith and be alive. To my mother Iman and father Nizar, whose support and encouragement keep me always goal-driven and determined.

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research that we all do here in MCV. My passion for science has grown tremendously and made me more determined to continue working in academia and medical sciences. The advanced knowledge I gained in VCU is the main driver for me to establish my own line of research back home. I would also like to thank The Hashemite University for supporting my graduate studies as without their financial and logistic support I would not have accomplished this.

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Abbreviations

53BP1	p53 Binding Protein 1
ADCC	Elimination by Antibody-Dependent Cell-Mediated Cytotoxicity
ALK	Anaplastic Lymphoma Kinase
AMPK	5' Adenosine Monophosphate-Activated Protein Kinase
ATG	Autophagy Related
ATM	Ataxia-Telangiectasia Mutant
ATR	Ataxia telangiectasia and Rad3 related
Baf	Bafilomycin A1
Bak	Bcl-2 Homologous Killer
Bax	Bcl-2-associated X Protein
Bcl-2	B-Cell Lymphoma 2
Bcl-w	Bcl-2-Like Protein 2
Bcl-XL	B-Cell Lymphoma-Extra Large
BrdU	5-bromo-2'-deoxyuridine
BTG1	B-Cell Translocation Gene 1
C	Control
C/EBP	Ccaat-Enhancer-Binding Proteins
C12FDG	5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside
c-AMP	Cyclic Adenosine Monophosphate

CDK	Cyclin-dependent Kinase
CDKI	Cyclin-dependent Kinase Inhibitor
cDNA	Cyclic Deoxyribonucleic Acid
Chk2	Checkpoint kinase 2
CO2	Carbon Dioxide
CQ	Chloroquine
CRC	Colorectal Cancer
CREB	cAMP Response Element-binding Protein
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/Cas9
CXCl1	Chemokine (C-X-C Motif) Ligand 1
CXCR2	C-X-C Motif Chemokine Receptor 2
CYR61	Cysteine Rich Angiogenic Inducer 61
D	Day
DAP3	Death-associated Protein 3
DAPI	4′-6-Diamidino-2-Phenylindole
DDR	DNA damage Repair Response
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay

ETO	Etoposide
FACS	Fluorescence-Activated Cell Sorting
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
H3K9Me3	Trimethylation of Histone 3 Lysine 9
HP1	Heterochromatin Protein 1
HSLCI	High-Speed Live Cell Interfermoetry
HSP90	Heat Shock Protein 90
IACUC	An Institutional Animal Care and Use Committee
ICD	Immunogenic Cell Death
IGFBP3	Insulin-like Growth Factor Binding Protein-3
IL1α	Interlukin-1 alpha
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-6R	Interleukin-6 Receptor
LC3B	Light Chain Microtubule-associated protein
LDH	Lactate Dehydrogenase
LLC	Lewis Lung Carcinoma
Mcl-1	Myeloid Cell Leukemia-1
MDM2	Mouse Double Minute 2 Homolog (MDM2)

MLL	Mixed Lineage Leukemia
MMP	Matrix Metalloprotease
mTOR	Mammalian Target of Rapamycin
NaCl	Sodium Chloride
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B
NK	Natural Killer
NOTCH1	Notch Homolog 1, Translocation-Associated (Drosophila)
NSCLC	Non-small Cell Lung Cancer
NSG	NOD Scid Gamma
OIS	Oncogene-Induced Senescence
p38MAPK	p38 Mitogen-Activated Protein Kinases
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate Buffered Solution
PCNA	Proliferating Cell Nuclear Antigen
PFA	Paraformaldehyde
PI	Propidium Iodide
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PTEN	Phosphatase and Tensin Homolog
qRT-PCT	Quantitative Real-Time Polymerase Chain Reaction
Rb	Retinoblastoma
RFP	Red Fluorescent Protein

RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SA-β-gal	Senescence-Associated β-galactosidase
SAHF	Senescence-Associated Heterochromatin Foci
SAMD	Senescence-Associated Mitochondrial Dysfunction
SASP	Senescence-Associated Secretory Phenotype
SCLC	Small-cell Lung Cancer
SD	Standard Deviation
SEM	Standard Error of the Mean
shRNA	Short Hairbpin RNA
siRNA	Short Interfering RNA
SQSTM1	Sequestosome 1
TGF-β	Transforming Growth Factor beta
TIS	Therapy-Induced Senescence
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WT	Wildtype

Abstract

Cellular senescence a specialized form of growth arrest that contributes to the pathogenesis of several aging-related disorders including cancer. While by definition tumor cells are considered immortalized, they can undergo senescence when exposed to conventional and targeted cancer therapy. Therapy-Induced Senescence (TIS) represents a fundamental response to therapy and impacts its outcomes. However, TIS has been considered a positive therapeutic goal since senescent tumor cells are expected to enter a state of permanent growth abrogation. In this work we examined the hypothesis that a subpopulation of senescent cells can re-acquire proliferative potential after a state of senescent dormancy, indicating that senescence is not obligatorily an irreversible process. Our observations indicate that H460 non-small cell lung cancer cells induced into senescence by exposure to etoposide, and enriched based on β-galactosidase staining and size, were shown to recover reproductive capacity, which was accompanied by resolution of the DNA-damage-response (downregulation of p53 and p21^{Cip1} induction), attenuation of the Senescence-associated Secretory Phenotype (SASP). To overcome the reservation that the newly dividing cells may not have been derived from the senescent population and in an effort to establish that escape from TIS is feasible, tumor cells induced into senescence by chemotherapy were enriched for senescence by flow cytometry; the subsequent division of senescent cells was demonstrable utilizing both real-time, live microscopy and High Speed Live Cell Interferometry (HSLCI). Furthermore, sorted senescent cells were observed to form tumors when implanted in immune deficient mice and with a significant delay in immunecompetent mice. As chemotherapy induced senescent cells have been identified in patient tumors, it is reasonable to propose that tumor cells that escape from senescence could contribute to disease recurrence. In addition, therapy-induced senescence could prove to reflect one form of tumor dormancy. Recently, ABT263 has been used

as a senolytic drug, effectively eliminating senescent cells from aging-related animal models. Here, we utilize ABT263 in a two-hit approach to eliminate senescent tumor cells that persistent after exposure to chemotherapy. ABT263 results in the killing of senescent tumor cells in a concentration-dependent manner and shifts the response towards apoptotic cell death. Furthermore, sequential administration of ABT263 interferes with the ability of senescent tumor cells to recover growth potential. These results indicate that senescent tumor cells can contribute to cancer relapse by acquiring proliferative properties and that senolytic therapy allows for the clearance of dormant senescent tumor cells and will potentially decrease cancer recurrence rates.

Chapter One: General Introduction

1.1. Historical Overview

The scientific perspectives on the replicative potential of cells in terms of cellular lifespan, aging and cancer have changed dramatically during the last century. This work should contribute to continuing efforts to understand the mechanisms that regulate stress, cell fate and responses to cancer therapy.

1.1.1. Early Viewpoints on Cell Immortality

Although it is well-established that somatic cells have a limited lifespan, this was not the predominant viewpoint on cultivated cells in the first half of the 20th century (1). Instead, it was thought that cells have the potential to divide indefinitely in culture, based in large part on the work of Nobel Laureate Alexis Carrel (2). In 1912, Carrel was able to establish a chicken cardiac fibroblast cell line *in vitro* that he and his colleague, Albert Ebeling, passaged in culture for more than 10 years (3). Carrel and Ebeling claimed that cells are immortal when isolated and allowed to grow under ideal culture conditions and that cellular aging was restricted to replicating cells in a multicellular organism (4). This proposition influenced other aging biologists like Raymond Pearl, who considered aging to be a process at the organismal level and thus, that cells can have an unlimited lifespan when grown separately from the living organism (2). Despite the growing consensus based on Carrel's research evidence, there were no laboratories that succeeded in growing cells indefinitely. For example, Haff and Swim attempted to grow rabbit cells in culture;

however, these cells died following 18 to 20 passages, a perplexing finding that was attributed to extraneous factors and technical complications (2). Similar findings were considered as unusual phenomena -- aberrations due to alteration in the cell culture media or confined to certain cell types (2).

1.1.2. The Hayflick limit

This longstanding paradigm of vertebrate cells growing indefinitely in culture was finally proven to be inaccurate in 1961 by Leonard Hayflick. Hayflick addressed this core issue by running a series of cell cultivations of normal human fibroblasts to show that these cells have a finite cell fate (5). For three years, Hayflick and his fellow Paul Morehead observed that fibroblasts eventually ceased to divide when passaged multiple times (5). Of course, at the time, Hayflick initiated these experiments with the mindset that Alexis Carrel was correct and hence, he was concerned that a technical issue concerning the preparation of his culture medium or dishes was the reason behind the inability of fibroblasts to continue dividing (6). To address this concern, he designed a co-culture experiment of *old* male fibroblasts (doubled for 40 times previously) with *young* female fibroblasts (doubled less than 10 times previously) (5). After a series of passages, the cell culture was almost entirely occupied by only the female cells, indicating that the old male cells had failed to continue proliferating (5). Certainly, this allowed Hayflick to confirm his preliminary findings that cells only divided for a certain number of times and that an experimental error was unlikely to account for his observation since the female cells were healthy and dividing. Initially, Hayflick described different phases of the life of a dividing cell in culture and stated that those which fail to proliferate after a number of doublings enter the phase III phenomenon[1] (5).

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[1] Phase I describes the initial culture, phase II describes doubling or proliferating cells while phase III describes growth arrested cells.

More importantly, Hayflick demonstrated that cell aging is not a product of time, as cryogenically preserved cells were able to divide normally even if kept frozen for years, indicating that cell aging is encoded genetically (7).

It is worth mentioning that Hayflick and Morehead's manuscript describing these findings was rejected by the Journal of Experimental Medicine, the journal that published most of Carrel's work, when they attempted to publish their findings. The rejection was based on the fact that their findings were not in line with the established dogma at the time (6). However, they succeeded in publishing their first report in the Journal of Experimental Cell Research, which later became one of the most highly cited papers in the history of modern science (5). Hayflick's findings invited other scientists to replicate the same findings and gradually a paradigm shift in cell biology took place. After a decade, Australian Nobel Laureate Macfarlane Burnet used the expression "the Hayflick limit" for the first time, which described the number of divisions a cell will undergo before it ceases to divide (8). Finally, multiple theories have been proposed to explain the mystery of Carrel's cells that divided for 34 years including that these cells might have undergone a replicative crisis or even transformation or perhaps were contaminated with an immortal cell line (9).

1.1.3 Telomere dysfunction and telomerase

Although Hayflick's findings were a breakthrough in cytogerontology, they were not explained until later in the 1970s. In 1972, James Watson described a phenomenon called the "end-replication problem" where DNA polymerases are incapable of fully replicating the lagging strand of a DNA double helix, always leaving a strip of unreplicated nucleotides at both ends of the linear molecule (10). Accordingly, the DNA molecule undergoes gradual shortening every time the cell

prepares to replicate its DNA in preparation for mitosis. This continuous attrition means that a small stretch of the genetic material will be lost with successive divisions, providing the first evidence that a deficiency in the DNA replication machinery could account for the inability of cells to divide indefinitely. It was later revealed that the linear ends of eukaryotic DNA are capped with short, repetitive sequences of nucleotides and that those "meaningless" repeats are lost as a sacrifice for the end-replication problem (11). These DNA caps were termed *telomeres* and their shortening eventually explained the inability of Hayflicks fibroblasts to continue dividing indefinitely (12). Telomere shortening was subsequently discovered to occur *in vivo* in tissue derived from aging humans (13).

The question that then comes to mind next is that of how immortal cells, such as pluripotent stem cells and cancer cells can actually divide indefinitely? It was later determined that the enzyme, *telomerase* is expressed in these cells and is responsible for elongating their telomeric ends (14,15). In fact, telomerase was shown to be expressed in almost 90% of cancers (16). Telomerase, or terminal transferase, is a reverse transcriptase enzyme that uses its own RNA template to elongate telomeres, thereby solving the end-replication problem (17). It was later shown that stable reintroduction of the telomerase catalytic subunit can stabilize the linear ends of normal human cells and is sufficient to considerably prolong their life span or even to achieve immortality (18).

The identification of telomeres and telomeric dysfunction was essential to understand the Hayflick limit. The telomeric ends of Hayflick's fibroblasts were gradually being eroded every time Hayflick passaged those cells and allowed them to divide. Consequently, we now fully understand that persistent telomeric dysfunction in the absence of telomerase robustly activates the DNA Damage Response (DDR) and leads to a stable, sustained growth arrested state routinely referred to as *cellular senescence* (19). We also now know that the phase III phenomenon fibroblasts that

Hayflick described were in fact "senescent" fibroblasts and that the finite lifespan of somatic cells is attributed to this one unique phenotype.

1.2 Cellular Senescence

1.2.1. The complexity of the definition

The word senescence is derived from the Latin word *senex* which refers to advanced age or an old individual. Traditionally, the term *cellular senescence* was coined to describe cells that ceased to divide following many doublings *in vitro* (7). However, gradually, the term took on further dimensions to describe a more complex cellular phenotype. Initially, the definition established an early and solid connection between the "old" or "aging" cells and their incapability to divide, in that persistent "senescent" cells must be in a growth arrested state. Furthermore, Hayflick concluded very soon after his initial experiments that phase III phenomenon cells undergo "irreversible functional decrements" that commit them into an "irreversible" cessation of growth (20). Hence, cellular senescence not only describes a phase of growth arrest that results from replicative exhaustion but that growth arrest must be permanent— the signature word in the definition of cellular senescence is "irreversible". Regardless of how a cell becomes senescent and what biological alterations that change entails, the change must be irreversible, and a reversion to the normal or pre-senescent stage is not feasible. It is difficult to pinpoint when exactly the irreversibility of senescence became the most essential characteristic of the phenotype, but there is a current overwhelming agreement that senescence describes an irreversible phenomenon and that is how it is distinguished from other cell fates (21).

Despite the fact that our current understanding of cellular senescence extends beyond the growth arrest described by Hayflick in the early 1960s, the insistence on the irreversibility of senescence

appears to be dogmatic. Judith Campisi, a lead scientist in gerontology and cancer research, describes senescence as an "irreversible arrest of cell proliferation (growth) that occurs when cells experience potentially oncogenic stress" (22). This definition was updated in 2016 but continued to include irreversibility of growth arrest as a hallmark of the senescent phenotype (23). Many others in the field seem to strongly agree. For example, Jerry Shay and Igor Roninson simply define senescence as a "signal transduction program leading to irreversible cell cycle arrest" (24). Clemens Schmitt refers to cellular senescence as an "irreversible cell-cycle arrest" which "reflects a safeguard program that limits the proliferative capacity of the cell exposed to endogenous or exogenous stress signals" (25). Collectively, these established opinions in the field place irreversibility at the heart of the definition of cellular senescence.

On the other hand, despite this general agreement on the irreversibility of senescence, there has been accumulating evidence that senescence might not be obligatorily a terminal cell fate. Drs. Lynne Elmore and my adviser David Gewirtz together published observations suggestive of the reversibility of senescence in tumor cells exposed to cancer chemotherapy, specifically doxorubicin (26). It is noteworthy in this context that it has become well-established, as we will discuss later in detail, that malignant cells retain the potential to undergo premature, or accelerated, senescence in response to different forms of stress, such as that induced by cancer chemotherapeutic drugs and radiation, which is the focus of this dissertation. Several laboratories have either shown or suggested that senescence might be reversible, and that under certain
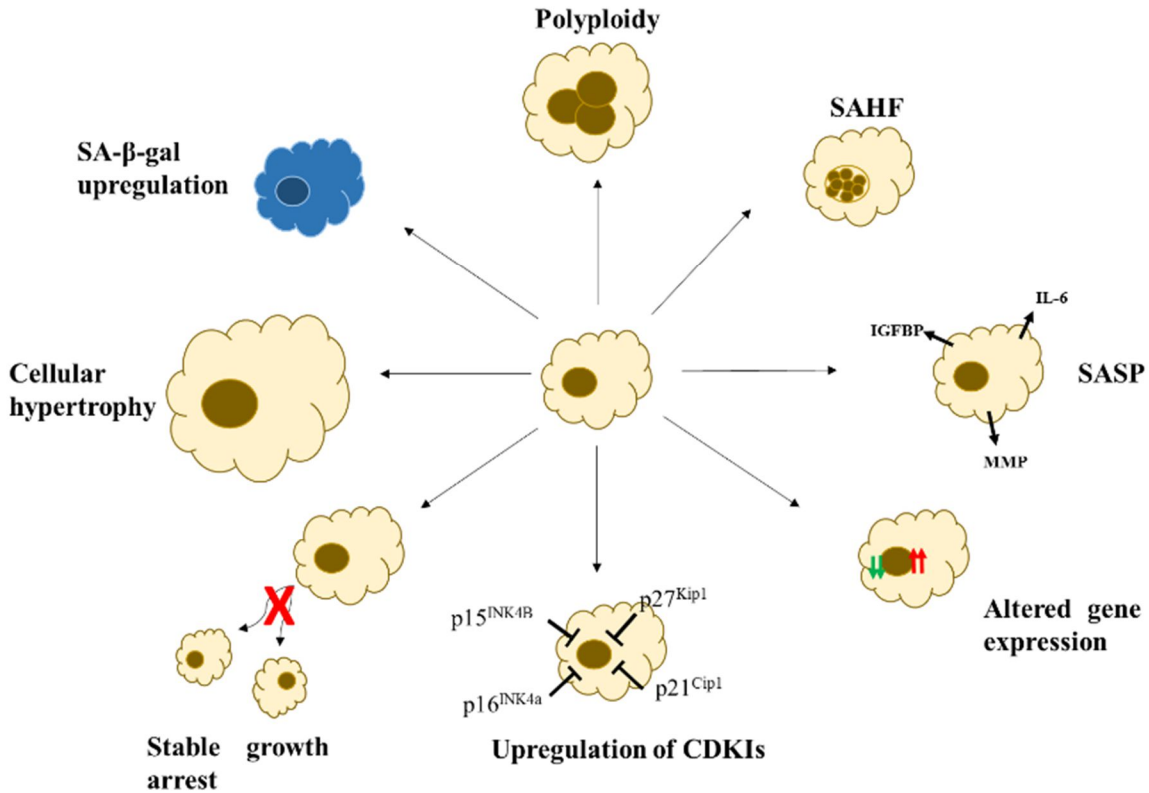
Figure 1.1. Hallmarks of Senescence. Despite the fact that permeant growth arrest and failure of progression through the cell cycle is the hallmark of senescent cells, the senescent phenotype encompasses a spectrum of distinct features. Senescent cells have a characteristic morphology. They become *large*, flattened with abundant cytoplasmic vesiculation. Senescent cells upregulate the lysosomal enzyme *SA-β-gal*. *Cyclin-dependent kinases* (CDKs) are also upregulated. In addition to undergoing vast alterations in gene expression, senescent cells develop also unique epigenetic changes collectively called the *Senescence-associated heterochromatin foci* (SAHF). Senescence has been closely associated with polyploidy or multinucealtion. Finally and most importantly, senescent cells engage into an active secretory process that involves the release of over 40 different cytokines, chemokines and other mediators to their surroundings, a phenomenon called the *Senescence Associated Secretory Phenotype* (SASP).

circumstances a cell can escape the permanent growth arrest after senescence induction, and that the terminology of "irreversible" may not be accurate. During the American Association for Cancer Research meeting in Chicago, Illinois in 2018, Rene Bernards, a prominent senescence expert, has openly argued in a senescence discussion panel that the senescent growth arrest should
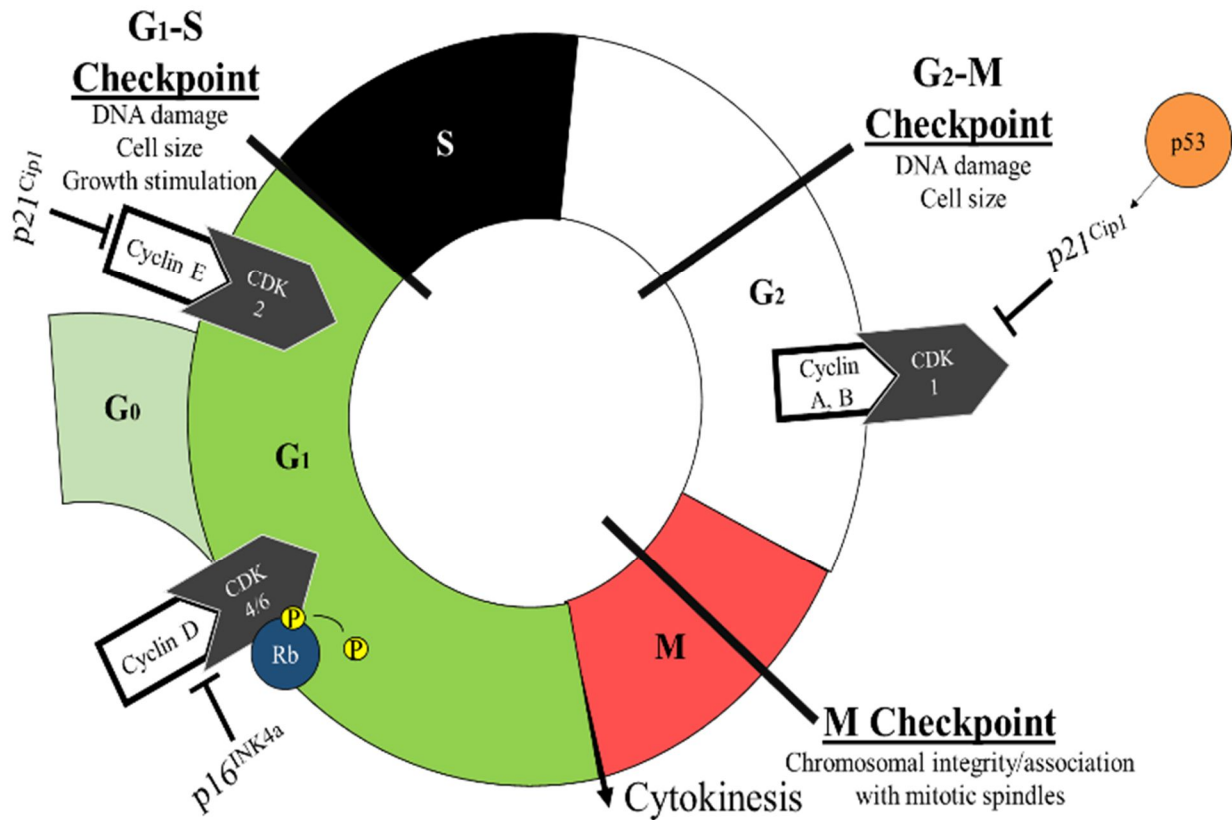
Figure 1.2. Cell cycle: phases, checkpoints and regulation. A schematic diagram illustrating different phases of the cell cycle. A cell spends the majority of its lifetime in the interphase (comprised of G_1, S and G_2 phases) before it prepares for mitosis and undergoes cell division (M phase). The progression through the cell cycle is governed by *cyclins* and *cyclin-dependent kinases* which mediate necessary phosphorylation events that allow the cell to transition through the next phase, such as the phosphorylation, and inactivation, of the retinoblastoma protein Rb. The action of these proteins is opposed by *cyclin-dependent kinase inhibitors*. p53 is a transcription factor that plays a major role in regulating the cell cycle and mediates the expression of $p21^{Cip1}$, a major cyclin-dependent kinase inhibitor. Several checkpoints ensure DNA integrity and monitor cellular size. If a cell is subjected to lack of nutrition or scarcity of growth simulation it reversibly exits the cell cycle into G_0 and becomes *quiescent*, until favorable conditions are restored.

be more precisely described as "stable" rather than irreversible. However, this new description connotes that the stability of senescence can be shaken. This new perception of what senescence actually represents is a central focus of this dissertation. In a later section, we provide a detailed analysis of some of the most important evidence in the literature that argues against the absolute irreversibility of senescence.

1.2.2. Features of the senescent phenotype.

Although a stable growth arrest is the hallmark of senescent cells, the senescent phenotype encompasses a spectrum of distinct features. These features represent dramatic alteration in gene expression that characterize the phenotype (**Figure 1.1**).

1.2.2.1 Growth arrest

When a mitotic cell is subjected to cellular stress, senescence is a potential outcome. The first sign of senescence induction is failure of progression through the cell cycle (27). The cell cycle describes a highly-organized sequence of events that lead to DNA replication and ultimately cell division into two daughter cells. In eukaryotic cells, the cell cycle consists of four distinct phases, in each of which the cell carries out specialized tasks (**Figure 1.2**). The **M** phase is the most considerable phase of the cell cycle where the cell undergoes *m*itosis. However, it represents only a short phase of the cell's lifespan as the cell generally spends more time in the Interphase which consists of G_1, S and G_2 phases. In G_1, or gap-1, the cell engages in high metabolic activity where it synthesizes a plethora of proteins and cellular suborganelles in preparation for the DNA synthesis which takes place in **S** phase. After DNA replication is completed, the cell enters into another gap phase, G_2, where it continues to grow, this time in preparation for mitosis. Since the cell undergoes DNA replication and doubles its chromosomal content in S phase, the DNA content varies from one phase of the cycle to another. For example, in G_1, and prior to DNA replication, the cell contains a diploid number of chromosomes (*2n*), while in G_2, after DNA replication the chromosomal number is doubled (*4n*).

Depending on the stimulus, cell type and which face of the cell cycle the cell is circulating in at the moment of stress, a senescent cell can have variable DNA content. Classically, human

fibroblasts undergoing telomeric dysfunction senesce with a G_1 DNA content (28). However, due to certain derangements in cellular stress response pathways, a human fibroblast can senescence with a G_2 DNA content as well (29). Similarly, a fibroblast accumulating oncogenic mutations can senesce with a G_2 DNA content (30). Moreover, tumor cells driven into extensive genomic instability induced by exposure to DNA damaging therapy often undergo a G_2-M growth arrest (31).

Progression through the cell cycle is tightly controlled by internal regulatory mechanisms and in response to critical extracellular stimuli. These stimuli are often growth signals that encourage the cell to continue proliferating. The main drivers of the cell through the cell cycle are serine/threonine protein kinases that are collectively called *cyclin-dependent kinases* (CDKs). The reason for the terminology is that these kinases depend on their activity on the binding of another group of proteins called *cyclins*. The third line of regulatory machines are called *cyclin-dependent kinase inhibitors* (CDKIs) which, as the name indicates, inhibit the necessary phosphorylation events carried out by CDKs, and thus, put brakes on further progression through the cell cycle. Several checkpoints monitor key process throughout the cell cycle such as cellular size (protein content and geometry), DNA replication, the lack of DNA damage and the integrity of the replisome and the mitotic spindle during division. These checkpoints are controlled by a complicated network of pathways that can enforce cell cycle arrest if a threatening defect (e.g., considerable DNA damage) occurs.

CDKIs play the main role in regulating the growth arrest of senescence, and are therefore, used as markers for senescence induction both *in vitro* and *in vivo* (32). In fact, the ectopic expression of these CDKIs, such as $p16^{INK4a}$ and $p21^{Cip1}$, on their own, can result in the induction of senescence (33). The role of these proteins in the induction and maintenance of senescence is indispensable,

although senescence can still be induced in the absence of particular CDKIs, for example, in tumor cells that suppress the expression of p16^{INK4a} during transformation but retain the potential to undergo accelerated senescence.

p16^{INK4a}, encoded in the *CDKN2A* gene, is a tumor suppressor and a CDKI that is closely associated with the senescent phenotype (34). In fact, p16^{INK4a} accumulates in the tissue of aging individuals, both in mice and humans (35,36). Moreover, p16^{INK4a} is induced in tissues exposed to ionizing radiation in parallel with the accumulation of senescent cells (37). As the cell progresses through G$_1$, cyclin D levels gradually increase so that it starts binding to the available CDK4 and CDK6, initiating a cascade of phosphorylation events that include the phosphorylation of the retinoblastoma protein (Rb). Rb phosphorylation (pRb) inactivates the protein, leading to the release of the transcription factor E2F which promotes the transcription of many genes necessary for progression from G$_1$ to S. p16^{INK4a} is often expressed late in response to telomeric or intrachromosomal DNA damage but leads to a robust inhibition of the CDK 4/6 and any further progression through G$_1$ through the hypophosphorylation (or the dephosphorylation) of Rb) (38). Finally, p16^{INK4a} expression is regulated by the binding of several transcription factors to the *CDKN2A* promoter, including PPARγ, AP1 and Sp1. (39,40). Furthermore, other epigenetic modulations such as demythelation of the *CDKN2A* promoter can also result in p16^{INK4a} and senescence induction (41).

 p16^{INK4a} expression is often suppressed in many pre-malignant or malignant cells (42,43). For example, the majority of pancreatic malignant cells lose the function of p16^{INK4a} by the time they are fully transformed (44). In fact, p16^{INK4a} mutations often occur early during transformation and result in the loss of an important barrier against cancer development (45,46). Despite the essential role of p16^{INK4a} in implementing the stable growth arrest of senescence, especially in aging

fibroblasts, mutational deletion of p16^{INK4a} or suppression of its expression by methylation in tumor cells does not actually mean that these cells will fail to undergo senescence in response to cellular stress; in fact many tumor cells retain the potential to undergo senescence in response to DNA damaging therapy (47). Indeed, this adds another factor of complexity to the senescence phenotype, as the absence of p16^{INK4a} might affect the stability of senescent tumor cells especially that it has been described to be a regulator of late maintenance of the cell cycle arrest rather than its induction (48).

p21^{Cip1} is another important CDKI that regulates the senescent growth arrest (49). p21^{Cip1} is capable of inhibiting multiple cyclins/CDKs complexes, thereby resulting in a growth arrest at different phases of the cell cycle. Despite its strong association with senescence, p21^{Cip1} is not considered sufficiently unique (in fact none of the senescence markers are) to solely describe the induction of senescence. For example, p21^{Cip1} can be induced during quiescence due to scarcity of growth factors (50). However, there is an extensive body of literature suggesting its essential role in senescence induction in tumor cells in response to DNA damaging therapy, especially since p21^{Cip1} is directly induced by p53 as part of the response to DNA damage (47). In fact, recent evidence has suggested that p21^{Cip1} is essential for the maintenance of the senescence phenotype by restraining pro-cell death pathways (51). Furthermore, p21^{Cip1} is responsible for regulating the response to DNA damage in senescent cells and its absence (e.g., by genetic knockdown) shifts the response to cellular stress towards apoptosis (51). In addition, deletion of the *CDKN1A* locus which encodes for p21^{Cip1} extends the lifespan of mice with dysfunctional telomeres, possibly due to the reduction in the number of accumulating senescent cells (52). Ultimately, p21^{Cip1} is a pivotal component of the senescent phenotype.

Finally, other CDKIs can potentially make substantial contributions to the induction and/or maintenance of senescence. For example, p27^{Kip1} is necessary for pRb-mediated senescence through the binding and inhibition of cyclin E-associated kinases in osteosarcoma cells (53), and p15^{INK4b} is known to accumulate in senescent T lymphocytes (54).

1.2.2.2 p53 and the activation of the DNA damage response

In the majority of cases, senescence is a direct consequence of severe DNA damage and persistent activation of the DNA damage response (DDR) (55). The DDR describes an integrated signaling pathway that detects DNA lesions and promotes their repair (56). The basic function of the DDR is to enforce cell cycle arrest until the defects in the DNA are repaired, after which the cell can resume proliferating. In senescence, severe DNA damage appears to induce a stable DDR and a more stable growth arrest. The DDR is a consequence of a variety of senescence-causing DNA damage events, including the classical telomere dysfunction in aging cells (57), exposure to genotoxic chemotherapeutics or radiation (58) as well as in cases of hyperreplication resulting from oncogene overexpression (30). While the DDR represents the first initiating event in the induction of senescence, and is therefore considered to diminish over time after the phenotype is established (59), recent evidence has suggested that the DDR is stably associated with senescence (55).

Large protein kinases, ataxia-telangiectasia mutant (ATM) and ataxia telangiectasia and Rad3 related (ATR), are activated by DNA damage; both proteins are recruited to the sites of DNA double-stranded breaks (**Figure 1.3**) (60). Once activated, ATM carries out a cascade of phosphorylation events, including the activation of the checkpoint kinase 2 (Chk2), and subsequently, increased transcription of p53 (60). Subsequently, p53 induces the expression of
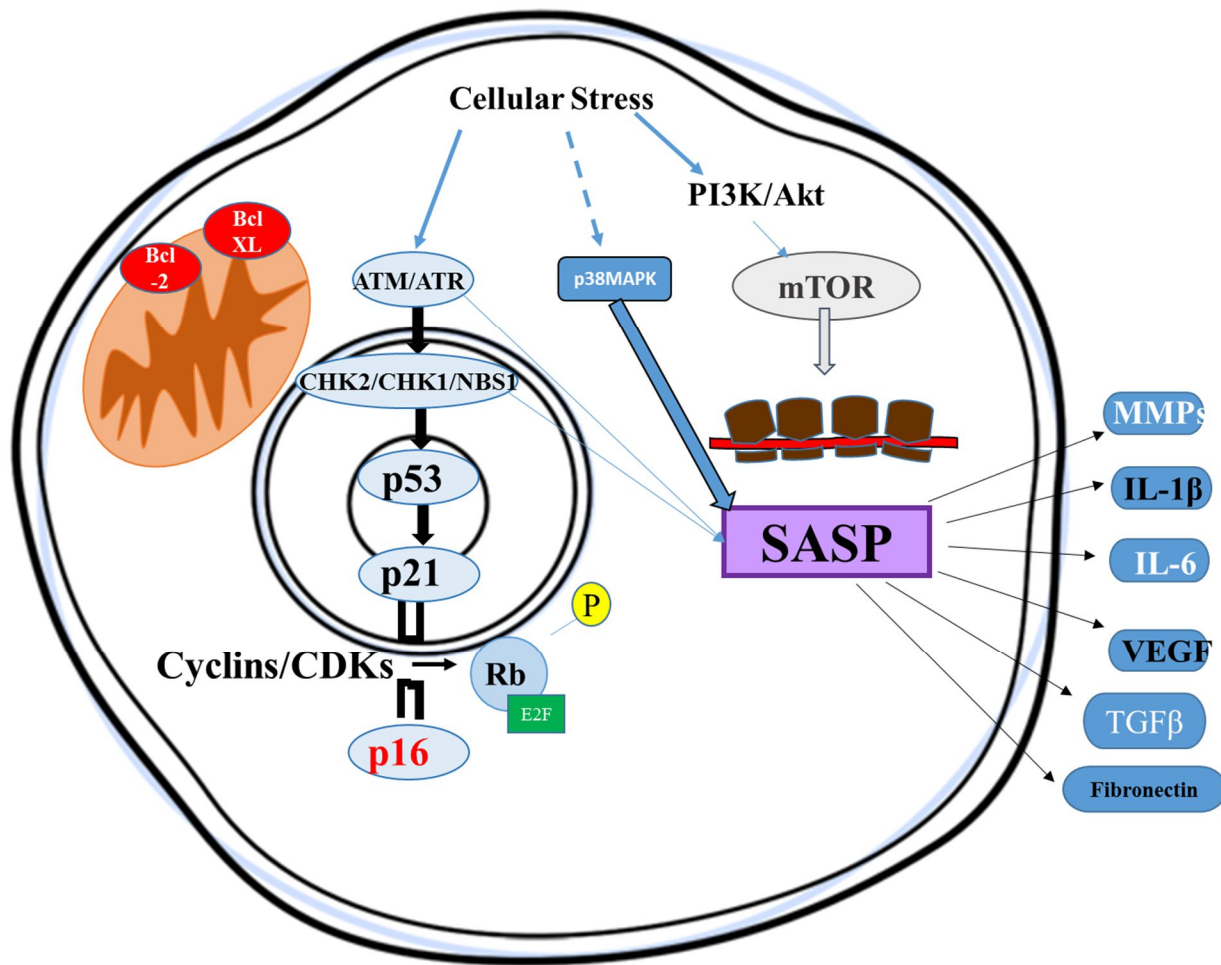
Figure 1.3. Regulatory pathways of cellular senescence. Regulation of cellular senescence is a complex and not completely understood process that involves the interaction of several pathways. Several cellular stresses result in the he induction senescence, most importantly, unrepaired DNA damage. DNA damage results in the activation of ATM and CHK2 of the DDR, followed by the activation of p53—p21^{Cip1} axis resulting in the Rb hypophosphorylation and activation, preventing progression into S phase The activation of p16^{INK4a} (and subsequent Rb hypophosphorylation) is an essential component in the maintenance of the senescent growth arrest. p16^{INK4a} is often mutated and inactivated in many tumor cells. Despite the stressed mitochondria, antiapoptotic factors of the Bcl-2 family stabilize the mitochondrial membrane and facilitate resistance to apoptosis. ATM and other regulators of the DDR contribute to the SASP. However, p38MAPK has been shown to control SASP independently of the DDR explaining the delayed induction of the secretory phase. p38MAPK is another key regulator of maintaining the senescent phenotype (promotes p53 and p16^{INK4a} activation) and its inhibition (or knockdown) is suspected to accelerate the capacity of cells to recover from senescence. Cellular stress can also result in mTOR (TORC1) activation which is involved in enhancing protein translation. Ultimately, multiple secretory factors (chemokines, cytokines, growth factors, structural proteins, etc) are secreted to the surroundings.

p21^{Cip1} which in turn inhibits the activity of cyclin-dependent kinases and results in growth arrest (47). At the same time as the cell undergoes cell cycle arrest, ATM activates repair mechanisms by recruiting multiple repair-associated factors to the damaged sites, primarily by the phosphorylation of the histone H2AX (γH2AX) which serves as a point of assembly of many of these repair complexes (60). The importance of the DDR in senescence induction is reflected by studies where its components have been disabled, which resulted in attenuation of senescence induction. For example, knockout of ATM in mice reduced the diabetes-associated accumulation of senescent endothelial cells in their main arteries (61), while ATM knockdown in breast tumor cells reduced the percentage of cells that undergo senescence in response to adriamycin genotoxic stress (62).

p53 plays a unique role in the regulation of the senescence phenotype (63). As discussed above, through its action as a transcription factor, p53 is able to induce the expression of CDKIs that mediate the senescence-associated growth arrest. Apart from its classical role in DNA damage repair, p53 exerts its tumor suppressive function via senescence induction in response to oncogenic stimulation (64). Accordingly, loss of p53 activity in Ras-overexpressing mouse embryonic cells allows them to bypass senescence induction (64). Mammary epithelial cells in mice where Ras is experimentally over-induced can develop cellular senescence as a barrier against transformation; however, in the case of p53 ablation, these cells can progress to generate hyperplastic lesions that can develop into tumors (65). In tumor cells exposed to DNA damaging therapy, p53 appears to be essential for senescence induction as tumor cell lines harboring mutant p53 failed to become senescent and underwent apoptotic cell death or mitotic catastrophe in response to the genotoxic stress (31). As part of the research in our group, we have shown that MCF7 breast tumor cells with silenced p53 fail to undergo senescence in response to adriamycin exposure (66). Breast tumor

samples from patients that have been exposed to cycles of chemotherapy show the accumulation of senescent cells that also show increased p53 staining (58). This important observation has also been described by our group (shown below). Despite this strong association between p53 and senescence, paradoxical evidence has been proposed where p53 is found to suppress the senescence program in cases where a cell is undergoing conventional growth arrest or quiescence, indicating the complexity of p53's role in senescence regulation (67). In those lines, the kinetics of p53 activation appears to play a role in determining cell fate in response to stress, i.e., whether the cell will undergo senescence or other forms of cell death (68). p53 is also implicated in several gene expression alterations, including the expression of many senescence-associated secretory factors which we discuss further below. Thus, p53's contribution to senescence extends beyond its original activation as a component of the DDR.

1.2.2.3 Resistance to apoptosis.

Apoptosis is the most studied cell stress response, describing a programmed cascade of cellular events that culminate in cell demise. Physiologically, just like senescence, apoptosis is a tumor suppressor mechanism that eliminates potentially cancerous cells; however, the difference is that apoptotic cells are cleared while senescent cells persist. In fact, senescent cells are by definition resistant to apoptosis (cell death). For example, human fibroblasts induced into senescence by oxidative stress can survive in culture despite death-inducing growth factor deprivation which was later attributed to the absence of nuclear insulin-like growth factor binding protein-3 (IGFBP3) (69). Senescent cells can also downregulate the expression of necessary apoptosis executioners such as caspase-3 or death-associated protein 3 (DAP3) (70–72). More importantly, senescent cells overexpress antiapoptotic proteins such as members of the Bcl-2 family shifting the balance towards survival (73). Senescent human fibroblasts fail to downregulate Bcl-2 in response to

death-inducing stimuli due to constant c-AMP/CREB stimulation, a pathway that positively regulates Bcl-2 (74). It is also evident that senescent cells upregulate other antiapoptotic members of the Bcl-2 family such as Bcl-w and Bcl$_{XL}$ (75,76). Despite these different explanations of how senescent cells resist apoptosis, the exact mechanism is still poorly understood, especially since non-senescent growth arrested cells can also exhibit apoptosis-evading behavior (77). Overall, resistance to apoptosis is one quality that explains how senescent cells remain stable in culture for prolonged periods of time and possibly persist in viable tissues.

1.2.2.4 Enhanced lysosomal biogenesis

Senescent cells remain metabolically active and undergo changes at the levels of subcellular compartments. A primary feature of senescent cells is the increase in lysosomal mass, an observation evident by the remarkable enhancement of acidic vesicles staining in senescent cells (78). Moreover, this accumulation of lysosomes or lysosomal dysfunction is a feature of aging tissue (79). This phenomenon has been tightly linked to senescence following the identification of increased expression of the lysosomal β-galactosidase enzyme by Dimri et al (80). The enzymatic activity of this enzyme is distinct in senescent cells as this lysosomal enzyme remains functional at unusually high pH condition (~pH=6). The Senescence-Associated β-galactosidase (SA- β-gal) enzyme is not detectable in quiescent or terminally differentiated cells as demonstrated by Dimri et al (80). The origin of the upregulation in SA- β-gal function comes from *GLB1,* which is the gene encoding for the lysosomal beta-D-galactosidase (81). Unlike SA- β-gal, the activity of β-D-galactosidase is typically detected at ideal lysosomal pH (~pH=4.5). Cells with depleted GLB1 mRNA fail to express SA- β-gal despite undergoing senescent-growth arrest after replicative exhaustion (81) . Thus, SA- β-gal reflects the upregulation in lysosomal activity which is probably

a consequence to the accumulation of damaged organelles which frequently occurs in senescent cells.

1.2.2.5 Gene expression alteration and the senescence-associated secretory phenotype

Senescent cells undergo extensive alterations in gene expression (82), changes that are not confined solely to genes involved in cell cycle regulation, but also include increased expression of a spectrum of secreted proteins (83,84). Specifically, senescent cells have been reported to secrete a spectrum of pro-inflammatory chemokines and cytokines that have paracrine tumor stimulatory effects (84) contributing to metastatic progression and age-related diseases even in younger cancer survivors (85).

The Senescence-associated Secretory Phenotype (SASP) can be a direct consequence of the activation of the DDR which is uniformly induced in tumor cells treated with chemotherapy or radiation (86,87) and can also result from oncogene-overexpression and hyper DNA replication (30). As discussed above, interference with DDR-associated proteins such as ATM or Chk2 attenuates the senescent response (62,86). p53 is not only a regulator of the senescent growth arrest but also partake in gene expression regulation of senescence. (88). While p53 is not an absolute requirement for cells to undergo senescence, loss of p53 function in senescent cells might actually facilitate recovery from senescence (89–91). This is particularly important given the fact that many tumor cells have mutations that affect the function of p53 (92). Conversely, loss of p53 function can result in enhanced SASP and its ability to drive pro-tumorigenic proliferation (93). The role of p53 in SASP regulation is further shown in senescent hepatic stellate cells implicated in hepatic cirrhosis where p53 ablation attenuates the inflammatory drive mediated by SASP (94). However,

the SASP can be precipitated independently of DDR, such as in the case of senescent cells that develop non-pathologically during embryogenesis (95) or during wound healing (96). Interestingly, SASP induced by wound healing after mechanical injury lacks IL-6, bFGF and TGF-β expression (96), whereas several of these factors are expressed after exposure of the lung to DDR inducing chemicals (97), or from DDR-inducing CYR61 exposure to fibroblasts (98). These observations demonstrate that the composition of the SASP can vary considerable depending on how the senescence is induced.

In addition to DDR and its signaling pathways, cell cycle regulators play important roles in regulating the SASP. Despite the fact that many tumor cells lose $p16^{INK4a}$ function during transformation, they retain the ability to develop the SASP upon DNA damage, most likely because SASP is regulated independently of cell cycle arrest (33,99). This dissociation between SASP and cell cycle regulators is also true for $p21^{Cip1}$ (33), although in certain scenarios where SASP is induced independently of DNA damage, $p21^{Cip1}$ knockdown can attenuate the secretory response (95).

The SASP is further regulated by p38MAPK which, in addition to p53 and $p16^{INK4a}$ activation, is responsible for an increase in the activity of NF-κB (100). p38MAPK is a primary responder to cellular stress and is activated in response to a variety of antitumor agents (101), while NF-κB is responsible for the transcriptional activation of many SASP components and is a chief regulator of TIS (102). In addition to NF-κB, the C/EBPβ transcription factor is necessary for the development of oncogene-induced senescence (OIS) in primary fibroblasts (103). The interplay between C/EBPβ and its heterodimeric partner C/EBPγ regulates the expression of multiple SASP genes (104). Lastly, mTOR appears to play a critical role in the regulation of the SASP as rapamycin promotes a robust suppression of inflammatory mediator release (105). In fact, mTOR

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inhibition selectively inhibits the translation of the membrane-bound cytokine IL1α, resulting in decreased NF-κB-driven expression of multiple SASP factors (106).

While senescence and SASP are consistently observed in the laboratory in cancer cells both *in vitro* an *in vivo* as a response to DNA damaging agents (107–109), certain targeted therapies can also induce senescence in tumor cells. For example, the anti-VEGF drug bevacizumab (or inhibition of the VEGFR2 pathway) was able to induce a modest senescent response in colon cancer cells, xenografts, as well as patients' tumors in a $p16^{INK4a}$ dependent manner (110,111). In this study, senescence was evaluated based on SA-β-galactosidase staining and $p16^{INK4a}$ expression; however, the ability of bevacizumab to induce SASP expression was not investigated. The effects of anti-VEGF agents on senescent tumor cells are interesting, since VEGF is a core element of the SASP. In fact, bevacizumab in combination with chemotherapy was associated with improved clinical outcomes in glioblastoma patients (112). However, it is not known whether this effect was attributed to enhanced senescence or due to blockade of VEGF as a SASP component.

Recently, aurora kinase inhibitors were shown to induce a robust senescent response in chronic myeloid leukemia, melanoma and non-small cell lung cancer cells (113,114). Moreover, CDK4/6 inhibitors such as palbociclib have also been shown to induce a pronounced senescence response in triple-negative breast cancer cells (115). While it is not certain if palbociclib can drive a secretory response in these senescent tumor cells, it was shown that chronic palbociclib treatment promotes senescence and a robust SASP in melanoma-associated fibroblasts which results in enhanced growth of multiple melanoma cell lines (116). This observation is particularly important, since CDK4/6 inhibition is not traditionally associated with DNA damage per se, suggesting a possibility for inducing SASP by alternative pathways (in this context, palbociclib has been shown to interfere with DNA damage repair only when tumor cells are exposed to radiotherapy (117)).

Finally, multiple SASP factors do appear to have the potential to fortify the senescent phenotype in a self-reinforcing autocrine fashion. For example, the chemokines receptor CXCR2 (IL8RB) closes the circuit of senescence induced by DNA-damage as it conveys NF-κB activation signals by multiple CXCR2-binding factors (118). Furthermore, as a component of the increased expression and release of multiple inflammatory mediators, senescent cells with activated mTOR can actually also show enhanced expression of cytokine receptors such as soluble IL-6R, supporting the premise that the tumor cells could be amenable to self-stimulation (119). Plasminogen activator inhibitor-1 is also a pivotal SASP component that is necessary for the induction and maintenance of the senescent phenotype in fibroblasts (120).

Despite the extensive body of literature relating to the SASP, it is uncertain whether the SASP contributes to the maintenance of senescence or promotes escape from senescence. In fact, the dynamic interplay of the different regulatory pathways of SASP can yield different functional outcomes. For example, the oscillation of NOTCH1 expression and signaling during transition into OIS can culminate into two distinct SASP profiles with variable effects (either an anti-inflammatory TGF-β or a pro-inflammatory SASP response) (121). To summarize, the outcome of the SASP is dependent on the heterogeneity of the senescent response (75), the profile of secreted factors dictated by the interplay of the SASP's regulatory pathways, cell type, temporal status of senescence and interaction with other components of the microenvironment (122).

1.2.2.5 Structural and morphological changes.

Senescent cells develop distinct morphological changes characterized by enlarged cellular size and flattening (123). These cells reach a critical cell mass beyond the point where cells usually undergo

mitosis. This is largely due to the activation of the mTOR pathway[2] and enhanced ribosomal protein biogenesis which is often seen in enlarged senescent endothelial cells (124). Also, senescent cells undergo dramatic changes in the cytoskeleton which account for the neuronal-like morphology of their cell bodies, an alteration dictated by ATF6α signaling pathway[3] (125). This change consists of dynamic structural rearrangements in the vimentin filaments which also undergo unique posttranslational oxidation that is specifically expressed in senescent fibroblasts (126). Furthermore, the composition of the plasma membrane in senescent cells is altered, acquiring increased caveolin-1 content, which is part of the caveolae cholesterol domains[4] (127).

Senescent cells also exhibit changes in the nuclear structure. For example, irradiated human fibroblasts that express SA-β-gal and growth arrest (but not SASP), have a reduced DNA content quite similar to pre-apoptotic cells, yet not sufficient to induce cell death (128). In addition, the nuclear lamina, a delicate meshwork lining the nucleoplasmic side of the nuclear envelope, undergoes characteristic changes during senescence (129). These changes include the formation of interaction points between damaged DNA foci and certain components of the lamina resulting in changes in the spatial organization of the nucleus (129). These interactions were found to play a pivotal regulatory role in senescence by controlling the expression of the repositioned genes (130). Furthermore, the nuclear periphery serves as a deck for the sequestration of many cytoplasmic proteins which might be related to derangements in the lamin protein family (131). Lamin B1, an essential component of the nuclear lamina, is downregulated in senescent cells, resulting in nuclear instability and chromosomal fragmentation (132). Lamin B1 loss occurs in

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[2] The mTOR pathway is involved in regulating several cellular processes including protein synthesis. mTOR activation has been shown to be critical for establishing senescence.
[3] ATF6α is an endoplasmic reticulum-associated transcription factor that is induced in response to cellular stress as part of the misfolded protein response.
[4] Caveolae are cholesterol-rich protein domains that are important component of cellular membranes of many cell types and play a critical role in extracellular signaling.

cells induced into senescence by replicative exhaustion or oncogene overstimulation, suggesting its universality as a senescence feature (132). Lamin B1 loss appears to be regulated primarily by the p53/p21^{Cip1} and p16^{INK4a}/pRB pathways, but not p38MAPK or oxidative stress pathway (132).

In addition to laminar changes, senescent cells exhibit dynamic alterations in the chromatin structure. In eukaryotic cells, chromatin is folded into a loose, less compact euchromatin, which permits active transcription of many genes, and a more condensed, closed heterochromatin, which is associated with a lower transcriptional drive (133). For example, when the cell decides to decrease the expression of a certain gene, the chromatin spatially reorganizes into heterochromatin which is thought to prevent the access of essential transcription factors, and thus, silencing of the gene (134). Senescent cells develop similar changes in their nuclei and develop what is collectively referred to as the Senescence-Associated Heterochromatin Foci (SAHF). This unique association of chromatic changes with senescence was first reported by Scott Lowe's group (135). Narita et al demonstrated that these foci of condensed chromatin are rich in characteristic histone modifications such as the heterochromatin protein (HP1γ) and histone H3 trimethylation on lysine 9 (H3K9Me3) (135). Furthermore, they demonstrated that these foci represent areas of repressed gene expression, in particular, genes regulated by the transcription factors E2F, thus linking the development of SAHF to the Rb pathway (135). In other words, upon senescence induction and activation of RB (hypophosphorylation of Rb), the chromatin condenses in regions containing genes associated with cell proliferation, preventing their expression, which is mediated by E2F. Hence, these changes are not strictly structural but appear to play an important regulatory role. Senescent WI38 fibroblasts undergoing oncogene-induced senescence and expression of SAHF including H3K9Me3 and H1Pα, β and γ, also contain the transcriptionally repressive variant of

histone H2A, macroH2A (136). SAHF can also contain other histone markers such as H4K20Me3 and a unique peripheral localization of H3K27Me3 at the nuclear rim (137,138).

In addition to the changes to the plasma membrane, nucleus and lysosomes, senescent cells also accumulate mitochondria. In fact, a major component of the accumulating dysfunctional organelles in aging cells are mitochondria with deranged membrane integrity (139). This probably happens due to a dysfunctional autophagic response occurring as part of the senescent phenotype; however, the connection between senescence and autophagy is not fully understood and is discussed later on (140). Overall, the increase in mitochondrial fusion and disrupted mitochondrial membrane potential contributes to the accentuated oxidative stress that accompanies senescence and possibly contributes to the stability of the growth arrest (139). This abnormal accumulation of mitochondria has been reported in many senescent cell types and in response to several senescence inducing stresses and is now collectively referred to as the Senescence-Associated Mitochondrial Dysfunction (SAMD) and as an additional feature of the phenotype (141).

1.2.4. Types of senescence

Senescence is classified into multiple types based primarily on the type of stressor. Senescence can be induced by a variety of stressful stimuli, including DNA damaging agents, which has been a focus of our work.

1.2.4.1 Replicative Senescence.

Replicative senescence is the well-established form of cellular senescence which was originally described by Hayflick (7). Replicative senescence is a primary cellular mechanism of aging and reflects the finite capability of a cell to proliferate. As mentioned earlier, replicative senescence is

the product of progressive telomere attrition (often described as telomere dysfunction) that occurs as a consequence of the end-replication problem (10,12). Telomeres are DNA segments that cap the linear ends of the DNA and consist of repetitive, meaningless stretches of nucleotides (17). In the non-replicating chromosome, telomeres arrange in a loop-like structure called the T-loop, in association with the DNA protective proteins, *shelterins* (142). Due to the end replication problem, which results from inability of DNA polymerase to replicate the end of the lagging strand, the telomeric length is shortened by around 150 bases with each cell division. Interestingly, the length of telomeres varies within the same cell, and the critical shortening of only one or two telomeres, rather than the average telomeric length, is sufficient to induce chromosomal instability (143).

The exact number of divisions that a cell can undergo varies between different cell types; however, once the telomeres reach a critical length, the resulting dysfunction triggers the DDR (144). This involves the phosphorylation of ATM and ATR with subsequent activation of p53 and other associated proteins, ultimately inducing senescence. The reason behind why cells respond to telomere dysfunction and activation of DDR with senescence rather than apoptosis is still poorly understood; however, this reiterates the fact that senescence is a response to genomic instability. In all cases, telomere shortening is not an unsolvable problem. Telomeres can be elongated by the reverse transcriptase, *telomerase* (18). However, most somatic cells do not express this enzyme and thus remain bound to a limited lifespan and undergo replicative senescence. While telomerase expression can successfully delay or bypass the onset of replicative senescence, it does not seem to affect senescence precipitated by non-telomeric DNA damage (66,145). Collectively, replicative senescence describes a stress response to telomeric DNA damage resulting in a stable growth abrogation in proliferating somatic cells and represents a fundamental basis of cellular and organismal aging.

1.2.4.2 Oncogene-induced senescence.

Oncogenes are mutated genes that can drive the transformation of a cell to become cancerous. Evolutionarily, several defense mechanisms have developed to combat the overexpression of oncogenes and prevent cancer occurrence. Interestingly, many cells also develop senescence as a response to oncogene overexpression. The first observation of oncogene-induced senescence was made in Scott Lowe's laboratory, where his group showed that fibroblasts expressing mutant Ras, a mitogenic signal transducer, undergo cellular senescence characterized by a permanent G_1 growth arrest (64). This form of senescence had similar features to replicative senescence and was regulated by p53 and p16^{INK4a} (64). However, it was not associated with telomere shortening or limited replicative potential of the fibroblasts, and thus was described as a *premature*, or *accelerated* form of senescence. Sequentially, oncogene-induced senescence was described to occur in response to multiple pro-proliferative oncogenes such as Raf and E2F-1 (146,147). Furthermore, senescence can also be induced by the loss of certain tumor suppressor genes, such as PTEN, representing a barrier against neoplastic potential (148).

This role of senescence in combating events leading to neoplastic transformation such as oncogene overexpression or tumor suppressor gene suppression set the evolutionary significance of senescence as a vital tumor suppressive mechanism (149). If fact, senescent cells accumulate in premalignant lesions, possibly as a first barrier to acute oncogenic aberrations (150). The classical example is the identification of senescence markers in naevi containing *BRAF* overexpressing melanocytes (151). In most cases, senescence is sufficient to block oncogenic transformation and results in a permanent abrogation of growth of a cell harboring an active oncogene; thus, bypassing oncogene-induced senescence is essential for malignant transformation. Accordingly, the co-expression of an additional oncogene, or a "second hit", is required for a cell to overcome the

oncogene-induced senescence and proceed towards becoming cancer (152). Finally, it is important to mention that oncogene-induced senescence is in fact a product of the activation of the DDR resulting from genomic instability induced by DNA excessive replication, which occurs as a result of oncogene-driven increase in cell proliferation (30). This again reiterates that senescence is a consistent cell response to oncogenic genomic alteration where its main role is to oppose malignant transformation as a failsafe tumor suppressor mechanism.

1.2.4.3 Therapy-induced senescence.

Early on, it was unexpected that tumor cells would undergo terminal senescence since they are immortal and possess the ability to divide indefinitely, at least in cell culture. This immortality is based, although not fully, on the fact that most tumor cells re-express telomerase which theoretically allows for an unlimited number of cell divisions by preventing telomeric dysfunction. However, the first clue that senescence induction is still feasible in immortal tumor cells originated from studies where human diploid fibroblasts underwent senescence due to non-telomeric DNA damage (153). This observation suggested that inflicting DNA damage in a tumor cell might induce a senescence-like phenotype. Early studies attempting to induce senescence in tumor cells utilized somatic fusion approaches where nuclei of normal cells were fused in tumor cells and resulted in growth arrest (154). These experiments suggested that the senescence genetic program is dominant and can be re-established even in immortal tumor cells. These studies were followed by a more focused examination of the role of certain senescence-associated genes in inducing the phenotype in tumor cells. For example, the re-induction of p53 expression in EJ bladder carcinoma cells that lack p53 resulted in immediate induction of p21^{Cip1}, subsequent G_1/G_2-M irreversible growth arrest as well as increased SA-β-gal expression (155). Using an inducible gene expression system, expressing functional Rb protein in Rb-defective breast, osteosarcoma and bladder tumor

cells resulted in a stable G_1 growth arrest and the induction of several other senescence-like features (156). Overexpression of p21^{Cip1} in H1299 p53-deficient lung tumor cells results in an irreversible growth arrest and the induction of multiple feature of senescence (157). These experiments further elucidated the pivotal role of these proteins in the induction and maintenance of senescence, but more importantly, demonstrated that senescence can still be induced in immortal tumor cells.

The first evidence of the ability of tumor cells to senesce in response to cancer chemotherapeutics was presented in nasopharyngeal carcinoma cells (CNE1 cell line) in response to the DNA-crosslinking agent cisplatin (158). After exposure to cisplatin, these cells demonstrated morphological changes consistent with senescence, underwent a stable growth arrest and expressed SA-β-gal and this resulting state was termed "senescence-like" phenotype since it was not precipitated by aging or inhibition of telomerase (158). Further studies on chemotherapy-induced senescence in tumor cells were published by the Igor Roninson group (31). In those studies, Chang et al performed an initial screen of the ability of several chemotherapeutic agents to induce senescence in 14 cancer cell lines based on SA-β-gal expression and the induction of growth arrest (31). At subtoxic concentrations, many of these drugs resulted in SA-β-gal expression and growth arrest in these cell lines, with topoisomerase inhibitors, such as doxorubicin, producing the strongest effect, and with microtubule poisons being the weakest (31). Soon after, multiple laboratories have shown the induction of senescence in multiple tumor cell lines in response to a wide variety of anticancer drugs such as etoposide, hydroxyurea, and camptothecin (159). Our laboratory has established that the induction of senescence in tumor cells by doxorubicin was independent of telomere dysfunction and was not affected by telomerase expression unlike the case in replicative senescence (66). Chemotherapy-induced senescence in

tumor cells was also associated with a robust secretory phenotype (160,161). Interestingly, senescence was also induced in tumor cells exposed to non-DNA damaging agents such as the growth factor, TGFβ, or retinoids (162,163). Similar to human or mouse fibroblasts, exposure of tumor cells to ionizing radiation results in accelerated senescence (164). In fact, tumor cells respond much faster and to lower doses of radiation in comparison with non-transformed fibroblasts, perhaps because of a higher susceptibility of tumor cells to radiation induced DNA damage. Lastly, it was established that senescence represents a fundamental response to DNA damaging anticancer therapy *in vivo* in cancer patients exposed to neoadjuvant cancer therapy (doxorubicin, cyclophosphamide and 5-fluorouracil) (58,165).

It is now well-established that exposure to a variety of cancer therapeutics or radiation precipitate senescence in tumor cells, and that senescence is a primary component of the outcome of these therapies (166). The senescent response is not limited to conventional DNA damaging agents, but can also be induced by several targeted cancer therapies such as the CDK inhibitor palbociclib (116,117), aurora kinase inhibitors (113,114) and epidermal growth factor receptor inhibitors (167). Therapy-Induced Senescence (TIS) also contributes to the effectiveness of cancer treatment and represents an important prognostic factor (168). Due to its importance in dictating therapeutic outcomes, TIS is the main focus of this dissertation.

1.3 Senescence vs. quiescence

Since both, senescence and quiescence, describe states of cell cycle arrests, it is quite important to discuss the major differences between the two phenotypes. In addition, there is a great confusion in the literature based on experimental findings where cells exposed to cellular stress undergo a transient growth arrest, are strictly defined as quiescent. The reason is that *quiescence* classically

describes a state of transient, reversible growth arrest where cells arrest temporarily with a G_1 DNA content (referred to as G_0 phase, **Figure 2**) to resume proliferation when favorable conditions are restored (169). The less durable nature of the quiescent growth arrest represents the major feature to be taken in consideration in making a distinction between senescence and quiescence. However, the complexity of both phenotypes extends beyond the stability of the growth arrest. For example, quiescence is often induced by stress stimuli that differ from senescence-triggering factors (discussed above). Such stimuli include primarily mitogenic deprivation or lack of growth factor stimulation, something that senescence appears to be independent of (170). Furthermore, cells can undergo quiescence as a results of over-confluence and the resulting contact inhibition or from loss of adhesion (171). Senescence on the other hand is tightly linked to oncogenic genomic instability. Thus, the mechanisms by which both phenotypes are induced are remarkably different.

For sure, the quiescent growth arrest requires regulation by CDKI and it is true that $p21^{Cip1}$ accumulates in quiescent cells; however, it has been recently proposed that $p21^{Cip1}$ is more important in inducing a more durable growth arrest, which often is associated with senescence (169). Moreover, $p16^{INK4a}$ appears to play no or minimal roles in quiescence in comparison to its exceptional importance in maintain the hypophosphorylation of Rb in senescent cells, and thus, the stability of the growth arrest (172). G_0 is not just an extended phase G_1. Major changes in genes expression distinguish quiescent cells from proliferating cells, or even, from senescent cells (173). Furthermore, quiescent cells, due mostly to lack of proliferative stimulation, reduce their metabolic drive, very often reflected by a decrease in mRNA translation (174) (although this has been controversial since several provocative reports have shown that quiescent cells are metabolically active (175)). However, senescent cells are metabolically active and maintain active protein synthesis which results in the secretion of several proteins as part of the SASP (176). Lastly,

senescent cells possess a distinct morphology characterized by cellular enlargement (mostly due to increased biomass but failure to divide) while quiescent cells have a more heterogeneous cell morphology. The major differences between both phenotypes are summarized in **Table 1.**

Because of all these phenotypic differences between the two phenotypes, limiting the main difference in the pattern of growth arrest is inaccurate. In fact, this approach has hindered novel propositions trying to investigate the instability of the senescent growth arrest in certain experimental system, simply because a less durable arrest must be quiescent. This circular argument usually ignores careful phenotypic evaluation of both phenotypes, where in certain cases, a senescence-triggering stimulus results in a reversible senescent arrest in the absence of quiescence-related features.

	Quiescence	Senescence
Causes	Mitogenic deprivation, contact inhibition, loss of adhesion	Telomere dysfunction, oncogene overexpression, exposure to DNA damaging agents
Growth arrest	Transient	Durable
Phase of cell cycle arrest	G_0	G_1 or G_2
Associated CDKIs	$p27^{Kip1}$, $p21^{Cip1}$	$P16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$, $P15^{INK5b}$, Rb, p53
Response to mitogenic stimuli	Responsive	Irrelevant
Protein synthesis	Decreased	Increased
Secretory Phenotype	No	Yes
Enhanced lysosomal biogenesis	No	Yes

Table 1. Comparison between senescence and quiescence. Both senescence and quiescence describe patterns of cell cycle arrest. However, each phenotype is characterized by different hallmarks. Fundamentally, the senescent growth arrest is more stable, while the quiescent growth arrest is often resolved once the triggering stress has resolved. Senescence has been classically described a response to telomeric attrition, amongst other causes, that all together result in the activation of the DDR, while quiescence is more associated with nutritional and growth factors scarcity. The DNA content of quiescent cells is that of G_1 (*2n*) while senescent cells can have a variable DNA content (*2n*, *4n* or more). The CDKI involved in regulating both phenotypes are similar except for $p16^{INK4a}$ which is more characteristic for senescence. Senescent cells are metabolically active and synthesize a plethora of secretory proteins while quiescent cells downregulate ribosomal translation. Alteration in the lysosomal biocontent in senescent cells is reflected by upregulation of SA-β-gal enzyme, something that is not detected in quiescent cells.

1.4. Experimental identification of senescence

Since the senescence phenotype displays a high degree of complexity, differences amongst varying cell lines and the lack of a single unique, universal marker, experimental identification of senescence, both *in vitro* and *in vivo* can be tricky. Therefore, the use of a single marker to firmly detect senescence induction in an experimental system is insufficient. In that, a comprehensive assessment of multiple features of the phenotype is highly encouraged (177). Furthermore, the lack of one or more features of senescence does not necessarily negate the existence of senescent cells in culture or in viable tissues. For example, as mentioned above, senescent cells exhibit remarkable enlargement in cellular size *in vitro*, a feature that can easily be detected by bright field microscopy; however, such changes are very difficult to detect in animal tissues or human samples.

1.2.4.1 Detection of DDR activation.

A comprehensive confirmation of senescence generally requires assaying for components of the DDR, the initial triggering event in senescence induction. This includes the detection of phosphorylated ATM or p53 and the resulting phosphorylation of H2AX (γH2AX); the latter is an established marker of the formation of DNA double stranded breaks (55). Another commonly used marker of the DDR that is often measured as an indication of senescence-associated DNA damage is the formation of p53 binding protein 1 (53BP1) foci (55). In fact, for a more rigorous assessment of the formation of DNA damage foci and activation of the DDR, colocalization of γH2AX and 53BP1 foci as detected by high resolution fluorescent microscopy is considered ideal (178). This relocalization of 53BP1 is indicative of the recruitment and initiation of DNA repair processes (179). It is important to note here that the detection of these markers is likely to be successful early on following the exposure of a proliferating cell to a senescence-inducing stimulus e.g., the

exposure of a tumor cell to ionizing radiation, a time period where many other senescence features are not yet established.

1.2.4.2 Identification of cell cycle arrest

The most typically assayed features of senescence are components of growth arrest. As mentioned above, senescent cells contain variable DNA contents depending on which phase of the cell cycle the blockade was enforced. Thus, a traditional analysis of the DNA content (cell cycle analysis) using a nucleic acid binding dye, such as propidium iodide, provides a useful mean to detect and characterize the phase of the growth arrest (180). The majority of tumor cells exposed to conventional cancer chemotherapy are arrested in the G_2-M phase as they undergo senescence (181). Again, this could vary depending on the DNA damaging agent used. The proliferation potential of cells can be studied by a colony-forming assay which measures the capacity of cells to form a viable cluster of cells; an impaired ability of stably arrested senescent cells, as well as the detection of DNA synthesis proliferation markers such as using the BrdU incorporation assay (182). BrdU, which is a synthetic analogue of thymidine, is actively incorporated into newly synthesized DNA once available in the surrounding of a proliferating cell. i.e., in the culture medium. Measurement of BrdU incorporation provides a useful tool to assess the proliferative status of cells, and thereby the lack of proliferation that accompanies the senescent cell cycle arrest (182). However, lack of such proliferative markers can also be evident in quiescent cells and the interpretation of such observations should be done carefully, again, and in combination with other senescence markers. Lastly, the detection of induced or activated CDKI is another commonly used means to assay for senescence. p16^{INK4a} is the classical CDKI that is very often measured in aging fibroblasts and appears to show uniqueness to the senescent phenotype (183). p21^{Cip1} induction is also frequently measured as part of the DDR as well as a CDKI that plays an important role in

senescence (51). However, p21^{Cip1} is less specific to senescence than p16^{INK4a} since it could be induced of cases of non-senescent cell cycle arrest and quiescence (172). Because many tumor cells suppress p16^{INK4a}, the detection of p21^{Cip1} is very important to identify senescence in these cells.

1.2.4.3 Measuring the upregulation of SA-β-gal

The most widely used marker for senescence is SA-β-gal (184). SA-β-gal activity can be detected at pH=6 of the staining solution (relatively high pH) containing a chromogenic substrate of the enzyme that is converted to blue, insoluble product after being metabolized by SA-β-gal (184). The resulting staining can be readily detected by light microscopy. Recently, a plethora of fluorogenic substrates of SA-β-gal have been developed which allow for more robust analysis of senescent cells, including fluorescent microscopic identification of senescent cells, flow cytometry based quantification of SA-β-gal positive cells and flow cytometry based purification of senescent cells (185). More importantly, the plasma membrane is highly permeable to these fluorogenic substrates, which allows for the detection of live senescent cells in culture in comparison with the classical histochemical staining that requires fixing the cells/tissues. SA-β-gal is positive in almost all senescent cells and has been the gold standard assay for senescence as its upregulation reflects enhanced lysosomal biogenesis as discussed earlier (78). Despite its wide use and reliability, using SA-β-gal as an absolute marker of senescence has some limitations. For example, SA-β-gal is not entirely specific, and cells that are handled badly in culture or grown at high confluence can express some SA-β-gal activity without being actually senescent. Also, SA-β-gal is not always detectable in pre-malignant lesion (e.g., melanocytic naevi) harboring abundant oncogene-induced senescent cells (186). Finally, despite the established changes mitochondrial function and cell

membrane structure that occur during senescence, these are not frequently assayed for in this context (32). However, recent reports identified senescence associated surface markers that could serve useful in the future (187).

1.2.4.4 Detection of SAHF

The epigenetic modulations and structural changes in chromatin can also be assayed for in senescent cells. These can be simply monitored using fluorescent microscopy and nucleic acid stains which reveal bright, condensed foci of DNA (135). This could be improved by measuring the formation of specific histone modifications that are part of the SAHF such as H3K9Me3 and HP1 modifications (177,188). However, these markers are not absolutely specific to senescence and in certain occasions can be restricted to certain forms of senescence as well (172). Specifically, they have been closely associated with oncogene-induced senescence (135). On the other hand, downregulation of Lamin B1 has been now established as a valid marker of senescence (132). Lamin B1 levels decrease rapidly within the first few days of exposure to cellular stress, a decline that is not often observed in quiescent cells (132).

1.2.4.5 Measuring the upregulation of SASP

The SASP is the primary functional marker of senescence and reflects the dramatic changes in gene and protein expression that occur as part of senescence. The SASP comprises different families of both soluble and insoluble proteins, such as cytokines, chemokines, growth factors, structural matrix proteins and proteases (85). Despite the variability in the SASP profile amongst different cell types, some SASP factors appear to be more consistently associated with senescence. IL-6 is a proinflammatory cytokine that has been frequently observed as part of the SASP induced in both human and mouse cells and in response to different senescence-inducing stimuli

(85,93,189). Both α and β subtypes of IL-1 are also common components of the SASP of several types of senescent cells (190,191). IL-8 is a soluble chemokine that is often detected as part of the SASP along with IL-1 and IL-6 where they all create a regulated signaling loop (192). Several other chemokines of both the CCL and CXCL families are often expressed as part of the SASP (193). In addition, senescent cells secrete soluble growth factors such as multiple isoforms of IGFBP family (194). Interestingly, senescent cells can robustly secrete different matric metalloprotease (MMPs) that actively modulate the surrounding connective tissue (195). In further contribution to extracellular matrix (ECM) modulation, senescent cells also secrete structural, insoluble large proteins such as fibronectin (196).

The measurement of the upregulation of multiple SASP components that represent different functional groups is highly recommended. For example, a routine analysis of the SASP would include a cytokine (e.g., IL-1β), a chemokine (CCL_2), a metalloprotease (MMP-3) and a soluble growth factor (TGFβ). This comprehensive analysis is often coupled with measurement of a CDKI expression level (e.g., $p21^{Cip1}$) as confirmation of senescence induction. Fortunately, measurement of gene expression levels of these different molecules at the message level (mRNA) is widely accepted as those levels consistently reflect the change in their protein levels (93). Thus, quantitative real-time polymerase chain reaction (qRT-PCT) is routinely and reliably used to identify the alteration in gene expression in senescent cells. However, in certain occasions, the measurement of the secreted protein levels of certain molecules such as IL-6 using enzyme-linked immunosorbent assay (ELISA) can be adventitious. Overall, including an analysis of SASP expression has become an important component of any senescence identification testing panel.

In conclusion, senescence is a complex phenotype that encompasses a spectrum of characteristic features, each one of them lacks universality or specificity to senescence. Accordingly,

experimental detection of senescence should always consider the measurement of multiple senescence markers for a more careful assessment. In addition, time after which a senescent-inducing stimulus is introduced intro a biological system should be always considered as different features of the senescent phenotype have variable temporal profiles. Lastly, the heterogeneity of the senescence presents as an additional complexity as the gene expression alteration can vary among different senescent cells that belong to the same biological environment (197). In this work, we utilize a combination of these markers to identify the induction of senescence in our experimental system.

Chapter Two: Reversibility of Therapy-Induced Senescence.

2.1. Introduction

As discussed in detail previously, Leonard Hayflick published his seminal work on the replicative lifespan of dividing cells, which has subsequently been established in the scientific literature as the Hayflick Limit (7). In summary, his finding that normal cells had a finite capacity to divide was subsequently shown to be a consequence of telomere shortening that acted as a counting mechanism to determine the number of divisions the cell had completed; when the telomere reached a critically short length, division was terminated and the cells entered a state of terminal senescence (198). Initially, Hayflick's findings were not readily accepted by the scientific community since they contradicted the paradigm of Nobel Laureate Alexis Carrel that established the replicative immortality of cells dividing in culture (4,199).

The concept of terminal senescence has largely withstood the test of time since senescence is virtually always defined as an irreversible form of growth arrest (28). Unfortunately, adherence to this paradigm may have interfered with the acceptance of findings in the literature that suggest that senescence may be reversible (91,200). In this chapter, we will describe this previous literature that provides observations suggestive of the reversibility of senescence, focusing exclusively on the senescence induced by cancer chemotherapeutic drugs and radiation, which is largely unrelated to telomere shortening (24). It is very important to note here that this proposition does not indicate by any means that senescent cells revert back to the non-senescent state completely in a similar fashion to a reversible chemical reaction; instead, the reversibility of senescence describes the

ability of cells to overcome the stable growth arrest and resume proliferation. The term "senescence" will always describe stable forms of growth arrest in most cases, especially when aging fibroblasts undergo replicative exhaustion, i.e., classical senescence. Accordingly, escaping senescence happens only in limited cases, such as during transformation when oncogene-induced senescence is bypassed by the accumulation of oncogenic mutations, or as we are proposing here, following exposure of genetically unstable tumor cells to senescence-inducing anticancer therapy.

There are several reasons why we think that experimental testing of the spontaneous escape of tumor cells from senescence is important to establish. While the intent of radiation and chemotherapy is to kill the malignant cell, generally through the promotion of apoptosis or other cell death pathways, it must be recognized that not all tumor cells will of necessity undergo cell death upon being subject to therapeutic strategies. Recognizing the heterogeneity of any tumor population, it is not difficult to imagine that there are some cells that are likely to evade the cytotoxic effects of treatment and undergo growth arrest as an alternative response. One type of growth arrest will be that of quiescence from which the cells can ultimately recover (201). In the case of irradiation or DNA damaging drugs, this is generally thought to occur when the DNA is repaired. However, in this context, a generally circular argument is often advanced that when tumor cells are shown to re-emerge from the growth arrested state, then by definition the growth arrest must have reflected quiescence rather than senescence, since senescence is virtually always considered to be irreversible (202). It is, however, now well-established in cell culture, in tumor bearing animal models, as well as in clinical samples, that senescence is not infrequently a primary response to therapy (58,181,203,204), and that senescence could possibly be reversible.

The consequences of Therapy-Induced Senescence (TIS) are not well understood. In terms of cell autonomous effects, a fundamental question is whether senescence is beneficial or undesirable

with regards to therapeutic outcomes. If TIS is truly irreversible, then this would be partially considered a desirable if not ideal outcome since the tumors driven into this state should never re-emerge (166). Moreover, it is further possible that, as suggested by some previous work, that senescent cells may be recognized and eliminated by the immune system, which would convert growth arrest into cell death (205). However, if TIS has a potential to be reversible, this could have serious clinical ramifications. That is, the senescence response may provide an avenue whereby tumors escape from drug and radiation killing only to remain dormant at either the primary tumor site or an occult niche after having metastasized. In addition, senescent tumor cells may represent a component of minimal residual disease that is often associated primarily with circulating tumor cells. This senescent cell population, which is generally considered to be resistant to apoptosis (21) could ultimately contribute to disease recurrence. Lastly, since, virtually by definition, dormant tumor cells in patients are occult and difficult to be identified (being few in number and inaccessible) (206), and mostly revealed in autopsies (207), one can only extrapolate from studies in cell culture and ultimately in animal models whether, in fact, senescent cells can give rise to resurgent tumors.

2.2 Evidence for the reversibility of Therapy-Induced Senescence (TIS).

As indicated above, the debate about the reversibility of senescence has suffered from the circular argument that if cells are observed to recover from a state of senescence, then the recovering cells could not have originated from the senescent population. Consequently, researchers have often resorted to terminology such as such as "senescence-like" and "pseudo-senescence" in reference to cells with characteristics of senescence that have escaped from prolonged growth arrest (208–

210). While this terminology is acceptable, it tends to minimize the validity of the observations that cells exhibiting certain features of senescence can overcome the durable growth arrest.

Early studies from our group have demonstrated that clinically relevant concentrations of adriamycin induce p53-dependent cellular senescence in MCF7 breast tumor cells (26). In this study, a small population of MCF7 cells recovered from acute adriamycin exposure and a senescence-resistant colony was isolated. This colony, which recovered from the senescent population, stopped responding to subsequent exposures to doxorubicin by undergoing senescence (26). In comparison with parental cells exposed to doxorubicin, the senescence-resistant colony expressed higher levels of proliferative cell cycle regulators, such as cyclin B1 and E2, PCNA and cdc2, following adriamycin treatment. In fact, continuous expression of cdc2 is more likely to account for the ability of these cells to evade senescence. Furthermore, this resistance was not due to decreased intracellular accumulation of adriamycin or because of an attenuated DDR since the resistant colony continued to respond to adriamycin by p53 and p21^{Cip1} induction (26) . These initial experiments suggested that certain tumor cells with altered genetic background can possibly evade senescence or at least acquire senescence-resistant traits.

A number of reports by the Wu group at the University of Washington have largely supported the ability of tumor cells to evade therapy-induced senescence (89,209,211). In one study, H1299 non-small cell lung cancer (NSCLC) cells, which are null in p53 and deficient in p16^{INK4a}, were induced into senescence by camptothecin and other chemotherapeutic agents such as etoposide and cisplatin (89). Since the choice of a cell line lacking pivotal senescence regulatory proteins could be a deficiency, the authors indicated that genes encoding for p53 and p16^{INK4a} are frequently mutated in clinical cancer and this model is representative for clinical cancer. In this case, senescence induction in H1299 cells was confirmed based on large, flat morphology, increased

granularity, retention of PKH2 (a cell membrane staining dye— retention is suggestive of lack of cell division) and finally, upregulated SA-β-gal expression. Interestingly, a small population of senescent cells were subsequently observed to regain proliferative capacity resulting in the formation of colonies 3 – 4 weeks after removal of drug; the frequency of escape was 1 in 10^6 cells suggesting that (*i*) the stability of the senescent growth arrest is the more predominant phenotype in agreement with the established conception of senescence and that (*ii*) escape of tumor cells from senescence is a rare event (89). In disagreement with the Elmore et al. study, where the senescence-resistant cells developed cross-resistance to multiple senescence-inducing drugs (26), the senescence-escaped cell colonies were found to have similar sensitivity to camptothecin as the parental H1299 cells, indicating that these cells did not arise from a senescence-resistant H1299 cell population (89). However, this escape from senescence was also associated with sustained expression of cdc2, suggesting that it plays an important role in evasion from senescence. Interestingly, cdc2 levels were elevated in clinical tumor samples from patients who received adjuvant chemotherapy and developed abundant tumoral senescence as marked by SA-β-gal staining (89).

Polyploidy, which reflects cells containing more than three times the number of haploid chromosomes, is a common feature of senescent cells. In a follow up study by the Wu group, Wang et al. demonstrated that many camptothecin-induced senescent H1299 cells are polyploid (209). Interestingly, 40% of these polyploid senescent tumor cells were able to uptake EdU several days after senescence induction, suggesting that they could still undergo DNA replication. Using live cell imaging and H2B-RFP labeling (that allowed fluorescence visualization of the nucleus), Wang et al. also showed polyploid senescent cells escaping from senescence. To confirm the live imaging study, camptothecin-induced senescent cells were sorted based on size and the large, polyploid

cells plated. Colonies were observed 7 – 10 days after seeding (209). Again, escape from senescence was found to be related to overexpression of Cdc2 in agreement with the previous observations (26,66). It was further demonstrated that therapy-induced cell senescence in patients with locally advanced NSCLC was associated with poor prognosis. Wang et al. found that survivin, which is downstream of Cdc2, is upregulated and plays a role in escape from senescence, as well as the subsequent viability of the escaped cells (211).

Polyploidy is likely to be a requirement for recovery from senescence since only multinucleated cells will have the capacity to generate replicating daughter cells. Studies by Mosienak et al identified polyploidy in HCT116 cells where senescence was again induced by doxorubicin (212). When polyploidy was abrogated, the cells were unable to escape from senescence. Moreover, MCF-7 cells that were shown to undergo senescence but not polyploidy upon doxorubicin treatment were unable to escape from senescence (212).

Was et al. also established the reversibility of senescence in their study where HCT116 cells were treated with 3 cycles of 24 h doxorubicin and 72 h drug-free treatment (213). Senescence was evident after 13 d based on flattened cell morphology, cell enlargement and increased granularity, SA-β-gal expression, as well as secretion of VEGF and IL8, components of the SASP (213). Here, also, senescent cells were shown to exhibit polyploidy. A 6-fold increase in cell number 2 weeks following removal of doxorubicin indicated an escape from senescence. Time-lapse imaging revealed proliferating cells in the polyploid, senescent cell population. Consistent with reports in literature of senescent cells exhibiting stem cell features (214,215), CD24+ (about 1.5% of cells) and NANOG exhibiting stem cell-like cells were also observed in the treated cell population (213).

Doin et al. demonstrated that cisplatin treatment both *in vivo* and *in vitro* results in an initial cessation of cell division and proliferation that is eventually succeeded by cells recovering from

growth arrest (216). Cisplatin treatment of PROb cells *in vitro* resulted in significant cell growth arrest but continued DNA replication. Again, live cell imaging using the fluorescent H2B-GFP showed the emergence of largely senescent giant polyploid cells which eventually disappeared due to depolyploidization and resumption of cell proliferation through atypical mitotic division (216).The observation of colonies of cells arising from the senescent cells lends further credence to the reversibility of senescence. When cisplatin was administered to PROb colon cancer tumor bearing BD-IX rats, there was significant tumor shrinkage characterized by giant polyploid cells, most of which stained positive for SA-β-gal (216). Tumors were observed to resume growth 1 month after drug administration during which time SA-β-gal was significantly absent, indicating that cisplatin treatment delayed but did not stop tumor growth (216).

The development of polyploidy followed by depolyploidization and atypical mitotic division was also demonstrated by Rohnalter et al. (217). In this study, carboplatin-treated SKOV3 ovarian cancer cells proceed through a series of stages that result in a chemoresistant phenotype (217). The multi-staged process included mitotic catastrophe, polyploidization, cell cycle arrest and senescence followed by the acquisition of stemness, increased cell divisions, depolyploidization, and eventually chemoresistance at 21 weeks following treatment. One of the most distinct features of this multi-staged process was the presence of giant cells that exhibit polyploidy. The process was accompanied by an initial increase in p21^{Cip1} that peaks at day 8 and begins to decrease as cell division increases and giant cell numbers decrease (217). The emergence of a subpopulation of cells that are capable of cell division and are chemoresistant is suggestive of the reversibility of carboplatin-induced senescence and the possible contribution of senescence to tumor resurgence or treatment relapse.

Sabisz and Sklandanowski studied senescence and escape of a subpopulation of A549 NSCLC cells (208). Here, senescence was induced with the topoisomerase II inhibitor etoposide. Similar to the Wang et al. study, about 20% of the senescent cells were found to exhibit polyploidy over the course of the study. Levels of p53 and p21^{Cip1} were also found to increase progressively while Cdc2 and cyclin B1 increased after 1-2 d and decreased beyond detection at day 5 (208). Following drug treatment for 5 days, cells were incubated in drug-free media and between 1 and 3% of the cell population was observed to regain proliferative capacity at 10 days post-treatment. Sabisz and Sklandanowski determined that about 1.1% of the treated cells expressed markers of cancer cell stemness (CD34 and CD117); given that this number was very similar to the senescence-escaped cell population, it was suggested that the two cell fractions could be the same, and that cells escaping senescence acquire stem cell-like characteristics (208). For example, a study by Achutan et al involving multiple breast cancer cell lines (MCF-7, MDA MB231, and T47D) and primary tumors also revealed that cells that escaped from doxorubicin-induced senescence could be from the cancer stem cell population (210). The small population of cells that escaped senescence were found to exhibit stem cell characteristics and to express increased levels of the stem cell marker CD133 (210).

The cornerstone of these studies is a recent report by Clemens Schmitt group. These studies were based on the emerging association between senescence regulatory pathways and stem cell functions or "stemness" (218). In agreement with previous observations, Milanovic et al demonstrated that a single exposure of Eμ-Myc – Bcl2-overexpressing lymphoma cells to adriamycin (0.05 μg ml^{-1}) resulted in a robust senescence induction (marked by over 80% SA-β-gal staining) and an accompanying increased expression of stem cell related genes as well as elevated activity of aldehyde dehydrogenase (ALDH) and ATP-binding cassette (ABC), both

associated with stem cell function (90). Importantly, enhanced stemness was not detected in cells exposed to the same concentration of adriamycin but that failed to undergo senescence (due to the absence of Suv39h1 enzyme which is responsible for the senescence-associated epigenetic signature H3K9Me3) (90). These studies established that enhanced stemness is a feature of chemotherapy-induced senescence in tumor cells. Using an inducible expression model for p53 and Suv39h1, the authors were able to deactivate these pro-senescence proteins and facilitate resumption of S phase activity after adriamycin-induced senescence (marked by EdU staining and gradual decline in SA-β-gal activity). Although the reversal of senescence was not fully spontaneous, the authors concluded that p53 inactivation is a common aberration in tumor cells and the possibility of evading the permanent cell cycle arrest is strong. In fact, they stated that "senescence is, in principle, a reversible condition, which becomes evident when essential senescence maintenance genes are no longer expressed" (90). Moreover, the cells that escaped senescence were able to undergo senescence again in response to adriamycin, indicating that no senescence-resistance phenotypes had developed. An additional interesting finding of this work is that cells that escaped TIS and acquired stem cell properties were more aggressive, forming rapidly growing colonies *in vitro* and more malignant tumors when implanted *in vivo* (interestingly, in immunocompetent mice). In summary, TIS results in extensive pro-stemness genetic reprograming that facilitates escape from the permanent growth arrest and possibly represents a component of relapsed cancer. Another important conclusion from this work is that tumor cells that escaped senescence are more malignant.

In support of the Milanovic et al study, Yang et al also demonstrated that A549 NSCLC cells exposed to adriamycin (100 nM) for 7 days can resume proliferation after 21 days of drug-free culture (219). However, unlike the studies in the lymphoma cells where p53 inactivation was

necessary for escaping senescence, Yang et al. demonstrated a spontaneous reversion of A549 senescent cells into the proliferative state (219). Lastly, the cells that escaped senescence exhibited more invasive and migratory properties.

These findings of reversible senescence are not limited to chemotherapeutic drugs. Senescence induced by ionizing radiation has also been found to be reversible in some studies(164,220,221). Chitikova et al. described the reversal of radiation-induced senescence in apoptosis-resistant E1A+E1B cells (221). Cells were shown to arrest in the G_2-M phase of the cell cycle while DNA replication significantly decreased 24 h following ionizing radiation treatment (221). Resumption of DNA replication occurred 48 h after treatment with an attendant polyploid cell accumulation. Polyploid cells were enlarged, flattened and expressed SA-β-gal, consistent with senescence. Cell proliferation following the induction of senescence was observed 7 d post-irradiation. Increased expression of stem cell markers NANOG and OCT3/4 was also observed with the proliferative recovery (221).

In our own studies with MCF-7 breast tumor cells, widespread senescence was observed as demonstrated by SA-β-galactosidase expression with 10 Gy ionizing radiation (164). Continuous culture of the senescent cells revealed proliferative recovery of the cells. We have also demonstrated in our work with DNA repair proficient and deficient HCT116 cells, that radiation-induced senescence associated cell growth arrest was ultimately reversible with or without Poly (ADP-ribose) polymerase (PARP) inhibition (220).

In summary, multiple observations in the literature point out with experimental evidence the potential of tumor cells to re-enter the cell cycle after senescence. However, these conclusions relating to the re-emergence of tumor cells from senescence have generally been based on studies in mass culture, where the origin of the replicating cells cannot be unequivocally attributed to the

senescent population. Furthermore, the interpretations of most of these observations were kept fairly conservative, largely to avoid generating provocative postulates that might appear to undermine the prevailing paradigm.

2.3 Dissertation Objectives

The main objective of this dissertation is to directly address the hypothesis that *a subpopulation of tumor cells induced into senescence by genotoxic conventional cancer therapy can re-acquire proliferative capacity after a period of cellular dormancy to form viable colonies and growing tumors in vivo*. The observational evidence should unequivocally demonstrate that any restoration of growth from a presumably senescent population should originate from a senescent cell and not from non-senescent cells that were either resistant to the anticancer agent or have been temporarily arrested without displaying senescence hallmarks. The specific aims for this work are:

Specific aim 1: *To induce a robust senescent phenotype in tumor cells based on the identification of multiple senescence hallmarks.*

For this aim, senescence will be induced in models of lung and colorectal cancer. Lung cancer and colorectal cancer represent the second and third most common cancers that affect both sexes; both types of cancer account for the majority of cancer-related deaths, with lung cancer being the most common cause of cancer death in the United States (222). Lung cancer is histopathologically divided into non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC represent the majority of lung cancer cases (~85%) and consequently, the majority of cancer-related deaths. Accordingly, we will be focusing on NSCLC and using primarily the H460 NSCLC (large cell carcinoma) and A549 NSCLC cell (adenocarcinoma) cell lines. Both cell lines are frequently used as models to study molecular processes involved with lung cancer. Lung cancer is

initially a silent disease which represents a diagnostic problem. Therefore, many cases of NSCLC are not diagnosed until later in the disease where most patients present with unresectable tumor. Consequently, the standard of care for the treatment of late-stage lung cancer is chemotherapy.

In patients with overexpression of the epidermal growth factor receptor (EGFR), which involves approximately 10-15% of the population, a number of new drug therapies have demonstrated various degrees of success (223). In this context, a number of other targeted therapies are under investigation with the goal of developing more individualized approaches based on both molecular and histological characterization (224,225). Of these novel therapies, drugs that target the Anaplastic Lymphoma Kinase (ALK) have shown significant reductions in tumor volume or maintenance of a stable disease state in a subset of lung cancer patients with rearrangements in the *ALK* gene; such fusion lesions render cells dependent on ALK signaling and susceptible to the actions of tyrosine kinase inhibitors (226). In almost all cases, ALK genetic rearrangements have been linked to mutations in other oncogenes such as EGFR and K-Ras and, interestingly, in patients who are mostly young and with low or no history of tobacco consumption (226,227). In addition, several immunotherapies have also been developed as second-line treatment for advanced cases that fail to respond to platinum therapy. Nivolumab, an anti PD-1 receptor monoclonal antibody, was approved by the FDA in 2014 and has shown acceptable clinical efficacy in patients with terminal squamous cell carcinoma and overall survival superiority over other checkpoint inhibitors (228,229). There is no evidence thus far that check point inhibition therapy will induce senescence in tumor cells, or that EGFR inhibitors can induce senescence in lung cancer cell lines *in vitro* (167).

Despite these considerable advancements in targeted therapy of lung cancer, the primary therapeutic strategy for most patients with advanced disease remains radiation therapy in

combination with cytotoxic chemotherapy, i.e. chemoradiation. The primary drugs currently used in chemoradiation are cisplatin and carboplatin (platinum-based, DNA cross-linking), paclitaxel and docetaxel (microtubule poisons) and etoposide (topoisomerase II inhibitor) (230). Microtubule poisons show higher efficacy against lung cancer; however, paclitaxel for example is a weak senescence inducer, mostly because it does not robustly activate the DDR and exerts its effects through disruption of mitosis (181). Cisplatin on the other hand has been shown to robustly induce senescence in lung cancer cell lines, especially at subtoxic concentrations (231). Topoisomerase poisons have been used as potent senescence inducers, not only in tumor cells, but in a variety of other cell lines (58). While adriamycin is the most established senescence-inducing agent, it is infrequently used as primary treatment of lung cancer, and thus, we will be focusing on etoposide, another topoisomerase II inhibitor, that can also robustly induce senescence in lung tumor cells (232). It is important to note here that the experimental use of such drugs will be completely for the purpose of inducing senescence in these lung cancer models rather than stimulating their clinical use, especially in cell culture studies. Lastly, senescence identification in the lung cancer cells will be evaluated based on available senescence hallmarks (discussed in chapter 1) relying heavily on SA-β-gal assay as it is the classical, most accepted approach to evaluate senescence (184).

Specific aim 2: *To directly test the possibility of escaping Therapy-Induced Senescence in vitro using both lung cancer and colon cancer cell lines.*

After establishing a senescent state in tumor cells, the tumor cells will be monitored over time for signs of proliferation. These observations will be recorded in mass, heterogeneous population of cells exposed to etoposide for senescence induction as well as in a senescence-homogenous population where senescent cells will be isolated using flow cytometry based on senescence

markers. Our approach will utilize flow cytometry based purification of highly senescent cells in combination with single cell analysis and direct cell lineage monitoring to ensure that any evidence of proliferation or escape from the cell cycle arrest is originating from a previously senescent cell.

Specific aim 3: *To test if senescent tumor cells can form viable tumors in vivo.*

An important aim of this work is to establish if a tumor cell recovering from senescence can form a viable tumor upon introduction into mouse. The impact of the immune system on the surveillance of senescent cells and their potential escape from senescence is profoundly important. It is now appreciated that select cytotoxic chemotherapies and radiation can enhance tumor cell immunogenicity by promoting immunogenic cell death (ICD) (233). A requirement of ICD is therapy induced autophagy which, when left unresolved, results in apoptosis. Under conditions where TIS results in an immune suppressive SASP, the benefits of ICD can be lost. This could happen frequently as therapy treated tumors will likely contain a mixture of cells undergoing immune stimulating ICD and immune suppressive TIS (234).

Generally, in those circumstances where therapy induces ICD, the immune response can eliminate the tumor cells (235). However, TIS is less deterministic as it can promote both pro- and anti-tumor immune activities (236). Central to the variability of the immune response to TIS is the SASP (85). The SASP is capable of attracting and further differentiating a variety of pro- or anti-tumor immune cells in the tumor microenvironment including natural killer (NK) cells, neutrophils, monocytes/macrophages, and T cells (237). How immune cells respond to the SASP depends on which chemokines are secreted, which in turn depends on how the tumor cell responds to chemotherapy (238). Depending on this response, SASP can lead to the elimination or the protection of tumors cells, where the latter could represent a key element for maintaining tumor cell dormancy and its potential recurrence (239). However, the consequences of TIS to the

antitumor immune response are less well characterized, largely due to difficulties in generating a pure population of senescent cells to study, in contrast to OIS, which has been studied in multiple contexts and has been reviewed extensively (240).

However, since tumor cell lines that we will be utilizing in our studies will be primarily of human origin, *in vivo* approaches will be restricted to immunodeficient animal models. The impact of the immune system on TIS is not a focus of this project, but the ability of senescent cells to form tumors following recovery will be tested experimentally.

Specific aim 4: *To test possible approaches to selectively eliminate senescent tumor cells in effort to enhance the efficacy of cancer therapy and possibly interfere with recovery from senescence.*

Recent efforts have shown that the selective removal of senescent cells from progeroid animals delays the onset of several aging phenotypes and ameliorates already established pathologies (241). This approach, which was based on a genetic model of a p16^{INK4a} suicide gene has been extended to identifying novel molecules that can eliminate senescent cells in a similar fashion; such drugs are referred to as senolytic agents (242,243). The term "senolytic" describes the ability of these drugs to selectively eliminate senescent cells. These promising efforts have been largely restricted to progeroid models (with little focus on senescent tumor cells) or to merely experimental therapies. Our goal here is to use these novel compounds, primarily the Bcl-2 inhibitor, ABT263 (Navitoclax), to eliminate senescent tumor cells that have been exposed to chemotherapy. Furthermore, we will be testing whether the use of ABT263 to eliminate senescent cells will attenuate the ability of tumor cells to recover from senescence.

Specific aim 5: *To investigated the impact of autophagy inhibition with the ability of cells to recover from Therapy-Induced Senescence.*

Autophagy has historically been considered as a defensive mechanism that is observed under conditions of cellular stress, responsible for metabolic compensation and organelle turnover (244). However, multiple other functions of autophagy have been characterized in different cancer cell lines, where it can play an anti-survival role or act as a cellular mechanism mediating sensitization to therapy (245).

Many of the chemotherapeutic agents that are commonly utilized in the treatment of non-small cell lung cancer and induce cellular senescence have been also shown to induce autophagy in parallel (140). Since that the metabolic stability of senescent cells is required for their survival, we will be testing the effect of autophagy modulation on senescence maintenance and, more importantly, whether recovery from senescence is dependent on autophagic metabolic regulation.

2.4 Summary

It is not difficult to imagine that the majority of tumor cells undergo cell death and recruit a robust immune response to cytotoxic therapies, leaving small and undetectable subpopulations of cells hiding in a dormant state. While having a pivotal impact on the natural history of cancers, we have a limited understanding of tumor dormancy. We know that it represents a phase where tumor cells persist in an occult state as part of the post-treatment residual disease (246). Furthermore, the mechanisms by which tumor cells transition into the dormant state, at primary or distant sites, and then restore growth capacity are still speculative (246). In addition, the stimuli that promote escape of dormant tumor cells remain to be identified. There is a general agreement that dormant tumor cells are in a quiescent state, simply because quiescence is, by definition, reversible (202). Ironically, all of the postulated mechanisms that would theoretically facilitate the recovery of dormant cells can be closely associated with senescence (202,247). For example, dynamic

alterations of the microenvironment and restoration of blood supply, critical events contributing to the capacity of dormant tumor cells to recover (248), are heavily influenced by SASP factors such as matrix metalloproteinases and angiogenic promoters such as VEGF. Moreover, as discussed earlier, senescent cells not only interact with, but also modulate the immune system, thus possibly contributing to evasion of immunosurveillance. In addition, senescent cells are resistant to apoptosis, metabolically active and can persist *in vivo* for prolonged periods.

A primary purpose of this work has been to propose the argument for senescence as model of tumor dormancy, wherein a subpopulation of cancer cells that escape cell death following bouts of cytotoxic therapy undergo senescence and persist for weeks or even months. These dormant, senescent cells secrete an array of soluble and non-soluble molecules that gradually alter the surrounding tissue and slowly promote angiogenesis (249). Eventually, a few senescent cells that manage to undergo proliferative recovery and escape immunosurveillance would be competent to take advantage of the changes in the ECM and the restored blood supply to re-initiate tumor formation. However, this proposed model requires the experimental validation that senescent tumor cells can actually evade the ***non-permanent*** growth arrest which is the main aim of this work.

Chapter Three: Recovery of tumor cells from a senescent state induced by etoposide.

3.1 Introduction

It is often thought that tumor cells exposed to chemotherapy or radiation all experience an identical fate; however, the response(s) are more likely to be heterogeneous (250). These can include cell death (often through various forms of apoptosis and/or mitotic catastrophe) as well as variable forms of growth arrest that include autophagy, quiescence and senescence (58,204,251–253). Not all of these processes will be strictly terminal, with the consequence that some tumor cells may survive and contribute to disease recurrence.

Cellular senescence is a well-established, highly-programmed response to various DNA damaging antitumor modalities wherein the cells enter into a durable and prolonged growth arrest that is generally considered to be irreversible (172,181,254). The senescent phenotype is characterized by a spectrum of distinctive features (32). These hallmarks include activation of the DNA damage repair response (255), enhanced lysosomal biogenesis as indicated by the upregulation of the classical Senescence-Associated-β-galactosidase (SA-β-gal) enzyme (78), extensive alterations in gene expression (82,83,256), and epigenetic modifications collectively referred to as Senescence-Associated Heterochromatin Foci (SAHF) (188,257). An additional hallmark of senescence is the Senescence-Associated Secretory Phenotype (SASP), wherein cells secrete a spectrum of chemokines and cytokines, many of which are thought to promote tumor growth (85). Quiescence is a more transient form of growth arrest from which cells are observed to rapidly recover,

presumably as a consequence of effective and efficient DNA repair that allows the cells to circumvent checkpoint-mediated arrest.

The durability of the growth arrest is a primary characteristic that distinguishes senescence from quiescence (172). Although a permanent abrogation of tumor growth (i.e. reproductive cell death) would be one desirable outcome of therapy short of over cell killing, there is evidence that senescent arrest in tumor cells is not uniformly durable For instance, it has long been evident that senescent cells retain inherent reproductive potential since transformation and immortalization involve escape from replicative or oncogene-induced senescence (258–260). Furthermore, in a recent report by Milanovic et al, senescence was noted to be inherently reversible only to be stabilized into a permanent state when the senescence maintenance program (stable cell cycle arrest, heterochromatin formation and repression of gene expression) is expressed (90). Moreover, those tumor cells induced into senescence by chemotherapy acquired aggressive, stem-cell like characteristics upon escape from the cell cycle blockade (90). More recently, breast tumor cells induced into a senescent-like growth arrest by MDM2 inhibitors were shown to re-acquire proliferative potential over time (261). In attempt to summarize these observations, a review by our research group provides an overview of the capacity of cells to escape from the three different forms of senescence (91). With regards to therapy-induced senescence (TIS), reports by our group as well as other laboratories have presented additional evidence in support of the premise that tumor cells in a state of senescence are not obligatorily in a terminally growth-arrested state (reviewed in chapter 2). However, conclusions relating to the reversibility of senescence have generally been based on studies in mass culture, where the origin of the replicating cells cannot be unequivocally attributed to the senescent population.

In this chapter, we present experimental evidence of senescence induction in tumor cells in response to etoposide exposure. We also provide a temporal analysis of senescent tumor cells growth and key molecular signatures following termination of treatment. Furthermore, we have attempted to overcome one of the primary limitations of previous studies (including our own) by utilizing two complementary experimental strategies. One was to utilize flow cytometry based enrichment to focus the analysis on a more homogeneous populations of senescent cells, and second was to monitor changes in single senescent cells using live microscopic imaging techniques. Lastly, we further implant the senescent-enriched cells into NSG mice to assess their capacity to grow out and form tumors *in vivo*.

We observe heterogeneous responses to the antitumor drug, etoposide, utilized at subcytotoxic concentrations. Some cells enter and remain in a senescence-like state for the duration of the experiment while some cells undergo cell death. However, a subpopulation of the cells expressing hallmarks of senescence and then enriched for SA-β-gal expression appears to be capable of overcoming a prolonged, yet, transient growth arrest to form proliferating colonies and viable tumors in mice. It could be and is, frequently argued that these cells were not actually senescent since they recovered proliferative capacity, but only expressed senescence markers and efforts to identify the factors that distinguish truly irreversibly arrested senescent cells from senescent like cells that generate proliferating progeny are under way. However, it appears possible that a senescence-like state that is capable of giving rise to actively dividing cells could represent one previously unrecognized form of tumor dormancy; furthermore, cells that arise from the senescent-like state and re-emerge into a proliferative state could also ultimately contributes to disease recurrence.

3.2 Experimental procedures

3.2.1 Cell Lines.

The H460 cell line was generously provided by Dr. Richard Moran. H460 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (SH30066.03, Thermo Scientific, MA, USA), 100 U/ml penicillin G sodium (15140–122, Invitrogen, CA, USA), and 100 μg/ml streptomycin sulfate (15140–122, Invitrogen, CA, USA) at 37º C and 5% CO_2. The HCT116 (BTG1-RFP) cell line was developed at the lab of Dr. Igor Roninson at The University of South Carolina. HCT116 cells were transduced with a lentiviral BTG1-RFP (dsRed) construct. Clonal cell lines were established from the BTG1-RFP transduced cells and clones that provided the strongest signal and induction by chemotherapy were identified. BTG1 was selected following a microarray analysis of genes that show altered expression in tumor cells that become senescent following exposure to chemotherapy (262). HCT116 were maintained in culture similar to H460 cells. Etoposide (VP-16-213) was obtained from Sigma-Aldrich, MO, USA (E1383).

3.2.2 Cell Viability and Clonogenic Survival.

Growth curves were determined by counting viable cells based on trypan blue exclusion at various time points after the treatment. Cells were harvested using trypsin, stained with 0.4% trypan blue (T8154, Sigma, MO, USA), and counted using a hemocytometer under light microscopy. The assay is based on the fact that viable cells have intact plasma membranes and thus impermeable to trypan blue. Colony forming assay measures the cell survival and the ability of cells to form viable colonies. For that, H460 cells were seeded at the indicated densities and allowed to proliferate. At

the indicated time points, cells were harvested, washed with PBS and fixed with methanol. Fixed cells were allowed to dry overnight and then stained with crystal violet (0.01%).

3.2.3 SA-β-galactosidase Staining/Enrichment.

Histochemical SA-β-gal labeling was performed as previously described by Dimri et al. (80). Images were generated using bright field by Olympus inverted microscope (Olympus inverted microscope IX70, 20x objective, Q-Color3™ Olympus Camera; Olympus, Tokyo, Japan). The $C_{12}FDG$ staining protocol was adopted from Debacq-Chainiaux et al. (185). At the indicated time points, cells were harvested, washed with cold PBS, and analyzed by flow cytometry (using BD FACSCanto II and BD FACSDiva software at the Virginia Commonwealth University Flow Cytometry Core Facility). Similarly for fluorescent microscopy, at the indicated time points, cells where exposed to bafilomycin A1 (100 nM) for 1 h, followed by incubation with $C_{12}FDG$ (33 μM) for 2 h. Cells were washed 3 times with PBS and nuclei were stained with Hoechst 33342 for 20 minutes in complete media. Microscopy was performed with an Olympus inverted microscope. For the enrichment assay, both H460 and HCT116 (BTG1-RFP) cells were seeded at $1\text{-}2 \times 10^6/150$ mm dish and cultured overnight before being exposed to etoposide (1 μM) for 24 h. On days 3 and 7 for H460 cells and day 3 for HCT116 (BTG1-RFP) cells, the cells were exposed to bafilomycin A1 (100 nM) for 1 h, followed by incubation with $C_{12}FDG$ in complete medium for 2 h. Cells were then harvested and sorted by flow cytometry based on parameters displayed in the supplementary data.

3.2.4 Confocal Microscopy

For γH2AX and H3K9Me3 staining, H460 cells were seeded in 4-well chamber slides (Nunc Lab Tek, Sigma-Aldrich, MO, USA). Cells were treated with etoposide (1 μM) for 24h and within 48

hours (or 6h for γH2AX), slides were processed for immunofluorescence. Cells were washed with 1X PBS and fixed with 1mL of ice-cold 4% paraformaldehyde (PFA) for 10 minutes. Excess PFA was removed by washing with PBS. Cells were then treated with a permeabilizing solution (0.5% Triton X-100 in PBS) for 10 mins and excess solution was removed by further washes with PBS. Cells were blocked using 1X PBS 1% Casein blocker (1610783, Bio-Rad, CA, USA) for 1 hour at room temperature. Subsequently, cells were treated with 250 µL of the appropriate primary antibodies (mouse monoclonal anti-γH2AX at 1:2500 (05-636, mouse, rabbit, Millipore, MA, USA) and rabbit anti-H3K9me3 (ab8898, Abcam, UK)) overnight at 4°C. After three cycles of washing with 1 mL of PBS for 10 min (for γH2AX staining, washing was performed using 1mL of 0.1% TritonX-100 in PBS), cells were incubated with 250µL of appropriate secondary antibodies: goat anti-mouse AF488 at 1:5000 (A21121, Thermo Fisher Scientific, MA, USA), goat anti-mouse CFL594 at 1:250 (sc-362277), goat anti-rabbit AF594 at 1:250 (35560, Thermo Fisher Scientific, MA, USA), for 3 hours at room temperature. Excess antibodies were removed by washing and cells were post-fixed using ice-cold 4% PFA for 10 min. For p21$^{Waf1/Cip1}$ staining, cells seeded in chambers were stained with anti-p21 antibody (610234, BD Biosciences, NJ, USA) as described above. After overnight incubation, cells underwent lysosomal alkalization by exposure to bafilomycin 1A (100 nM) for 1 h, followed by incubation with $C_{12}FDG$ (33 µM, D2893, Thermo Fisher, MA, USA) in complete medium for 2 h at 37°C. The nuclei were counterstained with Vectashield mounting medium containing 1.5 µg/mL 4′-6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories, CA, USA). Immunofluorescence was observed with the Zeiss LSM700 Confocal Laser Scanning Microscope equipped with a 63X, 1.4 NA oil immersion objective, located in the Virginia Commonwealth University Microscopy

Facility. Confocal images were obtained using a 405 nm diode laser (DAPI), a 488 nm laser (AF488) and a 555 nm laser (AF594, CFL594).

3.2.5 Live-cell Microscopy.

For live microscopy, only the high-C_{12}FDG-positive H460 cells were reseeded and monitored for 40 h at 5 days following flow cytometry sorting. Time-lapse imaging was performed using a Zeiss Cell Observer microscope (Carl Zeiss Microscopy, Thornwood, NY, USA), equipped with a Pecon stage incubator (set to 37° C, 5% CO2), an Axiocam MRm camera, and a Prior motorized XY stage (programmed to re-visit multiple selected sites in the culture dish). Phase contrast images were collected using a 10x / 0.3 NA Plan-Neofluar objective lens at 5 min intervals over a 48 h period. Approximately 10 fields of view were time-lapse imaged in the culture dish for each experimental trial. Images were collected and processed using Zen software.

3.2.6 HSLCI

The HSLCI protocol has been described in detail previously (263); briefly, a wide field phase detection camera is coupled to light emitting diode, motorized stages holding a 24 well plate, and a piezo actuated autofocusing system. The detected phase change in the light is then used to calculate the mass of single cells, and cell clusters. For the unsorted cell experiment, H460 cells were plated in a 24-well optical glass-bottomed plate (P24-0-N, Cellvis, CA, USA) at 1×10^4 cells/ml with a total of 1ml of medium (DMEM described above) in each well. Plated cells were allowed to adhere overnight. Cells were then exposed etoposide (to 1 μM) and put inside the HSLCI for imaging for 6 days at 10x or 20x, 37°C, and 5% CO2. Media was replaced after 24 hours of drug exposure and then every 48 hours after that. When using sorted cells, high-C_{12}FDG, low-C_{12}FDG and untreated control cells were plated and allowed to adhere overnight, followed by

monitoring in the HSLCI for 5 days at 10x or 20x at 37º C, and 5% CO_2. Media was replaced every 48 hours. A random 10% of locations were monitored from days four to six post dosing to observe mitosis. Only clusters in which all single cells were visible were counted.

3.2.7 Western Blotting

Western blotting was performed as previously described (253). Antibodies used: p21[Cip1] (BD Biosciences, 610234), TP53 (BD Biosciences, 554293) and GAPDH (Cell Signaling, 5174).

3.2.8 Annexin V staining.

At the indicated time points, cells were harvested, washed with PBS, and resuspended in 100 µl of 1x Binding Buffer (AnnexinV-FITC apoptosis detection kit; 556547, BD Biosciences, NJ, USA) with 5 µl of Annexin V and 5 µl of PI, then incubated at room temperature for 15 min in the dark. This suspension solution was then brought up to 500 µl using the same buffer and then analyzed by flow cytometry. Apoptotic cells express phosphatidylserine on the extracellular surface of the plasma membrane and thus, amenable for binding with annexin V. PI is a nuclear stain that cannot permeate the cell membrane of living cells. Cells stained with both annexin V and PI are late apoptotic cells that have disrupted plasma membranes.

3.2.9 TUNEL assay

For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, adherent cells were harvested and centrifuged at 10,000 rpm for 5 min onto slides (Shandon Cytospin 4, Thermal Electron Corp) 48 h after etoposide removal. Slides were fixed with 4% formaldehyde for 10 min and then washed with PBS for 5 min at room temperature. The cells were then fixed with a 1:2 dilution of acetic acid and ethanol for 5 min, followed by staining with a 1:1000 dilution

of DAPI at room temperature. Coverslips were sealed using clear nail polish and apoptosis was assessed by evaluating three fields per condition with an Olympus inverted microscope (20x objective, Q-Color3™ Olympus Camera, 488 nm filter and UV light, Olympus, Tokyo, Japan). TUNEL assay detects DNA fragmentation, a process that is frequently associated with apoptotic cells.

3.2.10 Cell cycle analysis

Cells were harvested, washed with cold PBS, then resuspended in a propidium iodide solution [50 µg/ml PI, 4 mM sodium citrate, 0.2 mg/ml DNase-free RNase A, and 0.1% Triton-X 100] for 1 h at room temperature, while being protected from light. Before analysis, NaCl was added to the cell suspensions to achieve a final concentration of 0.20 M. The cell suspensions were then analyzed by flow cytometry.

3.2.11 qRT-PCR.

H460 cells were exposed to etoposide (1 µM) for 24 h and then harvested at the indicated time points. Total RNA was isolated from the cell pellets following the manufacturer's instructions with the RNeasy kit (QIAGEN, Germany). cDNA samples from different cancer cell lines were synthesized from the same starting material of total RNA following the manufacturer's instructions (ExiLERATE LNA™ cDNA synthesis kit, Exiqon, Germany). All samples were amplified in triplicates using SYBR® Green probes from Exiqon in an ABI-7900HT model of real-time PCR machine (ExiLERATE LNA™ qPCR, SYBR® Green master mix kit). Primers were purchased from Exiqon.

3.2.12 Animal Studies

NOD/SCID/Ifrg2r-/- (NSG) mice were purchased from Jackson laboratory and housed under pathogen-free conditions according to the Virginia Commonwealth University IACUC guidelines. To test for recovery potential of senescent cells, enriched high-C_{12}FDG-positive H460 cells (1×10^6) were suspended in PBS and injected into both the right and left flanks of 6-8 week old male NSG mice (n=6). Tumor volume was measured every two days with calipers (tumor volume was calculated using the following equation: V = length × width 2 × 0.5).

3.3 Results

3.3.1 Etoposide induces senescence in H460 NSCLC cell line.

Our initial goal is to establish an experimental system where we establish a stable senescence response in tumor cells using cancer chemotherapy. As discussed earlier, this experimental effort will be focused in H460 NSCLC cells using the topoisomerase II poison. Utilizing different treatment regimens, etoposide was able to induce a senescent growth arrest marked by increased expression of SA-β-gal enzyme and development of characteristic morphological features (enlargement, flattening and vesiculation) (**Figure 3.1**). These regimens included (*i*) a single etoposide exposure for 24 hours at 1 μM concentration, (*ii*) a single etoposide exposure for 24 hours at 8.7 μM concentration (which represents a clinically relevant concentration) (*iii*) five exposures to etoposide at 1 μM concentration each for 24 hours and every 5 days and (*iv*) continuous exposure for 7 days. Using this screening, we decided to utilize the 1 μM concentration for the majority of the studies in this project since it extensively induced senescence (SA- β-gal expression) in the H460 cell line with minimal cell death as identified by TUNEL assay and Annexin/PI staining (**Figure 3.2**). This allowed us to establish a model where senescence is the primary response to treatment.

In addition to increased SA- β-gal expression and the characteristic morphology, etoposide exposure induced other senescent hallmarks. First, etoposide exposure resulted in increased formation of phosphorylated H2AX (γH2AX) and 53BP1 foci indicative of the formation of DNA double stranded breaks and the activation of the DDR (**Figure 3.3A**). Etoposide-induced DNA
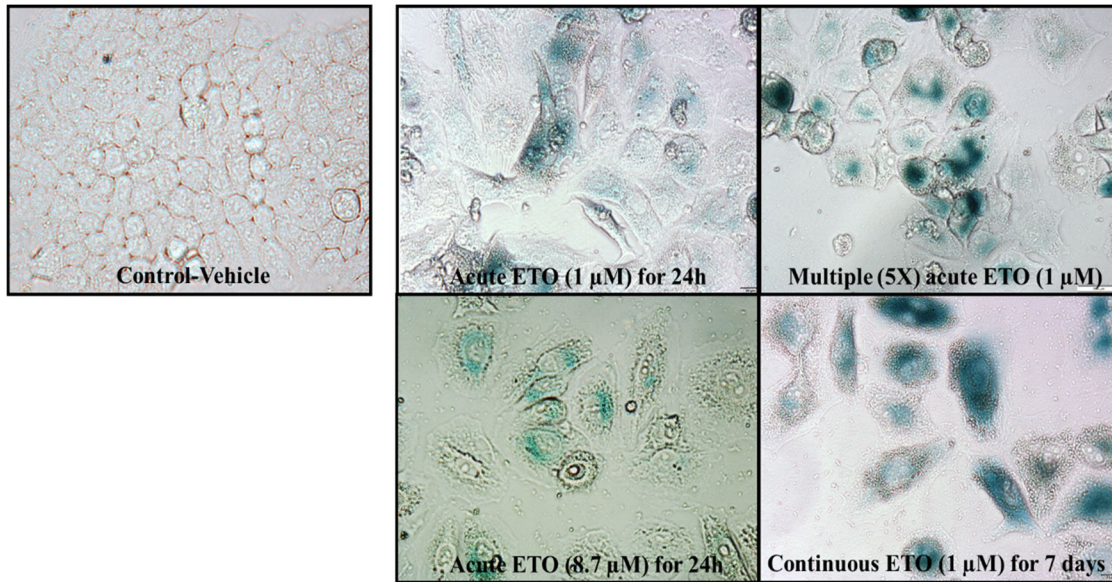
Figure 3.1. Upregulation of SA-β-gal in H460 cells following etoposide exposure. Parental H46o cells are round, small and grow in cauliflower-shaped colonies. Control H460 cells show little or no SA-β-gal staining when cultured at subconfluent densities. After etoposide exposure, H460 cells exhibit a large, flattened morphology and develop increased SA-β-gal expression which is evident by the blue staining in this figure. Different etoposide concentrations or treatment regimens resulted in the induction of SA-β-gal. All images were taken at the same magnification using 20x objective and bright field microscopy.
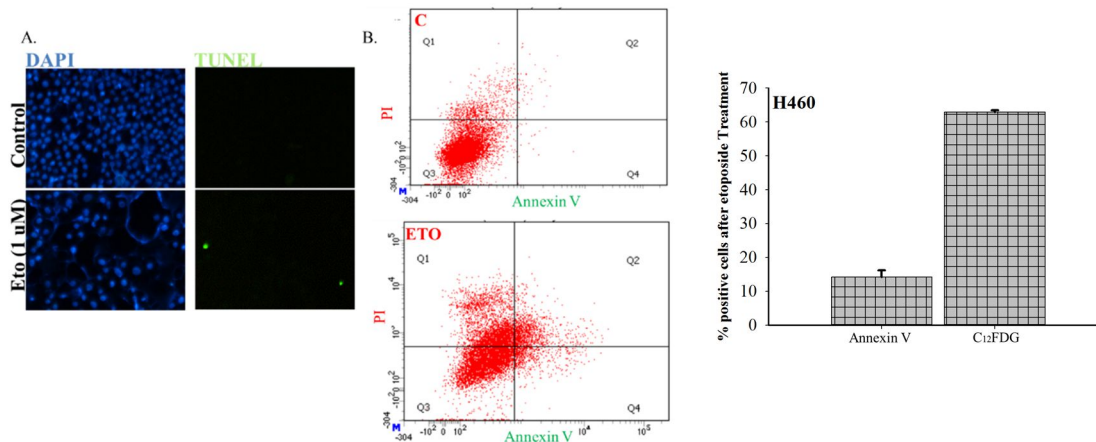

Figure 3.2. Exposure to etoposide (1 µM) results in minimal apoptosis. A. TUNEL assay shows minimal staining for apoptosis 72 hours after etoposide (ETO, 1 µM) exposure in H460 in comparison with control. **B.** Annexin V/Propidium iodide (PI) staining of H460 cells at 72 hours after etoposide (1 µM) exposure. Cells in Q1 are PI positive and reflect cells with disrupted plasma membrane. Cells in Q4 are annexin V positive and reflect cells that have flipped phosphatidylserine on their outer membrane i.e., early apoptosis. Cells in Q2 are positive for both stains and described as late apoptotic. The bar graph shows a quantification of the percentage of apoptotic cells in comparison to SA-β-gal positive cells following etoposide treatment suggesting that senescence is the predominant response.

double stranded breaks were detected as soon as 6 hours of exposure and persisted for 12 hours which was followed by repair leaving residual persistent damage (**Figure 3.3B**). Activation of the DDR resulted in the induction of the p53—p21^{Cip1} axis as marked by the increased expression of p21^{Cip1} at the message and protein levels (**Figure 3.3C**). Due to homozygous deletion of the *CDKN2A* gene that encodes for the CDKI p16^{INK4a} in H460 cells it appears that the etoposide growth arrest in these cells is primarily mediated by p21^{Cip1} (264). p21^{Cip1} is capable of enforcing a stagnant growth arrest as part of senescence induction by inhibiting critical CDKs required for progression in the cell cycle. Accordingly, within 24 hours following etoposide exposure, most H460 cells arrested with a G$_2$ DNA content (**Figure 3.4A**). This growth arrest was evident by the inability of etoposide-treated cells to proliferate and form colonies in comparison to the rapidly growing control cells that were not exposed to etoposide (**Figure 3.4B**).

Next, we tested whether etoposide resulted in gene expression alterations. After 72 hours of etoposide exposure, the expression of a number of SASP components was significantly increased. Specifically, the expression of IL-1β and IL-8 as well as CXCL$_1$ (**Figure 3.5A**). Interestingly, the results of three independent experiments failed to show an increase in the expression of IL-6 following senescence induction by etoposide. Despite the fact that IL-6 is a frequent component of the SASP, the variability in gene expression between different cell lines is common as it appears that SASP can have multiple signatures (75). Finally, we monitored histone modifications that reflect epigenetic modulations that are closely associated with senescence, namely, H3K9Me3. H3K9Me3 marks focal condensation of chromatin, which when associated with senescence is collectively named SAHF. Etoposide resulted in the formation of numerous H3K9Me3 foci in H460 cells (**Figure 3.5B**).
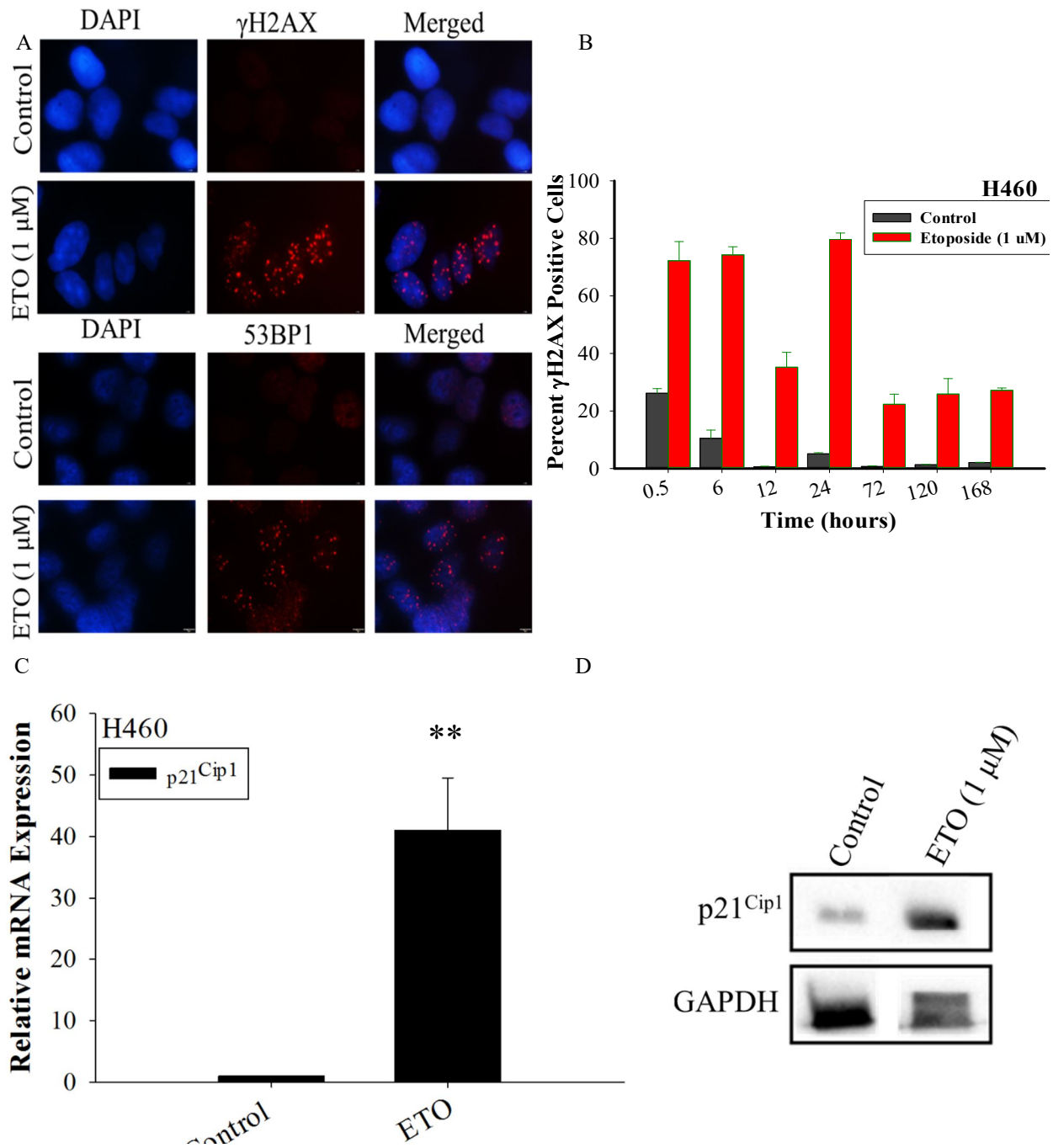
Figure 3.3. Exposure to etoposide (1 μM) activates the DDR. A. Wide-field fluorescent microscopy images of both γH2AX and 53BP1 6 hours following etoposide exposure (ETO, 1 μM). All images were taken at the same magnification using a 100x oil immersion objective. Nuclei are stained with DAPI. **B.** Quantification of γH2AX staining using flow cytometry over time after etoposide exposure. **C.** qRT-PCR quantification of the expression level of p21^{Cip1} 72 hours in H460 cells following etoposide exposure. Statistical analysis was performed by one-way ANOVA with Tukey post-hoc test. **p$<$0.01 **D.** Western blot demonstrating the increased protein level of p21^{Cip1} 72 hours in H460 cells following etoposide exposure.
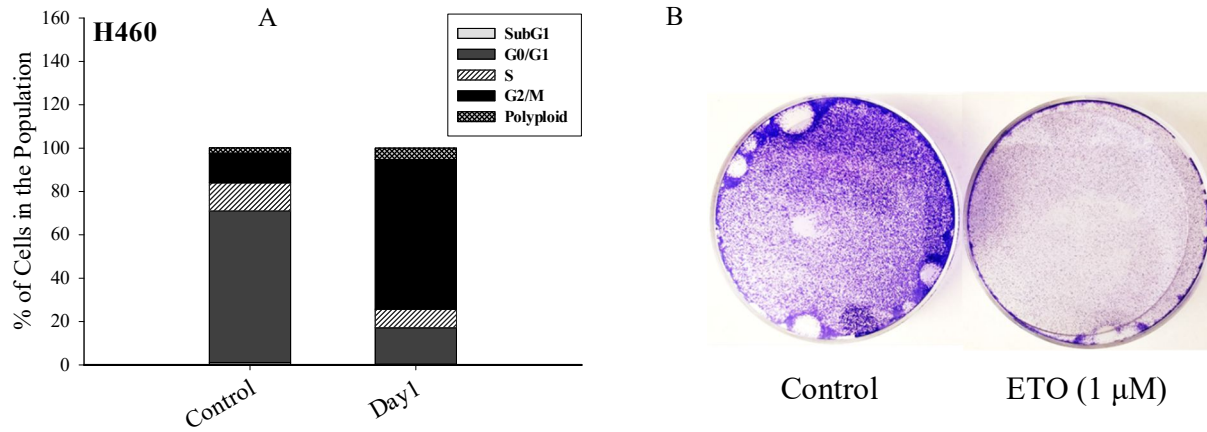
Figure 3.4. Exposure to etoposide (1 μM) results in a G$_2$-M growth arrest in H460 cells. A. Cell cycle analysis showing that the majority of H460 cells exposed to etoposide (1 μM) have a G2 DNA content indicative of growth arrest at the G$_2$-M transition point 24 h after drug treatment. **B.** Abrogation of growth in the etoposide-treated H460 cells in comparison with control, proliferating cells. Cells were seeded at the same density.
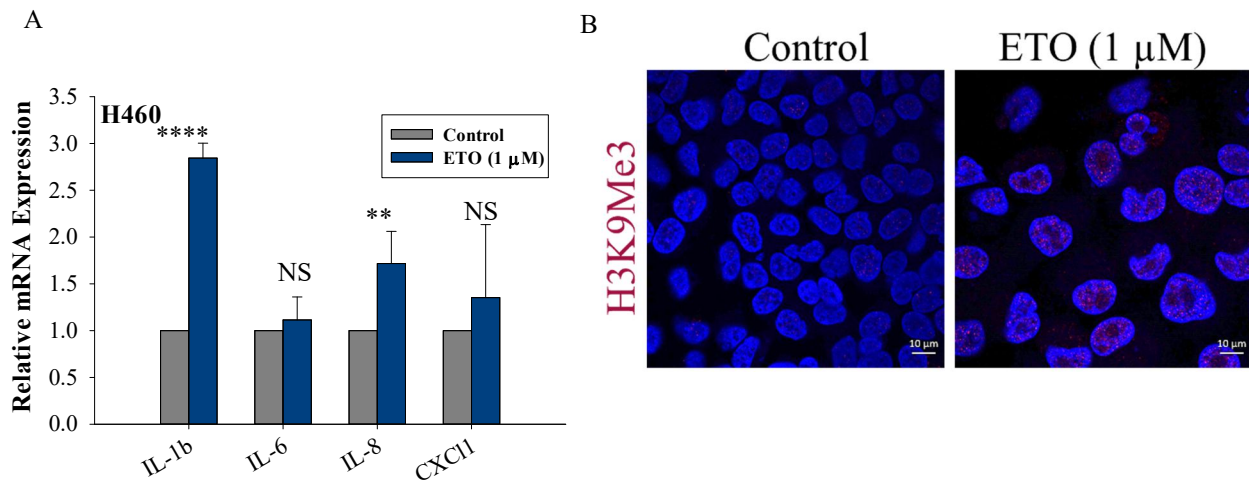
Figure 3.5. Changes in gene expression of SASP factors and SAHF formation. A. qRT-PCR quantification of the expression of multiple Senescence-associated Secretory Phenotype (SASP) factors (IL-1β, IL-8 and CXCL1) in H460 at day 1 after etoposide (1 μM) exposure. Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed by one-way ANOVA with Tukey post-hoc test. **p<0.01, ***p<0.001, ****p<0.0001. **B.** Immunofluorescent staining of H460 cells showing the formation of H3K9Me3 foci (red). H3K9Me3 images were obtained by confocal microscopy (63x objective).

Collectively, etoposide effectively induced senescence in H460 cells based on suppressed proliferation, cell cycle arrest, activation of the DDR, upregulation of SA-β-gal, SASP induction and formation of SAHF.

3.3.2 Etoposide-induced senescence in H460 NSCLC cells is followed by proliferative recovery.

To evaluate the growth pattern of etoposide-treated H460 cells that exhibit senescence features, we monitored their growth temporally. After etoposide exposure, H460 underwent growth arrest initially as expected, but that growth arrest was not durable i.e., it was followed by restoration of growth capacity which we refer to as proliferative recovery (**Figure 3.6A**). Furthermore, the recovering cells were able to generate colonies after days of senescence-associated growth abrogation (**Figure 3.6B**). We also observed a similar growth pattern using the multiple treatment regimens with etoposide (1 μM) as well as the near-clinical plasma concentration of etoposide (8.7 μM) (**Figure 3.6C and D**). These observations suggests that the etoposide-induced growth inhibition is not terminal despite the induction of multiple senescence markers in the majority of the treated cells.

To determine if the change in growth pattern was accompanied by alteration in the senescent phenotype, we assessed the expression of several senescence markers over time following the removal of etoposide. First, to obtain an objective assessment of the number of senescent cells, we utilized the SA-β-gal fluorescent substrate, C_{12}FDG, which allows for: (*i*) microscopic identification of SA-β-gal positive cells where other senescence labels can be combined. For
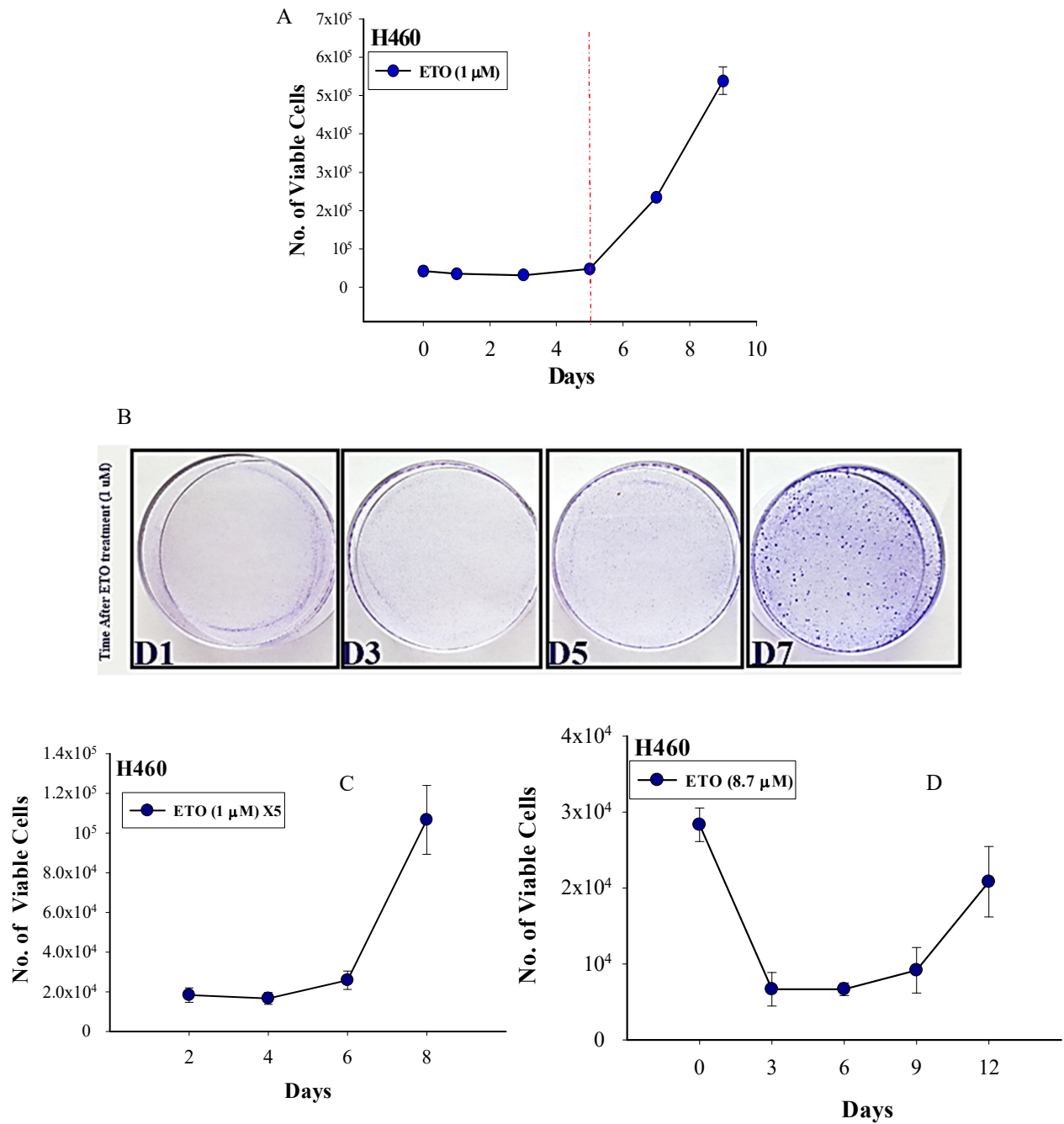
Figure 3.6. Proliferative Recovery after etoposide exposure. A. Growth arrest and recovery of H460 and exposed to etoposide (1 μM) for 24 h (day 0) as determined by the number of viable cells measured at the indicated time points by trypan blue exclusion. **B.** Recovery of colonial proliferation after etoposide-induced growth arrest in H460 cells. **C and D.** Growth curves of H460 cells exposed to etoposide at the indicated concentrations. In C, H350 cells were exposed to etoposide 5 times (X5) each for 24 hours.

example, both SA-β-gal and p21^{Cip1} or H3K9Me3 expression can be evaluated simultaneously, (ii) flow cytometry based quantification of the SA-β-gal positive senescent cells. Following etoposide treatment, as presented previously, H460 cells upregulated SA-β-gal and express higher levels of p21^{Cip1} (**Figure 3.7A**). Using flow cytometry, we were able to quantify the percentage of SA-β-gal positive senescent cells over time. The initial upregulation of SA-β-gal at day 1 and 3 after etoposide treatment was followed by a gradual decline in expression levels back to control levels by day 7, which coincides with proliferative recovery (when the cells start to grow back) (**Figure 3.7B**).

Next, we evaluated the cell cycle distribution over time; the number of cells arrested with a G$_2$ content within 72 hours of etoposide treatment was gradually decreasing, suggestive of resolution of the growth arrest (**Figure 3.7C**). Furthermore, proliferative recovery was accompanied by a gradual decrease in the expression of p21^{Cip1}, consistent with the resolution of the growth arrest (**Figure 3.7D and E**). Finally, monitoring the expression levels of IL-1β, IL-8 and CXCl$_1$, the expression of SASP was also declining over time as the cells were recovering proliferation. Collectively, these results suggest the gradual resolution of the senescence phenotype in H460 cells induced into senescence by etoposide.

3.3.3 Evidence for proliferative recovery based on SA-β-gal enrichment and live cell microscopy.

Proliferative recovery from senescence growth arrest by H460 and HCT116 (BTG1-RFP) cells was further monitored by staining with crystal violet and C$_{12}$FDG. Cells reverted from an enlarged, highly-vesicular (abundant cytoplasmic granules) morphology to a more round, smaller phenotype; the cells further demonstrated reduced C$_{12}$FDG staining (**Figure 3.8**). However, one

A

Control   ETO (1 µM)

C12FDG/p21^Wafl/Cip1

B

H460

Percent C$_{12}$FDG positive Cells

Control | Day1 | Day3 | Day5 | Day7

C

H460

% of Cells in the Population

Control | Day1 | Day3 | Day5 | Day7

Time after ETO (1 µM)

SubG1
G0/G1
S
G2/M
Polyploid

D

Relative mRNA Expression

H460

p21$^{Cip1/Wafl}$

****

**

Control | Day1 | Day3 | Day5 | Day7 | Day9

H460   C   Day1 Day3 Day5 Day7 Day9

p53

p21

GAPDH
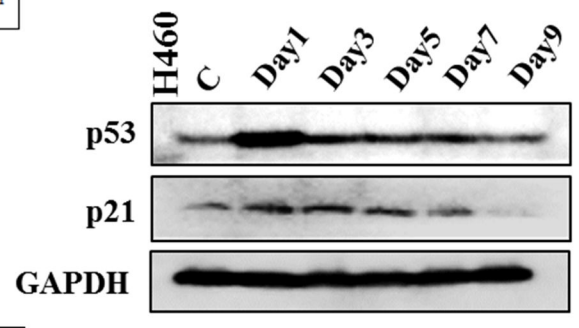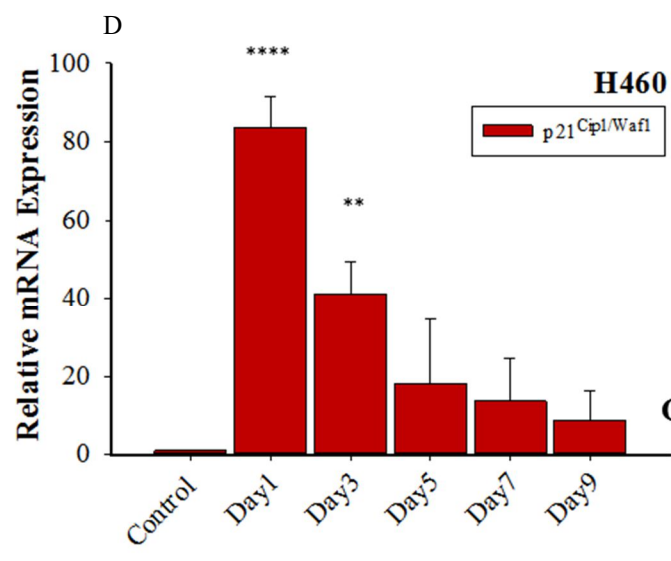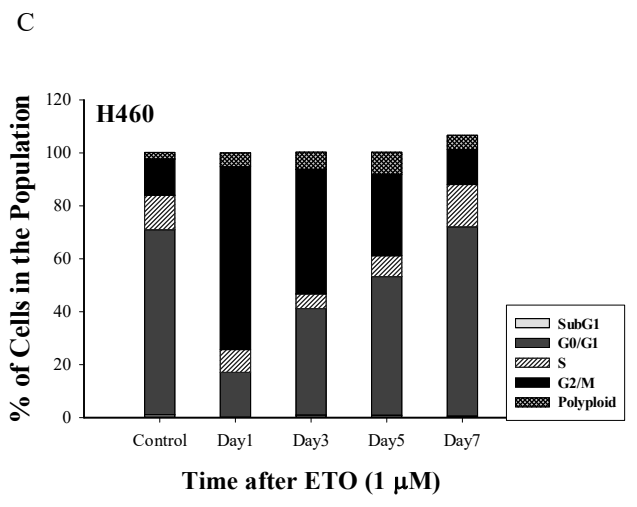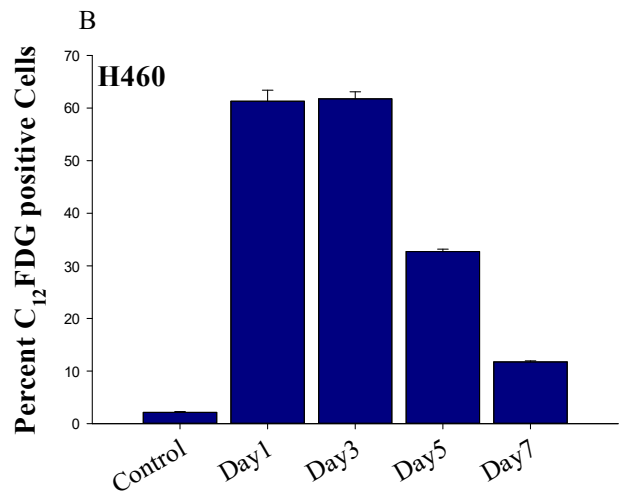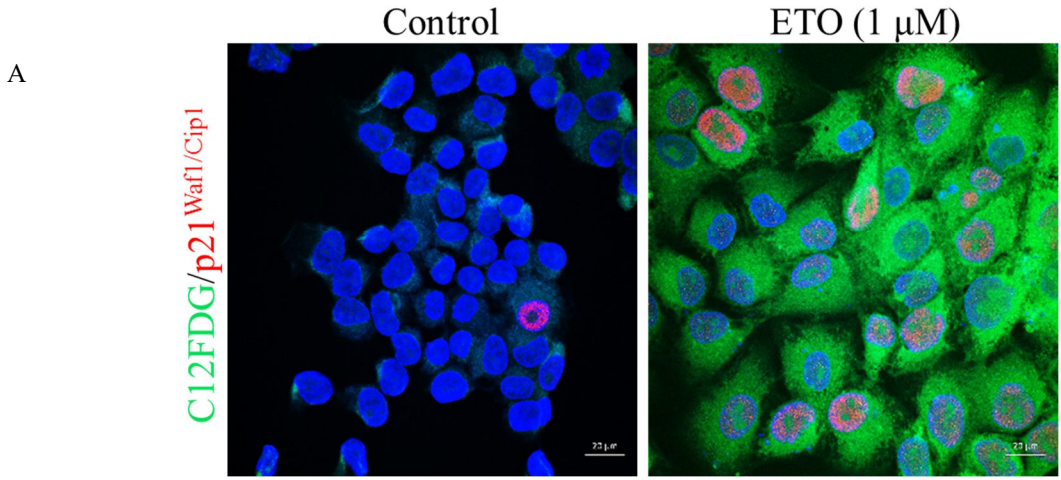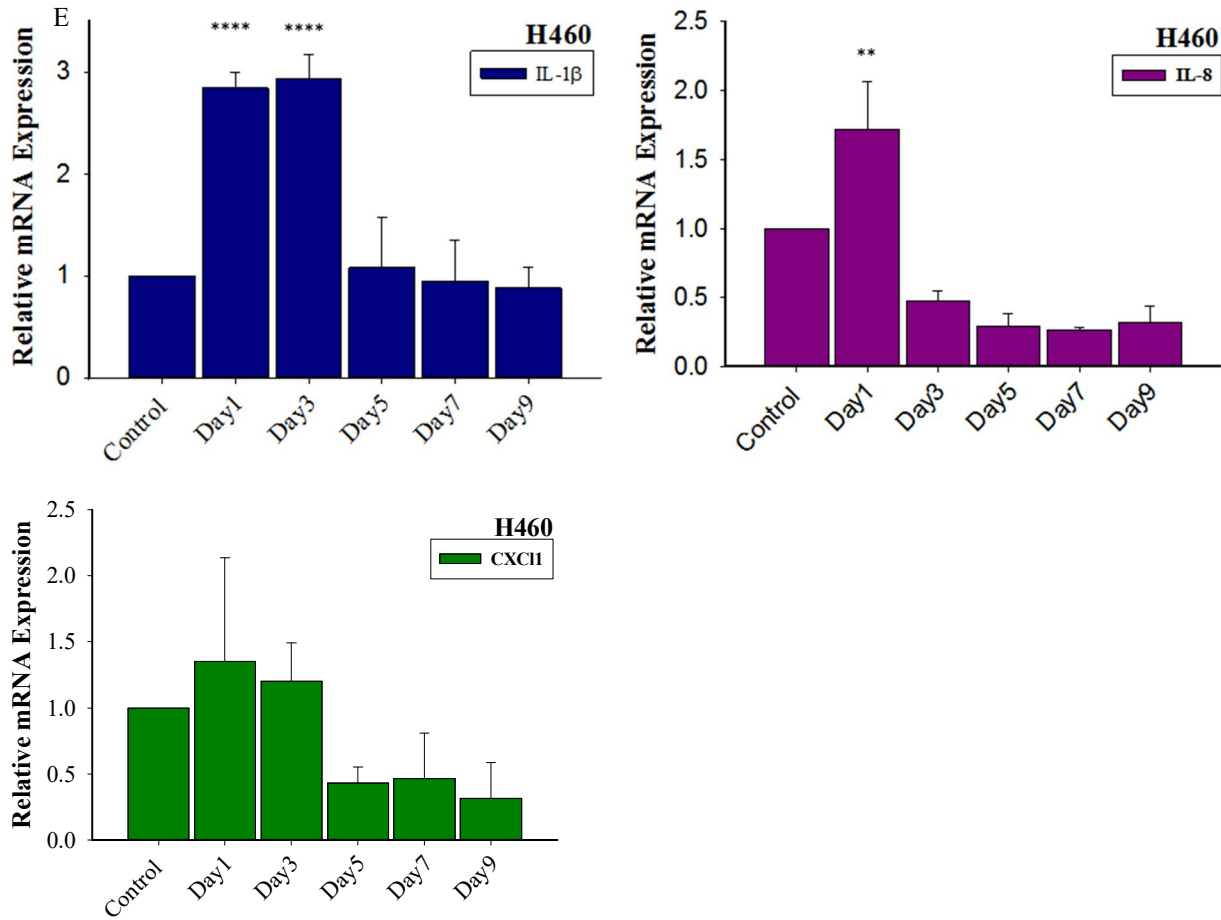
Figure 3.7 Proliferative recovery from etoposide treatment of H460 and HCT116 cells is associated with resolution of some senescence-associated features. A. Confocal microscopy and immunolabeling for p21^{Cip1} (red) and C$_{12}$FDG (green) (63x objective) after etoposide (1 μM) exposure in H460 cells. **B.** Flow cytometry quantification of senescence based on C$_{12}$FDG staining (SA-β-gal positive cells) over the indicated time points after etoposide (1 μM) exposure suggestive of senescence induction and resolution. **C.** Cell cycle distribution of H460 cells after etoposide (1 μM) exposure at the indicated time points. **D**. qRT-PCR quantification of the expression of p21^{Cip1} message and Western blotting of both p53 and p21 ^{Cip1} in H460 cells following etoposide (1 μM) exposure at the indicated days. **E.** qRT-PCR quantification of the expression of multiple Senescence-associated Secretory Phenotype (SASP) factors (IL-1β, IL-8 and CXCL1) in H460 following etoposide (1 μM) exposure. Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed by one-way ANOVA with Tukey post-hoc test. **p<0.01, ***p<0.001, ****p<0.0001.

interpretation of the previous data could be that the proliferating cells were not derived from the senescent-like population, but were derived from cells that escaped the full effect of etoposide treatment and were not detected until they achieved a critical mass. To address this potential drawback to our studies, cells induced into the senescence by etoposide were labeled with $C_{12}FDG$ followed by flow cytometric cell sorting and subsequent reseeding of the highest $C_{12}FDG$-positive (SA-β-gal positive) subpopulations. Enrichment was followed by post-sorting confirmation by analyzing $C_{12}FDG$ staining of the purified population and then single cell phenotypic validation using imagery flow cytometry and labeling for additional senescence markers (SA-β-gal, p21^{Cip1} and γH2AX) (**Figure 3.9**).

 As was observed for the heterogeneous population generated in response to etoposide treatment, these highly $C_{12}FDG$-positive H460 cells were also able to recover proliferative capacity (**Figure 3.10A**). Additional studies were designed to evaluate the capacity of senescent-like cells *that maintained prolonged SA-β-gal activity* (essentially a sustained senescence) to recover proliferative capacity. High $C_{12}FDG$-positive H460 cells (enriched for senescence at 7 days following etoposide exposure) were also able to recover proliferative capacity (**Figure 3.10B**). These highly $C_{12}FDG$-positive cells also demonstrated the capacity for colony formation in a cell density-dependent manner (**Figure 3.10C**). As might have been anticipated, a large proportion of these senescence-sorted cells remained in a growth-arrested state for days following enrichment, indicating that only a subpopulation of senescent cells re-emerges from the growth-arrested state (**Figure 3.10D**).
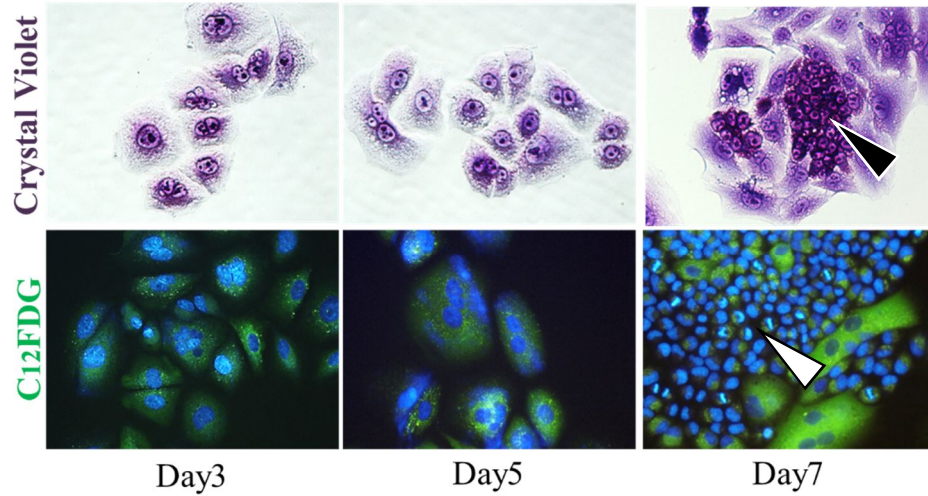
Figure 3.8. The recovering population from etoposide treatment of H460 cells resembles parental cells. *Upper panel:* Crystal violet staining after senescence induction in H460 cells (on days 3, 5 and 7). *Lower panel*: $C_{12}FDG$ staining (SA-β-gal on days 3, 5, and 7.) Arrow heads point to the recovering population. (20x objective).
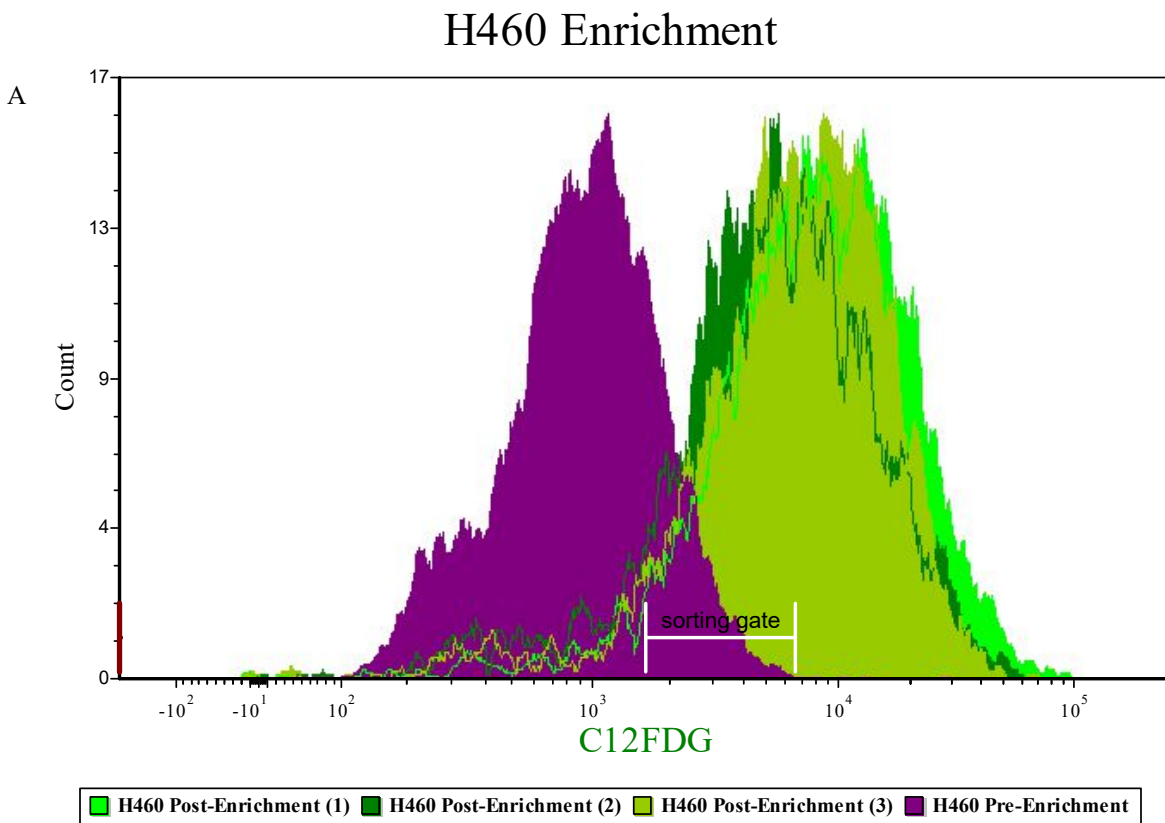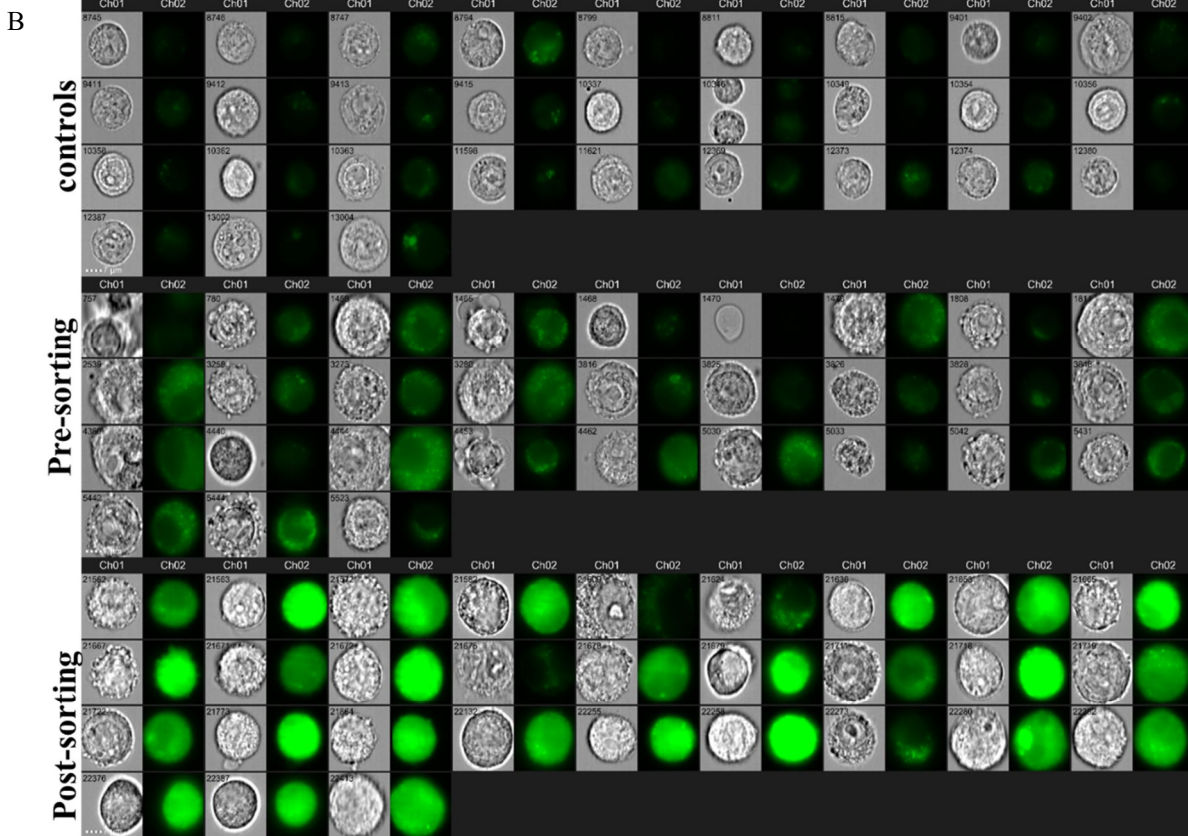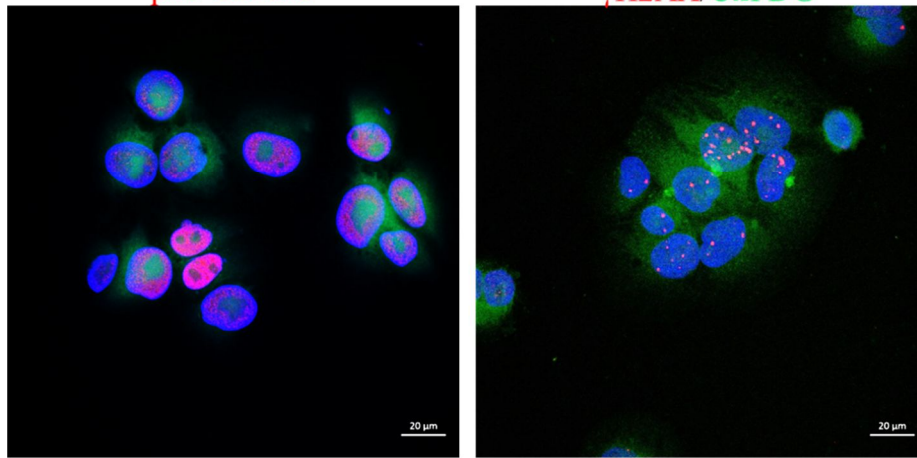
Figure 3.9 Post-enrichment validation of sorted H460 cells. A. Flow cytometry profile for H460 cells before and after enrichment. Cells were then harvested under sterile conditions and enriched based on the gating for cell size and $C_{12}FDG$ staining using BSC Aria- BD FACSAria™ II High-Speed Cell Sorting. Purple histogram is reflects the profile of $C_{12}FDG$ staining in etoposide-treated H460 cells prior to enrichment, while the three green histogram are for three representative samples of the profile of $C_{12}FDG$ staining following enrichment. **B.** Post-enrichment analysis of control, etoposide- treated pre-sorted and sorted H460 cells showing $C_{12}FDG$ staining using AmnisImageStreamX Mark II™ flow cytometry **C.** Confocal microscopy images of high-$C_{12}FDG$-positive H460 cells following enrichment, showing $C_{12}FDG$ staining, p21^{Cip1} and γH2AX. Scale bar: 20 μm.

To definitively distinguish tumor cells that might have *initially* evaded etoposide-induced senescence from cells that developed a senescence-like state, but recovered proliferative (self-renewal) capacity, the sorted high $C_{12}FDG$-positive H460 population was reseeded and monitored by live cell imaging using phase contrast microscopy. The temporal response after etoposide-induced senescence was found to be quite heterogeneous. Over the period of observation (40 hours), the majority of the senescent cell population persisted in a growth-arrested state, while a few cells appeared to undergo apoptosis (or possibly mitotic catastrophe). However, occasional hypertrophic, flattened cells that had remained in an arrested state were found to be capable of recovering the ability to divide; **Figure 3.10E** shows representative images of the monitored population over the indicated time periods which illustrates spontaneous mitotic events (tracked by arrow heads). Taken together, these results indicate that *only a subpopulation* of senescent cells re-emerges into a proliferative state.

As a complement to the real-time imaging studies presented in the previous section, we utilized High Speed Live Cell Interferometry (HSLCI) to evaluate changes in biomass kinetics and single cell area in response to etoposide. HSLCI uses interferometric microscopy to perform rapid optical biomass measurements to quantify cellular response to drugs (263). LCI has been previously used to profile drug-induced growth arrest with picogram sensitivity and drug profiling in melanoma and breast cancer models (263,265,266). Like other quantitative phase imaging techniques, HSLCI is non-invasive with no fluorescence or dye labeling (263). **Figure 3.11A** shows a cluster of three cells exhibiting a classic senescence-like phenotype with quite extensive morphological changes,
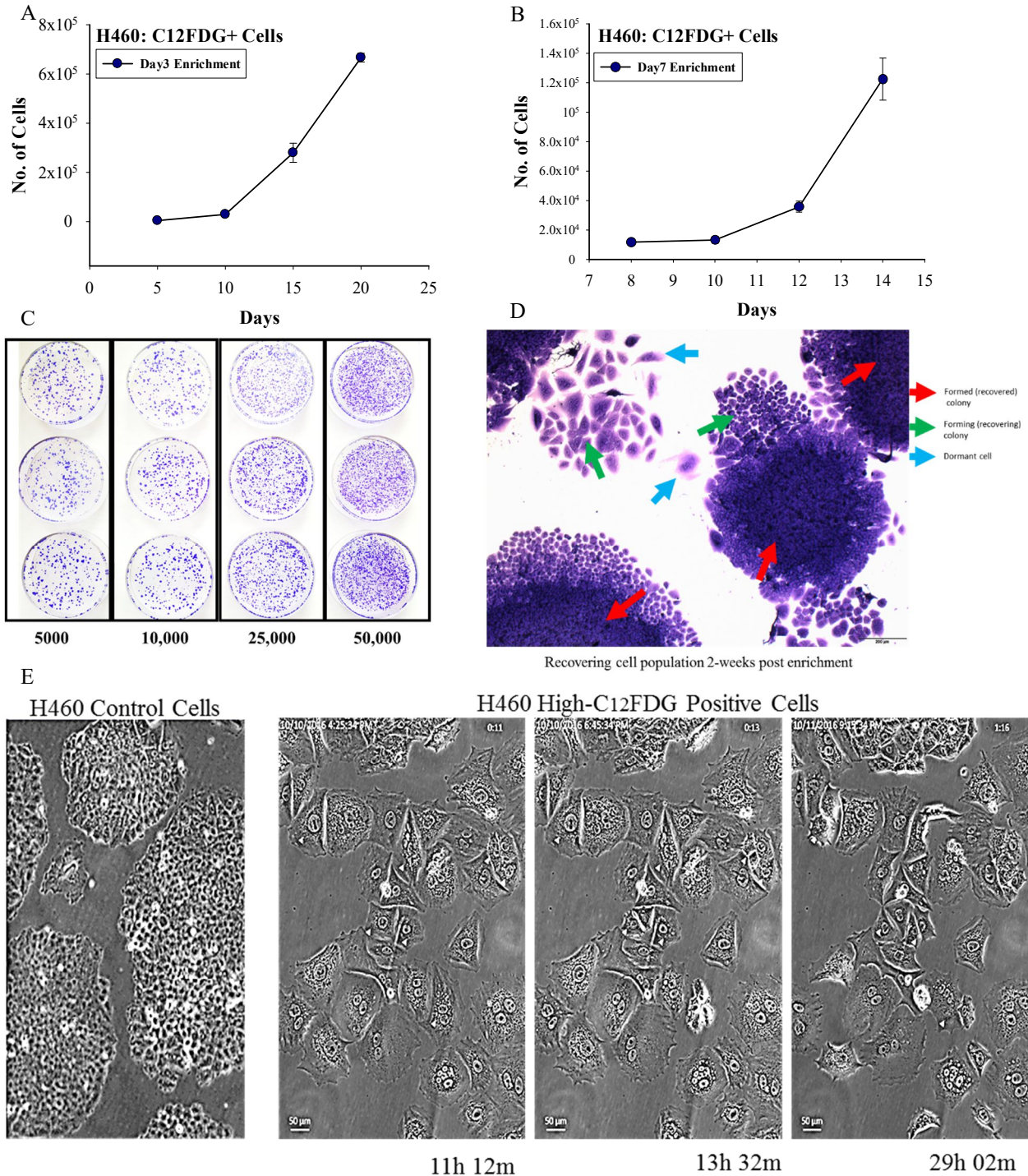
Figure 3.10 Proliferative recovery of enriched senescent H460 cells. A and B. Growth curves for high-C_{12}FDG-positive H460 (enriched on day 3 and 7 cells after flow cytometry enrichment. X axis indicates time points relative to etoposide (1 µM) exposure. **C.** Colony forming assay showing cell-density dependent restoration of growth of high-C_{12}FDG-positive H460 cells. **D**. Representative images of the recovering high-C_{12}FDG-positive H460 cells showing variable cell fates. (Scale bar: 200 µm) **E.** Time-lapse live-cell microscopy of H460 high-C_{12}FDG-positive cells performed for 40 h at 5 days following flow cytometry enrichment. Arrows indicate mitotic events.

specifically flattening marked by increased cell area and mass *without division* (**lower panel of Figure 3.11A**). To investigate this observed change in single cell size at a population level, mass and area measurements of control and etoposide-treated single cells were taken on day 4 post etoposide exposure (**Figure 3.11B**). The etoposide-treated cells were comprised of a heterogeneous population that differed markedly in mass and area, consistent with the heterogeneous response to drug exposure suggested by the previous experiments. As can be seen in the population histograms (**Figure 3.11C**), a subpopulation of cells in the etoposide treated group remained the same size as control cells while the majority of the population increased in size and area. For example, in the same image in **Figure 3.11B** of the etoposide treated, ***but unsorted*** population, cell *b* has a mass and area of 853 pg and 423 um^2, respectively, which is comparable to control cell *a* (618 pg, 207 um^2) while (senescent) cell *c* has a mass of 4981 pg and an area of 2646 um^2. (**Figure 3.11B**).

Flow cytometry based enrichment for SA-β-gal coupled to HSLCI, generated a profile of mass and area for the different populations. Representative example cells of the low $C_{12}FDG$ population, cell *d* (727pg, 279 um^2)*,* and high $C_{12}FDG$ population, cell *e* (2515 pg, 2250 um^2)*,* reflected the observation that the high-$C_{12}FDG$ cells increased in size while the low $C_{12}FDG$ maintained the size of control cells despite treatment with etoposide. **Table 2** provides a summary of the median mass and area for control cells, unsorted cells after etoposide treatment, and high and low $C_{12}FDG$ populations. On a population level, control cells had a median mass of 917 +/- 79 pg and a median area of 431 +/-71 um^2 while the mass of unsorted etoposide-treated cell population had a median mass and area of 1799 +/-149 pg and 1355 +/-117 um^2, respectively (p<0.001, **Figure 3.11C**). High- $C_{12}FDG$ cells (i.e. after sorting) had a median mass of 2491+/- 171 pg, and median area per

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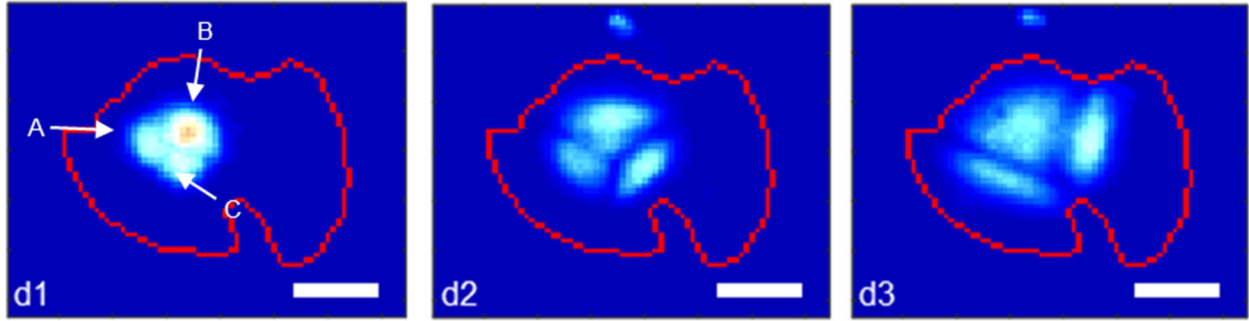
A

Mass (pg) values over time			
	Day 1	Day 2	Day 3
A	1282	1378	2340
B	2339	2995	4333
C	1042	1609	2879

Area (μm^2) values over time			
	Day 1	Day 2	Day 3
A	828	1215	2286
B	1269	2169	3321
C	648	1287	2349

B

C

Vehicle

ETO 1 µM

low-C$_{12}$FDG

high-C$_{12}$FDG

D

E
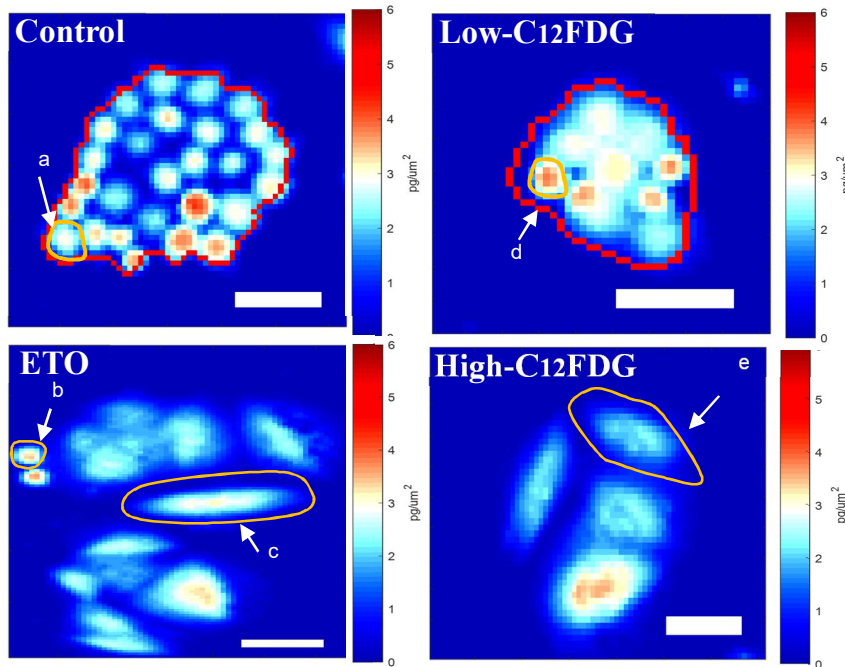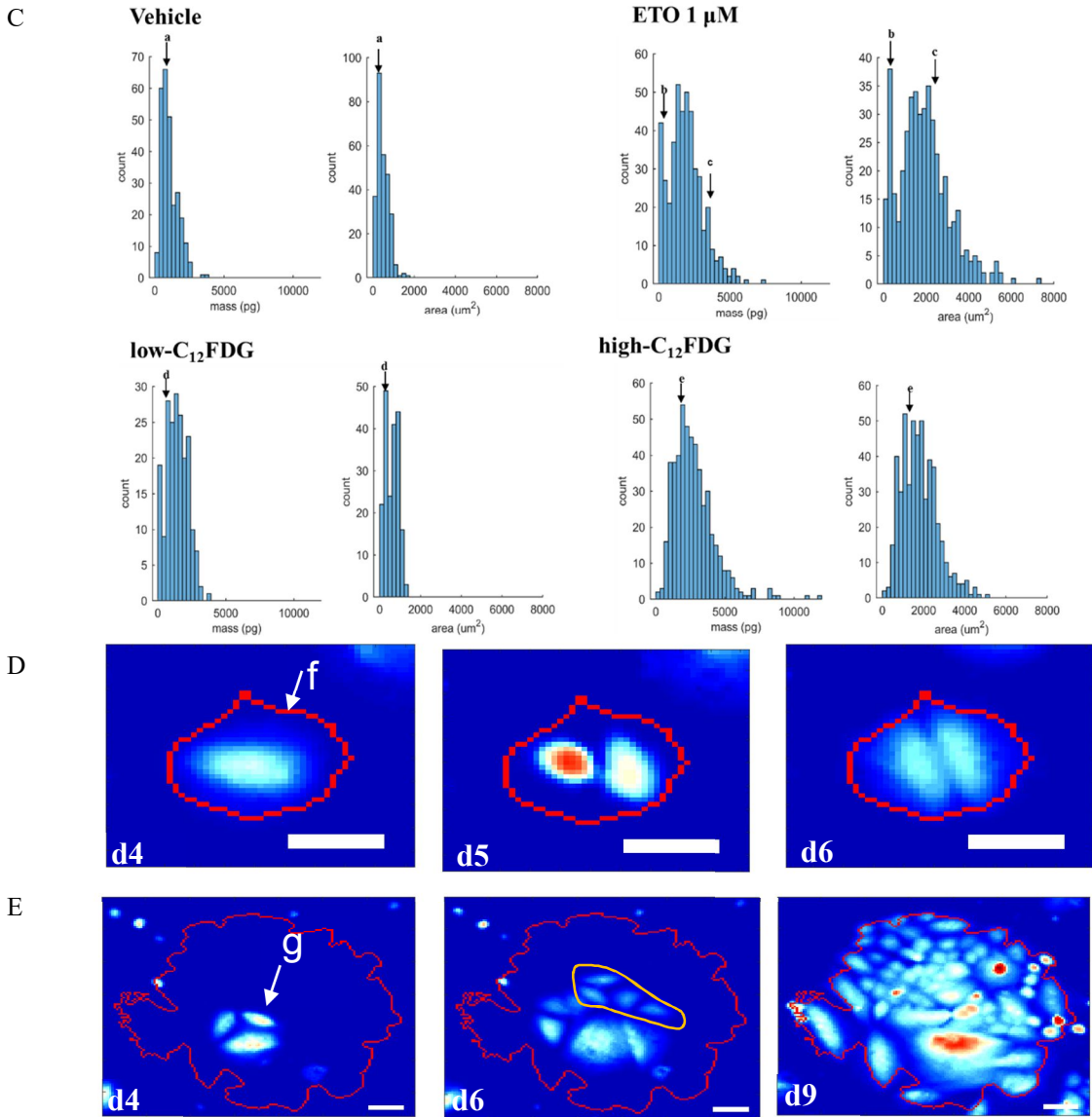
Figure 3.11. **HSLCI analysis of etoposide-treated H460 tumor cells shows increases in mass and area, and division of high-C$_{12}$FDG cells**. **A.** HSLCI biomass images of the cluster pictured. This cluster is three cells that do not grow in number over a period of three days after etoposide treatment while exhibiting increase in mass and area over time. The corresponding change in mass and area of each cell labeled A, B, and C over time is in the inset table below the images. All scale bars are 45 um. **B.** At day 4 post treatment, the cell population exposed to etoposide (n=447) had a mixed population with some single cells with higher mass, and larger area per cell as compared to the control population (n=272) while others maintained similar sizes to control population. In a separate experiment, etoposide treated cells were sorted into verified senescent-like (high-C$_{12}$FDG) and non-senescent (low-C$_{12}$FDG) populations prior to HSLCI measurement. Senescent-like high-C12FDG cells (n=505) were more massive and larger on average than non-senescent low-C$_{12}$FDG cells (n=199) which had similar size to the control population. **C.** The single cell mass and single cell area of etoposide treated (n=447) and C$_{12}$FDG-high cells (n=505) were significantly larger than vehicle (n=272) or C$_{12}$FDG-low (n=199) (C) cells. Letters refer to the locations of cells from figure 5 in the histograms. **D and E**. examples of two large etoposide-treated, enriched high-C$_{12}$FDG cell which divides over day 4-6 and 4-9 after etoposide treatment, respectively. Cell *g*'s progeny are circled in orange. All scale bars are 45 um. Red outlines indicate clusters that were tracked.

cell of 1683 +/- 131 um^2, while in the low-C$_{12}$FDG population of cells, median mass was 1386 +/- 147 pg and median area was 627 +/- 99 um^2 (p<0.001, **Figure 3.11C**). These observations indicate that etoposide treatment results in a dramatic dysregulation of cellular biomass; the majority of cells are large in size consistent with the cellular flattening that characterizes the senescence-like phenotype, while the minority of cells that maintain biomass regulation (i.e. that do not enter into senescence after exposure to etoposide) have low-C$_{12}$FDG expression (**Table 2**).

The senescence enriched etoposide-treated cells gained biomass slowly, occasionally followed by cell division. Two examples of large, SA-β-gal positive cells dividing are shown in **Figure 3.11D and E.** The mass and area of the single cells shown to divide are, prior to division, much greater than single cells measured in the control or low-C$_{12}$FDG populations (**Figure 3.11D and E**). For example, the masses of cells f (3519 pg) and g (2245 pg) are four and two standard deviations from the mean of control cell mass respectively. Similarly, the areas of f (2133 um^2) and g (1495 um^2) are over three standard deviations of the mean control cell area. Similar to the decline in SA-β-gal activity as proliferative recovery progress, the progeny of cell g lose the classic enlarged senescent phenotype as cells become smaller in consecutive generations (**Figure 3.11E**). Collectively, these data demonstrate that senescent-like H460 cells undergo spontaneous divisions at a single cell level, indicating that SA-β-gal-positive enriched, senescent-like cells are not terminally arrested and can resume mitotic proliferation.

	Median mass (pg)	Median area (μm^2)
Control	917 +/- 79	431 +/-71
ETO	1799 +/-149	1355 +/-117
Low-C$_{12}$FDG	1386 +/-147	627 +/- 99
High-C$_{12}$FDG	2491+/- 171	1683 +/- 131

Table 2. **HSLCI biomass and area measurements of pre and post sorted H460 cells.** Post sorting high-C12FDG cells (n=505) exhibit higher mass and area than the post-sorted low-C$_{12}$FDG cells (n=199) and the pre-sorted etoposide-treated cells (n=447).

3.3.4 SA-β-gal positive cells have the potential to form viable tumors in a mouse model.

To determine whether senescent-like cells have the capacity to generate tumors *in vivo*, enriched, high C_{12}FDG-positive H460 cells were subcutaneously injected into NOD/LtSz-scid IL2R gamma null (NSG) mice. Within approximately 2 weeks of injecting a senescence-enriched tumor cell population, these mice showed evidence of tumor development (**Figure 3.12**).
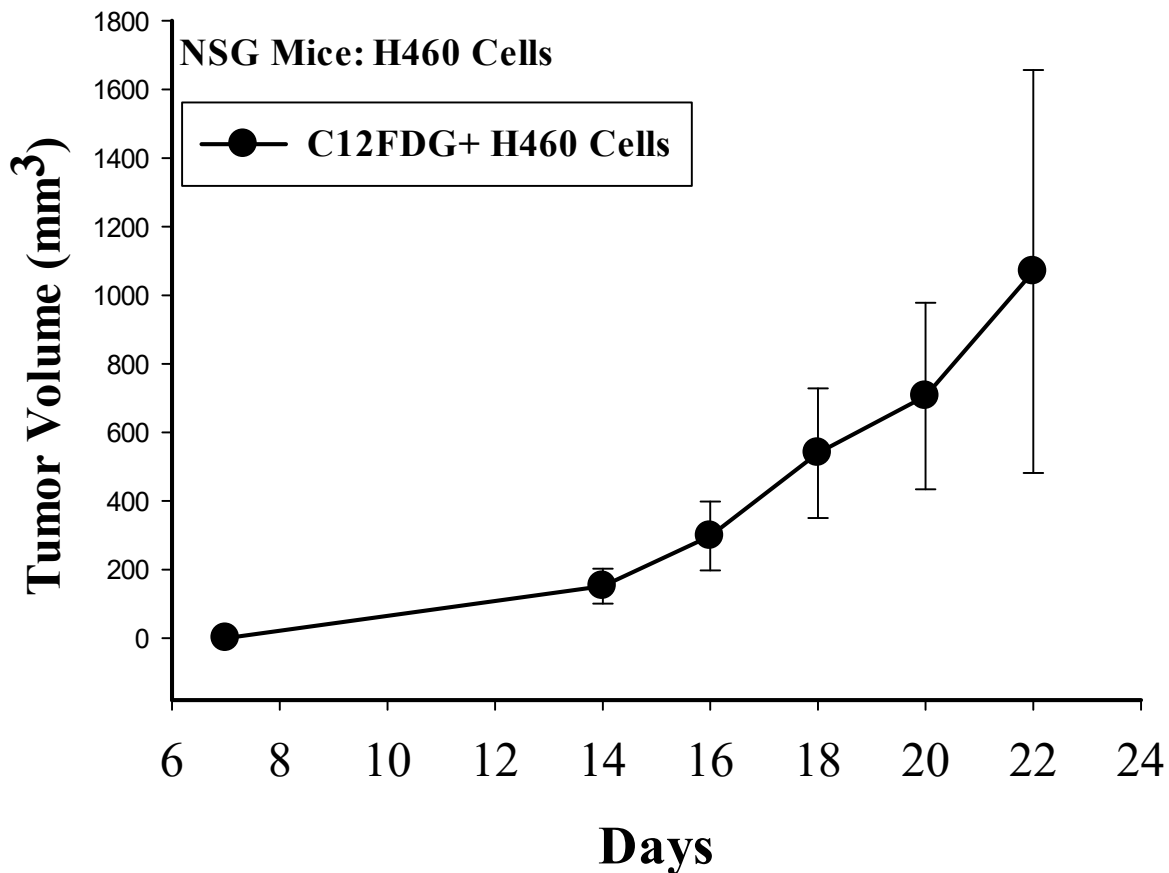

Figure 3.12. **SA-β-gal positive cells have the potential to form viable tumors in a mouse model**. Tumor formation following transplantation of senescence-enriched H460 cells in NSG mice (n=6), indicating their ability to form proliferating tumors in vivo. Data are expressed as mean ± standard error of the mean (SEM).

3.3.5 Recovery from etoposide-induced senescence in HCT116 CRC cancer cells.

In parallel with the studies in the H460 NSCLC cell line, we have utilized a similar experimental approach in HCT116 colorectal cancer cells exposed to etoposide. HCT116 cells were obtained from Dr. Igor Roninson's laboratory at the University of South Carolina which were derived by lentiviral transduction of HCT116 cells with the construct expressing dsRed from the promoter of BTG1 gene (RFP-BTG1). BTG1 (B-cell translocation gene-1) is a tumor suppressor gene that possesses multiple antiproliferative and cell cycle regulatory functions (267). BTG1 has been identified as a marker of senescence based on microarray analysis of alteration in gene expression following the exposure of tumor cells to doxorubicin (262). Moreover, the association between senescence and BTG1 was independent to cell cycle regulators such as p53 or $p21^{Cip1}$ suggesting that it is regulated separately (262). The expression of BTG1 was also shown to be associated with senescence induced by doxorubicin in HT1080 osteosarcoma cells (data not shown). Here we utilize BTG1 as a surrogate for senescence and an additional marker to enrich for chemotherapy-induced senescent cells.

First, BTG1 expression is increased in cells that also upregulate SA-β-gal in response to etoposide exposure, indicative of both senescence induction and the association of BTG1 with senescence in HCT116 cells (**Figure 3.13A**). Similar to our observations in the H460 cells, etoposide-treated HCT116 (BTG1-RFP) cells undergo a senescent growth arrest followed by proliferative recovery which was also observed within 5-7 days following treatment (**Figure 3.13B**). While the BTG1 reporter construct is strongly expressed initially following etoposide treatment, its expression declines gradually over time, again suggesting reversal of senescence. This reversal of senescence

is accompanied by a generally similar pattern in the cell cycle profile, as well as in the expression levels p21^{Cip1} and other components of the SASP (**Figures 3.13C, D and E**). Here, IL-1β and IL-8 expression was significantly increased at Day 3 after drug exposure and, similar to the data generated in the H460 cells, gradually declined over time. While IL-6 levels did not change (similar to H460 cells), CXCL1 expression was initially increased in association with senescence followed by a gradual decline.

Similar to the H460 cells, a single-cell enrichment protocol was utilized to isolate highly senescent cells after etoposide treatment. Again, enrichment involved rigorous gating for the highest 30% of the C$_{12}$FDG–positive (SA-β-gal-positive) and enlarged cells, in addition to the expression of RFP-BTG1. The dual expression of C$_{12}$FDG and RFP-BTG1 was confirmed post-sorting (**Figure 3.13F**). Enriched HCT116 cells were monitored in culture and found to regain proliferative capacity in a similar fashion to enriched H460 cells (**Figure 3.13G**). Collectively, this evidence shows that etoposide-induced senescence is not obligatorily permanent in another tumor cell model.

B **HCT116 BTG1-RFP** — No. of Viable Cells vs Days, ETO 1 μM

C **HCT116** — Mean Fluorescence Intensity, BTG1-RFP (Control, Day1, Day3, Day5, Day7)

D **HCT116** — mRNA Relative Expression, p21$^{Cip1/Waf1}$ (Control, Day1 ****, Day3 ****, Day5 *, Day7, Day9)

E **HCT116** — mRNA Relative Expression, IL-1β (Control, Day1, Day3 ****, Day5 ****, Day7 ****, Day9 *)

HCT116 — mRNA Relative Expression, IL-8 (Control, Day1, Day3 ****, Day5 ****, Day7, Day9)

HCT116 — mRNA Relative Expression, CXCL1 (Control, Day1, Day3 **, Day5, Day7, Day9)

F

G

Figure 3.13. **Proliferative recovery from etoposide-induced senescence in HCT116 cells**. **A.** Wide-field fluorescent microscopy of BTG1-RFP (red) and $C_{12}FDG$ (green) in HCT116 cells after etoposide (1 μM) exposure. Scale bar: 20 μm. **B.** Growth arrest and recovery of HCT116 (BTG1-RFP) cells exposed to etoposide (1 μM) for 24 h (day 0). **C.** Flow cytometry analysis of BTG1-RFP expression over time following etoposide (1 μM) exposure. **D.** qRT-PCR quantification of the expression of p21Waf1/Cip1 and multiple senescence-associated secretory phenotype (SASP) factors (IL-1β, IL-6, IL-8 and CXCL1) over time following etoposide (1 μM) exposure. Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed by one-way ANOVA with Tukey post-hoc test. *$p<0.05$, ** $p<0.01$, ****$p<0.0001$. **E.** Widefield fluorescent microscopy images of HCT116 (BTG1-RFP) cells following enrichment showing both RFP and C12FDG staining. Scale bar: 200 pixel. **G.** Growth curves for $C_{12}FDG$/RFP- dual positive HCT116 (BTG1-RFP) cells after flow cytometry enrichment. X axis indicates time points relative to etoposide (1 μM) exposure.

3.4 Discussion

A long-standing paradigm in the scientific literature has been that senescence is an irreversible form of growth arrest (24,268). However, a number of previous reports in the literature, including work from our own laboratory, have suggested that some subpopulations of cells demonstrating characteristics of senescence have the potential to recover proliferative capacity by escaping or reverting from the senescent state (26,89,212,213,216,220,269,270). Consequently, in addition to the overall heterogeneity of tumor cell response to chemotherapy and radiation that can involve different modes of cell death such as apoptosis, necroptosis, ferroptosis, mitotoic catastrophe and a cytotoxic form of autophagy, as well as conventional growth arrest (251,252), and growth arrest mediated by autophagy and senescence (253), it appears that cells that express markers of senescence may also demonstrate heterogeneous responses (75). What we observe in our real-time imaging work is that while some cells that appear to be senescent remain in a prolonged state of growth arrest, senescent cell retain the capacity to re-emerge into a proliferative state.

In addition to the heterogeneity observed in the real-time imaging studies of cells enriched for senescence, our high-speed live cell interferometry (HSLCI) confirmed that the arrested cells exhibited increased cell mass and area consistent with cellular senescence(271). Furthermore, the ability of cells induced into senescence to form tumors was established in immune deficient mice. In this context, studies of the contribution of the immune system to the recognition and elimination of cells induced into senescence by chemotherapy and radiation are in progress.

The HSLCI technology, as applied in the current work, provides a novel perspective on the growth kinetics of tumor cell lines, specifically the relationship between cellular proliferation rate and the mass - area. The control cell lines and the subpopulation of etoposide treated cells that does not

undergo senescence-like changes exhibit high proliferation rates, while maintaining similar morphologies and similar mass and area. That is, under normal circumstances, cells do not grow beyond a certain mass-area without division being triggered. However, the etoposide-treated cells demonstrating senescence apparently have this relationship of proliferation with mass-area altered such that the cells continue to gain mass and grow in area without proliferation being triggered. However, this is not an indefinite process. While the bulk of the senescent cells remain in a growth arrested state, consistent with the paradigm that senescence is "irreversible", subpopulations of the senescent cells recover proliferative function.

Although the factors that allow for division of this subset of cells expressing senescence markers are ongoing, it has been suggested that these cells may acquire stem cell characteristics (90). Many tumor cells develop a high degree of genomic instability and eventually inactivate or otherwise lose the function of tumor suppressor genes such as p53 and p16^{INK4a}, which are regulatory elements of senescence (272,273). We further cannot predict whether the cells that have escaped from the senescence-like state will indefinitely maintain replicative capacity for subsequent generations, or will ultimately die. However, previous studies have suggested that the recovering cells may be more aggressive than the parental cell population (219). Furthermore, the capacity of cells derived from the senescent population to form tumors in animals suggests that tumorigenic potential is maintained. Here, it should be noted that we have also observed a similar capacity of breast tumor cells induced into senescence by doxorubicin to form tumors (a separate project in the lab).

Efforts to identify the factors that distinguish irreversibly arrested senescent cells from senescent cells that generate proliferating progeny are under way. We postulate that senescence that is

capable of giving rise to actively dividing cells could represent one previously unrecognized form of tumor dormancy; furthermore, cells that arise from the senescent-like state and re-emerge into a proliferative state could also ultimately contributes to disease recurrence.

There is strong evidence of senescence induction in the tumors of patients that have received chemotherapy or irradiation (58,93). In one study, senescence induction following neo-adjuvant chemotherapy was associated with a poor outcome in cancer patients(168). This effect might prove to be linked to the SASP, which has been suggested to contribute to adverse effects of therapy, poor outcomes, and possibly disease relapse (274). A direct clinical correlate of the model reported here is the emergence of therapy induced acute myelogenous leukemia in patients undergoing treatment with etoposide (and other topoisomerase II inhibitors). These patients typically present with rapidly progressive disease bearing translocations of the mixed lineage leukemia (MLL) gene, with multiple translocation partners. It is known that the hematopoietic progenitors develop DNA damage in response to topoisomerase II inhibitors, and that they present with a latency of 1-2 years following chemotherapy exposure (275,276). Based on the findings reported in this work, it may be postulated that these progenitors develop a senescent state in the immediate aftermath of chemotherapy, from which they recover with MLL and possibly other genetic lesions once the chemotherapy is stopped. Eventually those progenitors with genetic lesions conducive to survival of the transformed cells develop full blown hematological malignancy. Collectively, this growing evidence strongly suggests that the induction of senescence in tumors of patients receiving chemotherapy is not necessarily a beneficial outcome of therapy.

3.5 Future studies

Despite successfully providing evidence on the ability of senescent tumor cells to recover, it is recognizable that further work is required to expand on these observations. First, the changes that accompany recovery from senescence need further investigation. This requires molecular characterization of the recovered senescent population in comparison to the parental cell line, primarily, changes in gene expression profiles. Furthermore, recent evidence has suggested that cell populations originating from senescence acquire more aggressive traits (90,219); therefore, assessment of the differences in invasive, migratory and response to therapy of the recovered population needs further study. The development of more malignant phenotypes that respond poorly to conventional therapy is another indication that senescence could be a major component of tumor dormancy since recurrent cancer is refractory to most treatments. Finally, this approach should assist in identifying signaling pathways that facilitate the recovery of senescent tumor cells. This requires single cell analysis of senescent tumor cells to discover molecular signatures that could help in the prediction of recovery from senescence, given that only a subpopulation of senescent cells is capable of escaping.

Senescent cells are removed from the body by immune cells (237). In this work we have demonstrated the ability of senescent tumor cells to recover *in vivo* in an immunocompromised animal. This observation provides the incentive for investigating the ability of senescent cells to recover from senescence in an immunocompetent animal, where the immune cells could either eliminate or keep senescent cell in check. Moreover, it is important to determine if the recovered cells acquire the ability to escape immunosurveillance, a question that has not yet been addressed so far. Again, these expectations further support the premise that senescence is a form of tumor dormancy.

Ultimately, an experimental model of tumor dormancy following chemotherapy and based on senescence could be developed. For that, we suggest designing an animal model where senescent cells accumulating following the administration of chemotherapy can be tracked *in vivo*. This requires the labeling tumor cells with fluorescent labels integrated with one or more senescence markers and complemented with *in vivo* bioluminescence imaging. Moreover, development of a transgenic tumor cell line based on the suicide gene approach should be ideal to study the effect of eliminating these senescent cells on the likelihood of recurrent metastasis development. Suicide gene models rely on the ability of an inert compound to induce cell death in cells that become sensitive based on the expression of certain markers, such as the expression of p16I^{NK4a}.

Chapter Four: Interference with recovery of tumor cells from a chemotherapy-induced senescent-like state by the senolytic, ABT-263

4.1. Introduction

Senescence is a fundamental mechanism of aging and a powerful tumor suppressor mechanism that prevents the replication of genomically unstable cells (149). Senescent cells accumulate both in pre-malignant lesions and as a main component of tumor composition in patients (277). Furthermore, as discussed earlier, senescence is induced in tumor cells as a consequence of both DNA damaging and targeted cancer therapies, making senescence a primary response to cancer treatment (159,203). Although chemotherapy- and radiation-induced senescence have been studied for decades, it remains uncertain how the senescent response contributes to (or interferes with) disease control (278). Senescence has been proposed as a favorable outcome of cancer treatment since tumor senescent cells are in a growth-abrogated phase that halt tumor proliferation. Moreover, senescent cells can elicit an immunesurveillant response resulting in their clearance from the body (279). Accordingly, development of novel therapeutics that can induce senescence in tumor cells has been encouraged (166,203). This established opinion that senescence is a desirable goal of therapy can be derived, for example, from studies where senescence was associated with increased radiosensitivity in nasopharyngeal carcinoma cells, or where overexpression of SA-β-gal was associated with a reduction in the recurrence of prostate cancer.

However, this rationale has always overlooked the extensive genetic heterogeneity of tumor cells as well as the transcriptomic cell-cell differences amongst a uniform senescent population (75,197), thus, excluding the potential that the senescent growth arrest might not be obligatorily permanent. So far, we have presented experimental evidence and similar observations from the literature on examples where cells expressing the hallmarks of senescence re-entered a proliferative state. In addition, there is a growing rationale that the accumulation of senescent cells is negative for multiple reasons: (*i*) senescent cells are resistant to apoptosis and persist viable and metabolically active in the body. This applies also to senescent tumor cells, thus, senescence might provide means for cancer to remain dormant. (*ii*) The accumulation of senescent cells in the body is associated with a chronic inflammatory state mediated by the SASP (85,280) which is implicated in many other pathologies (281). More importantly, senescence, and the chemokines and cytokines secreted as a consequence of the SASP have deleterious paracrine influence that promotes several adverse effects associated with conventional chemotherapy including relapse (274). In addition, a pro-tumor SASP response could lead to a suppressed immune response and the maintenance of dormant tumor cells (282–284). (*iii*) Finally, recent studies have reported that cells derived from a senescent tumor population are often more aggressive than the original population which would be consistent with the difficulties encountered when treating recurrent cancer (90,219). Collectively, these recent updates indicate that senescence is an initially favorable response to therapy by delaying tumor progression, but an ultimately deleterious therapy outcome.

Recent efforts have shown that the removal of senescent cells from aging animals delays the onset of several aging-associated phenotypes and ameliorates already established pathologies (241,285). This approach, which was based on a genetic model of a $p16^{INK4a}$ suicide gene has been extended to identifying novel molecules that can eliminate senescent cells in a similar fashion; such drugs

are referred to as *senolytic agents* (243). These promising efforts have been largely restricted to progeroid models (with little focus on senescent tumor cells) or to merely experimental therapies. For example, an experimental cytotoxic drug delivery system based on the upregulation of SA-β-gal cleared senescent tumor cells induced by the CDK4/6 inhibitor, palbociclib, and resulted in dramatic tumor xenograft regression (286). In this context, a powerful model to eliminate senescent cells is based on the use of the established Bcl-2 inhibitor ABT263 (navitoclax) which was shown to clear senescent cells from animals subjected to total body radiation, resulting in rejuvenation of their hematopoietic functions (76,287). ABT263 was also shown to improve radiation-induced pulmonary fibrosis in a mouse model by eliminating senescent cells (288). ABT263 is currently being tested in dozens of clinical trials making it already available in the clinic. Although ABT-263 has impressive therapeutic effects as a monotherapy for lymphoid malignancies (289), it has been recently been replaced with ABT-199, which is more selective against Bcl-2, which is the major anti-apoptotic protein active in leukemias (290). ABT-263 is now actively being tested for activity against solid tumors in combination with other chemotherapies because of its ability to target BCL_{XL}, another member of the Bcl-2 protein family (www.clinicaltrials.gov).

While drugs such as ABT-263 are usually administered together with the chemotherapy(291), we are proposing a novel therapeutic approach utilizing the senolytic potential of ABT263, in that, once the tumor cells have entered into an established state of senescence, ABT263 shall be used as a "clearing" agent to eliminate the residual senescent tumor cells in order to interfere with disease recurrence (**Figure 4.1**). Given the sensitivity of senescent cells to senolytics, drugs such as ABT-263 are also likely to be most effective following the use of chemotherapeutic agents that either promote primarily senescence or that leave a residual surviving senescent cell population.

In addition to ABT-263, several other senolytic agents have recently been identified, most prominently the HDAC inhibitor panobinostat (292), and the HSP90 inhibitor 17-DMAG (293). Each of these compounds is approved for use in investigational clinical trials either alone or in concert with traditional chemotherapies (294,295). However, the most effective scheduling to maximize their clinical benefits against solid tumors remains to be determined. It is possible that drug efficacy would be maximized by treating patients when their tumors have achieved peak levels of senescence (either as a primary response or in residual cells). This would reduce the total exposure to senolytic agents, possibly allowing the use of effective lower doses, and for extended periods of time.
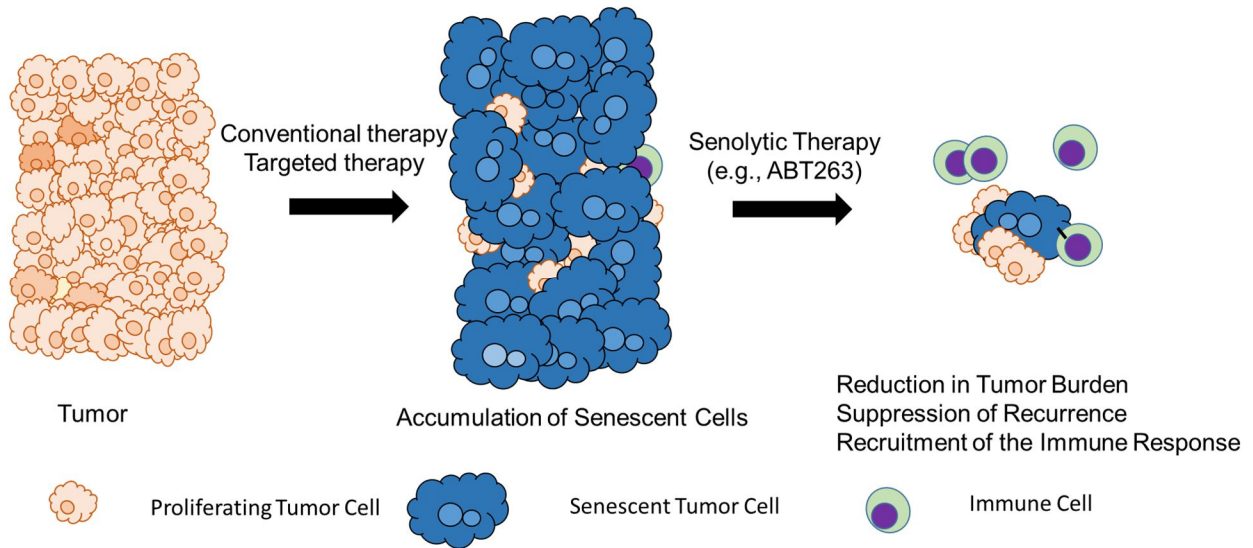
Figure 4.1. The use of senolytic therapy to eliminate senescent tumor cells. A model illustrating the proposed therapeutic approach. Tumors exposed to conventional/targeted therapies accumulate senescent tumor cells that persist after the end of treatment. These cells contribute to adverse effects of therapy and can contribute to cancer relapse. Sequential administration of ABT263 and other senolytic agents results in the selective elimination of residual senescent tumor cells and thereby tumor regression, delay in cancer recurrence and recruitment of an immunosurveillant response.

4.2 Experimental procedures

4.2.1 Cell Lines.

The A549 cell line was generously provided by Dr. Charles Chalfant (Virginia Commonwealth University). The H460 and the HCT116 (WT and p21^{Cip1} knockout) cell lines were generously provided by Dr. Richard Moran and Dr. Lisa Shock (Virginia Commonwealth University). Lewis lung carcinoma (LLC) cells, which originated in the lungs of a C57BL mouse, were a generous gift from the laboratory of Dr. Andrew Larner (Virginia Commonwealth University). A549, H460 and LLC cells were cultured in DMEM, while HCT116 cells were cultured in RPMI, all supplemented with 10% (v/v) fetal bovine serum (SH30066.03, Thermo Scientific, MA, USA), 100 U/ml penicillin G sodium (15140–122, Invitrogen, CA, USA), and 100 μg/ml streptomycin sulfate (15140–122, Invitrogen, CA, USA) at 37º C and 5% CO2. Cells were grown at subconfluent cultures and passaged 3 times weekly.

4.2.2 Cell Viability and Clonogenic Survival.

Growth curves were determined by counting viable cells based on trypan blue exclusion at various time points after the treatment. Cells were harvested using trypsin, stained with 0.4% trypan blue (T8154, Sigma, MO, USA), and counted using a hemocytometer under light microscopy. The assay is based on the fact that viable cells have intact plasma membranes and thus impermeable to trypan blue. Colony forming assay measures the cell survival and the ability of cells to form viable colonies. However, instead of classically seeding a low number of cells and allow for colony formation, A549 cells were seeded in a heavy monolayer before being induced into senescence by

etoposide. At the indicated time points, cells were harvested, washed with PBS and fixed with methanol. Fixed cells were allowed to dry overnight and then stained with crystal violet (0.01%).

4.2.3 SA-β-galactosidase Staining.

Histochemical SA-β-gal labeling was performed as previously described by Dimri et al. (80). Images were generated using bright field by Olympus inverted microscope (Olympus inverted microscope IX70, 20x objective, Q-Color3™ Olympus Camera; Olympus, Tokyo, Japan). For the *in vivo* SA-β-gal staining, the harvested tumors were immediately snap frozen in liquid nitrogen and then infiltrated with sucrose solution (5%) at 4º C overnight. Next, the tumors were embedded in O.C.T. compound (Sakura, 25608-930) and stored at -80 º C. Frozen sections were performed in the Virginia Commonwealth University Microscopy Core using Thermo FSE cryostat. The samples were then stained similar to fixed cells.

4.2.7 Western Blotting

Western blotting was performed as previously described (253). Antibodies used: Bcl-2 (Sigma, B3170), Bcl-X$_L$ (Cell Signaling, 2764S), p53 (Santacruz, sc-126), p21^{Cip1} (BD Biosciences, 610234), Bax (Cell Signaling, 2772T), Bak (Cell Signaling, 6947S), Noxa, (Invitrogen,, MAI-41000), Mcl-1 (Enzo, ADI-AAP-240F), (c-caspase (Cell Signaling, 9664S) and GAPDH (Cell Signaling, 5174).

4.2.8 Annexin V staining.

At the indicated time points, cells were harvested, washed with PBS, and resuspended in 100 µl of 1x Binding Buffer (AnnexinV-FITC apoptosis detection kit; 556547, BD Biosciences, NJ, USA) with 5 µl of Annexin V and 5 µl of PI, then incubated at room temperature for 15 min in the dark.

This suspension solution was then brought up to 500 µl using the same buffer and then analyzed by flow cytometry. Apoptotic cells express phosphatidylserine on the extracellular surface of the plasma membrane and thus, amenable for binding with annexin V. PI is a nuclear stain that cannot permeate the cell membrane of living cells. Cells stained with both annexin V and PI are late apoptotic cells that have disrupted plasma membranes.

4.2.9 TUNEL assay

For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, adherent cells were harvested and centrifuged at 10,000 rpm for 5 min onto slides (Shandon Cytospin 4, Thermal Electron Corp) 48 h after etoposide removal. Slides were fixed with 4% formaldehyde for 10 min and then washed with PBS for 5 min at room temperature. The cells were then fixed with a 1:2 dilution of acetic acid and ethanol for 5 min, followed by staining with a 1:1000 dilution of DAPI at room temperature. Coverslips were sealed using clear nail polish and apoptosis was assessed by evaluating three fields per condition with an Olympus inverted microscope (20x objective, Q-Color3™ Olympus Camera, 488 nm filter and UV light, Olympus, Tokyo, Japan). TUNEL assay detects DNA fragmentation, a process that is frequently associated with apoptotic cells.

4.3 Results

4.3.1 Elimination of senescent A549 NSCLC cells by ABT263.

To study the potential of ABT263 to eliminate senescent tumor cells, we established a system using A549 NSCLC cells exposed to etoposide either continuously for a week (at 2 μM concentration) or using short-term exposure to the near-clinical (8.7 μM) concentration. Both treatment regimens resulted in the induction of a robust senescent response in A549 cells, marked by extensive upregulation of SA-β-gal (**Figure 4.2A**). In addition, etoposide treatment resulted in a stable growth arrest, as the cells halted growth during the treatment period and at least 2 weeks afterwards.

Next, we exposed senescent A549 cells to increasing concentrations of ABT263. At the same time, we exposed proliferating, non-senescent A549 cells to the same range of ABT263 concentrations. For a drug to exert a senolytic activity, by definition, it should induce cell death selectively in senescent cells, with no or little effect in non-senescent cells. ABT263 produced a concentration-dependent decrease in the survival of etoposide pre-treated A549 cells with almost no cytotoxic effect on proliferating non-senescent cells (**Figure 4.2B**). This observation indicates that ABT263 produced selective elimination of senescent A549 cells consistent with its established role of eliminating senescent fibroblasts in aging models.

We determined that the decrease in survival resulting from exposure of senescent cells to ABT263 is due to induction of apoptosis, as demonstrated by the significant increase in annexin 5 staining and the cleavage of PARP, both representing classic apoptosis markers (**Figure 4.2C and D**). Again, in agreement with the survival results, ABT263 produced only a marginal increase in annexin V staining in proliferating, non-senescent A549 cells.
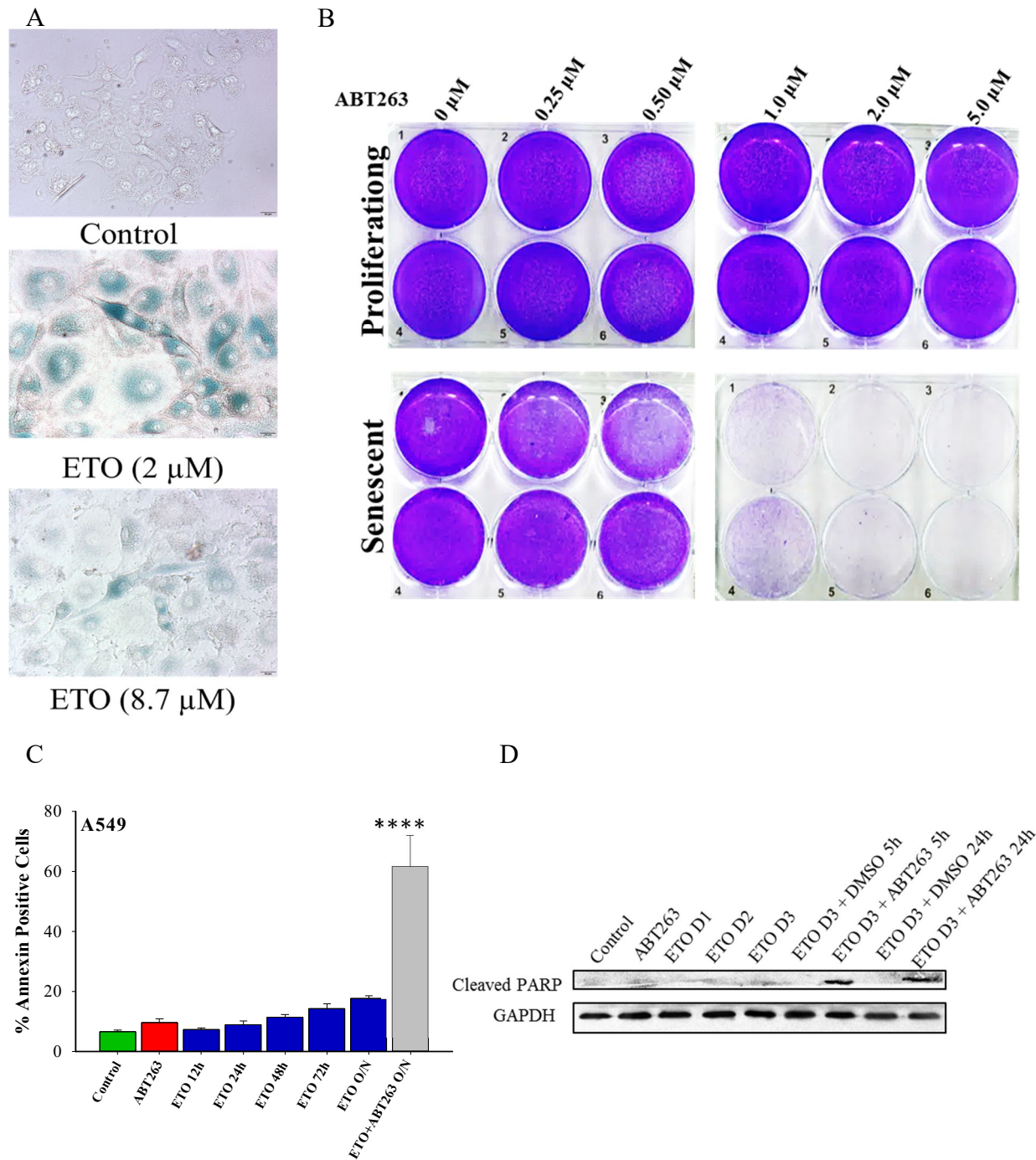
Figure 4.2 Senescent A549 cells are amenable for clearance by ABT263. A. Images are for histochemical SA-β-gal staining indicating senescence induction following both treatments in A549 cells. **B.** Elimination of senescence-like A549 tumor cells induced by etoposide (2 μM for 7 days) by multiple concentrations of ABT263 (48 h exposure). Figure also shows the effect of ABT263 on proliferating, non-senescent cells. **C.** Assessment of apoptosis by annexin V staining in etoposide-treated cells over time indicating increased cell death only in the senescent population after overnight (O/N) ABT263 exposure. **D.** Western blotting showing PARP cleavage in senescence-like cells following ABT263 exposure.

Because interfering with recovery from senescence would be an additional advantage of using senolytics, we moved to test the effect of ABT263 on the temporal growth of A549 cells after etoposide exposure. Again, we used an identical regimen where we expose cells to etoposide followed by the addition of ABT263. ABT263 had no effect on the growth of parental, non-senescent A549 over time (**Figure 4.3A**). Consistent with senescence induction, A549 cells exposed to etoposide (either 2 μM or 8.7 μM) exhibited a stable growth arrest pattern that was sustained for days even following the removal of etoposide from the culture medium (**Figure 4.3B**). The addition of ABT263 resulted in a dramatic decrease in cell viability in etoposide pre-treated cells. This decrease in viability was due to the decreased number of SA-β-gal cells following ABT263 exposure (**Figure 4.3B**). Interestingly, the small population of cells that persisted after ABT263 treatment are SA-β-gal positive with morphological characteristic of senescence, suggesting that a single bout of ABT263 is incapable of completely eliminating a senescent population of tumor cells. Furthermore, this remaining senescent population gradually undergoes proliferative recovery (**Figure 4.3C**).

In effort to interfere with proliferative recovery, we exposed the surviving population with a sequential, second bout of ABT263 48 hours after the first exposure. The second dose of ABT263 resulted in a further decrease in cell number suggesting that the residual senescent population remains sensitivity to ABT263 (**Figure 4.4A**). This result indicate that sequential exposures of senescent tumor cells could interfere with proliferative recovery. As might have been anticipated, the senolytic ability of ABT263 diminishes over time as the senescence-like state is resolved, suggesting that ABT263 is most effective immediately after the induction of senescence (**Figure 4.4B**).
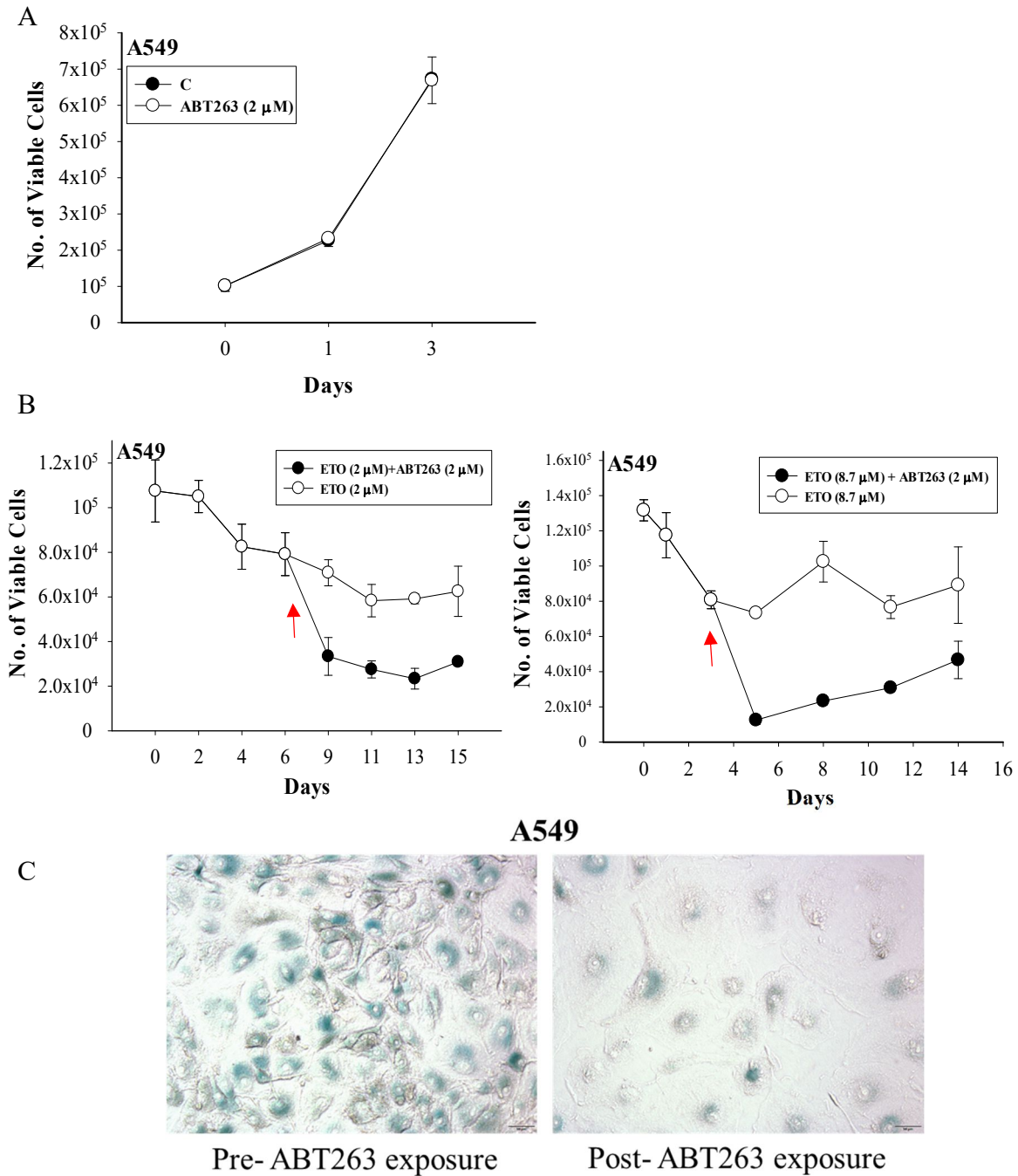
Figure 4.3. Temporal growth curves before and after ABT263 exposure . A. Growth curves of proliferating, non-senescent A549 cells exposed to ABT263 (2 μM). **B.** Growth curves of A549 cells after induction of senescence by etoposide (2 μM or 8.7 μM) followed by ABT263 (48 h exposure). Red arrows indicate when ABT263 was administered. X axis refers to days relative to etoposide exposure. **C.** Images for histochemical SA-β-gal staining before and after treatment of A549 cells to with ABT263 (2 μM).

A

B

Time indicates when ABT263 was administered
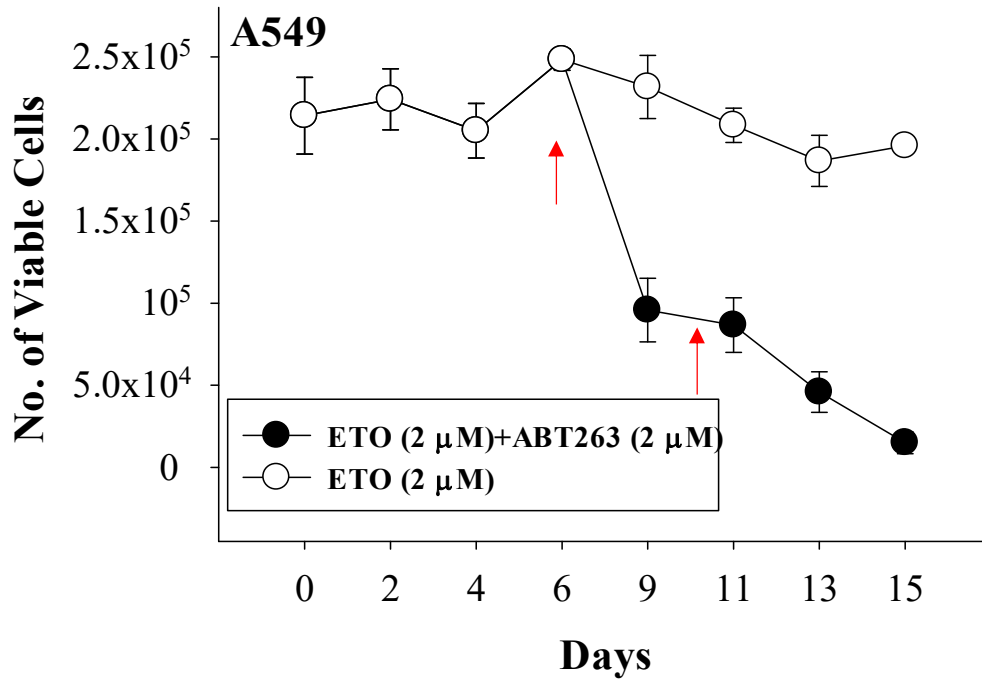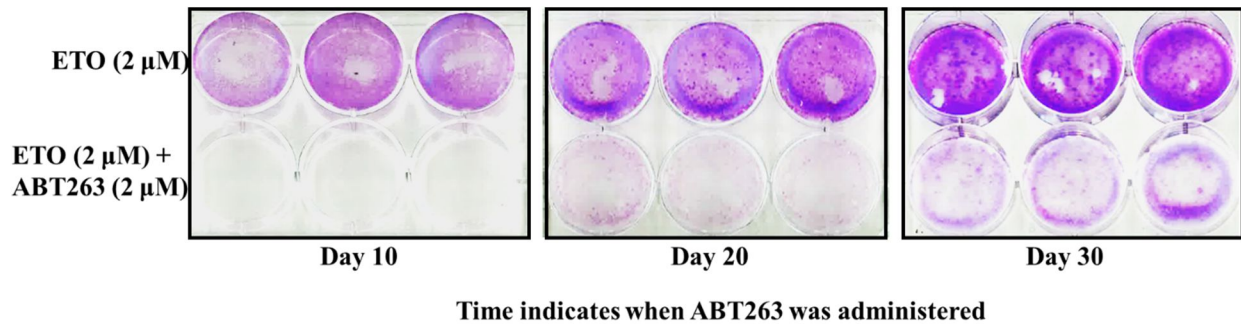
Figure 4.4. Interference with proliferative recovery of senescent A549 by two exposures to ABT263. A. Growth curves of A549 cells after induction of senescence by etoposide (2 μM) followed by two doses of ABT263 (48 h exposure). Red arrows indicate when ABT263 was administered. X axis refers to days relative to etoposide exposure. **B.** Attenuation of the senolytic activity of ABT263 over time after etoposide removal from the culture medium. Time indicates when ABT263 was administered relative to the end of 7 days exposure to etoposide (2 μM).

4.3.2 Senolytic activity of ABT263 in senescent A549 cells is mediated by Bcl-X$_L$

Cells respond to stressful stimuli by activating a tightly controlled machinery of signaling and effector molecules that lead to cell death, or *apoptosis*. Apoptosis represents a fundamental defense barrier against the accumulation of damaged cells and is an important homeostatic mechanism (296). A classic example is the induction of apoptosis in skin epithelial cells developing UV-induced DNA damage, where failure of these cell to activate their suicide program contributes to their transformation into squamous carcinoma of the skin (297). Accordingly, evasion of apoptosis, or "resisting cell death", despite harboring lethal mutations, represents an important hallmark of cancer cells (298). Resistance to apoptosis develops due to derangements in its regulatory pathways, namely imbalances in the interaction between pro- and anti- apoptotic molecules.

For years, a main focus of study in the biology of cancer was the B-cell lymphoma 2 (Bcl2) protein family which plays a pivotal role in the regulation of apoptosis through the intrinsic (mitochondrial) pathway (299). In healthy cells, complex interactions between members of the Bcl-2 family maintain the integrity of the mitochondrial membrane, preventing the release of apoptotic molecules from the mitochondria. This function is maintained by the "anti-apoptotic" members of the family, namely, Bcl-2 itself, Bcl-X$_L$, Bcl-w and Mcl-1. On the other hand, the "pro-apoptotic" members are either activators, such as Bid and Bim, or effectors, such as Bax and Bak, where the latter two are capable of dimerization or breaching the integrity of the outer mitochondrial membrane, thus, initiating the cell death cascade. Bcl-2 and the other anti-apoptotic molecules can directly interact with Bax and Bak to prevent the initiation of apoptosis.

Accordingly, overexpression of Bcl-2 can facilitate the acquired resistance to cell death stimuli that occurs during transformation. In fact, as was described soon after its identification, Bcl-2 mediates survival in hematopoietic cells allowing for other oncogenic drivers, such as c-myc, to promote accelerated proliferation (300). Similarly, Bcl-X_L has been established to have an important role in promoting tumor progression and stemness, not only in hematologic malignancies but also in melanoma and glioblastoma (301), while Bcl-w contributes to the pathogenesis of multiple lymphoid cancers (302).

Interestingly, senescent fibroblasts which exhibit a unique resistance to apoptosis, fail to downregulate Bcl-2 in response to stress, indicating a key role of members of the Bcl-2 family in the stability of senescent cells (74). Furthermore, Bcl-2 is necessary to mediate the cell cycle arrest induced by DNA damage or serum starvation in senescent fibroblasts (303). However, other reports have indicated that senescent cells develop apoptosis resistance independently from Bcl-2 function, suggesting a complicated role of Bcl-2 family in senescence (73,304). Furthermore, elimination of senescent IMR-90 human fibroblasts (induced by radiation, replicative exhaustion or oncogene overexpression) was dependent on Bcl-X_L and Bcl-w rather than Bcl-2 (76). Interestingly, upon assessment of Bcl-2, Bcl-X_L and Mcl-1 proteins in A549 cells exposed to etoposide and undergoing senescence, Bcl-2 expression was not evident (**Figure 4.5A**). This suggested the involvement of the other targets of ABT263 in mediating its senolytic activity in senescent A549 cells. Thus, we attempted to silence the other target og ABT263, Bcl-X_L, using short interfering RNA (siRNA) immediately after the removal of etoposide from the culture medium (similar to when we add ABT263) (**Figure 4.5B**). Bcl-X_L knockdown resulted in a decrease in senescent cells viability in a similar fashion to ABT263, suggesting that ABT263 senolytic effect could be mediated by Bcl-X_L (**Figure 4.5C**).
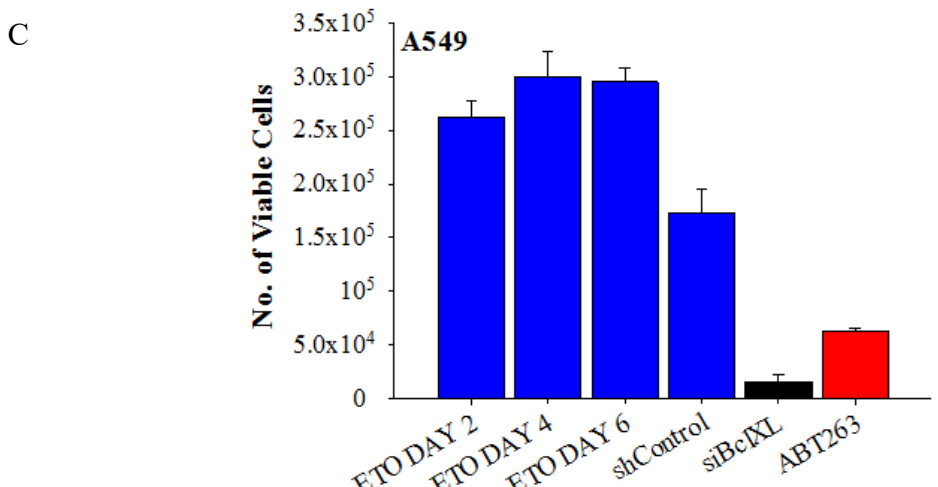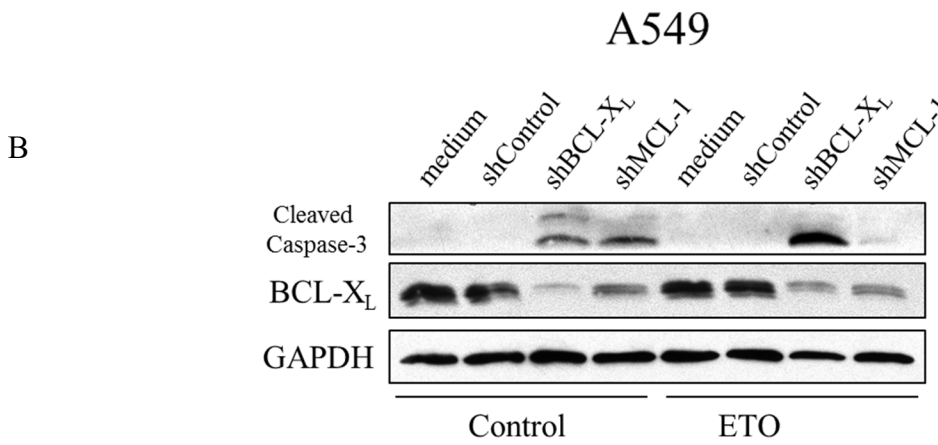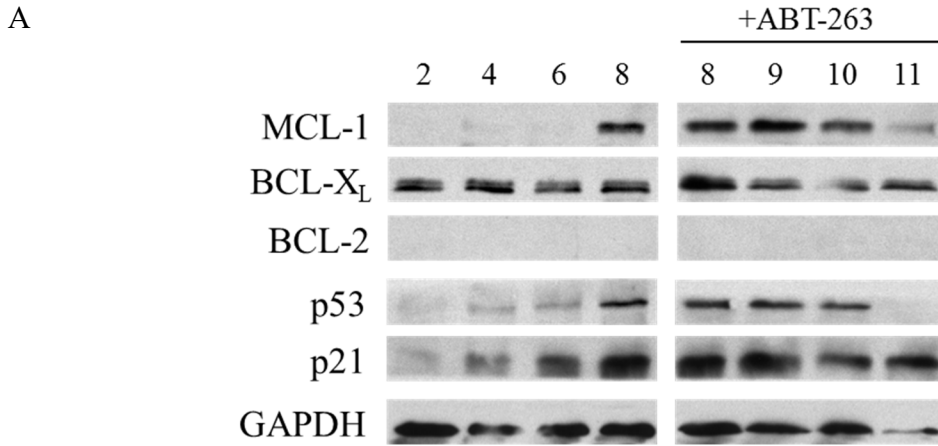
Figure 4.5. Senolytic activity of ABT263 in senescent A549 cells is mediated by Bcl-X$_L$. A. The expression of anti-apoptotic BCL-2 family proteins in etoposide-induced senescent cells A549 cells were treated with etoposide for 8 days to induce senescence, then ABT-263 was added for 48 hours **B.** The expression of BCL-XL and cleaved caspase-3, indicative of apoptosis, was determined by Western blots after BCL-X$_L$ knockdown. **C.** A549 cells were treated with etoposide for 6 days. Then, lentiviruses harboring shBCL-X$_L$ or shControl were infected overnight. Two days later, numbers of viable cells were determined by trypan blue exclusion.

4.3.3 ABT263 exerts senolytic activity against senescent H460 and LLC cells

To examine the whether ABT263 is capable of exerting its senolytic activity in other lung cancer cell lines, in a similar fashion to senescent A549 cells, H460 cells induced into senescence by etoposide were exposed to ABT263 and were shown to be sensitive as well, as marked by reduced cell viability (**Figure 4.6A**). In addition, the surviving population consisted of senescent, SA-β-gal positive cells, suggesting that they might be amenable to a second bout of ABT263 (**Figure 4.6B**). We also examined the effect of ABT263 in a Lewis lung carcinoma cells (LLC, murine lung cancer cell line) induced into senescence by etoposide (**Figure 4.6C**). Again, ABT263 was able to reduce the viability of these cells (**Figure 4.6D**).

4.4 Discussion

The contribution of senescence to the pathogenesis of multiple age-associated pathologies is the main driver for the development of drugs that can preferentially eliminate senescent cells. In the seminal experiments, the elimination of a small portion of the accumulating senescent cells in an aging mouse using a transgenic suicide gene model resulted in a remarkable improvement in healthspan (241). Consequently, efforts have been focused on developing senolytic agents; compounds that selectively clear senescent cells without compromising the viability of their healthy neighbors (281). This approach is primarily based on promoting apoptosis in senescent cells, which otherwise
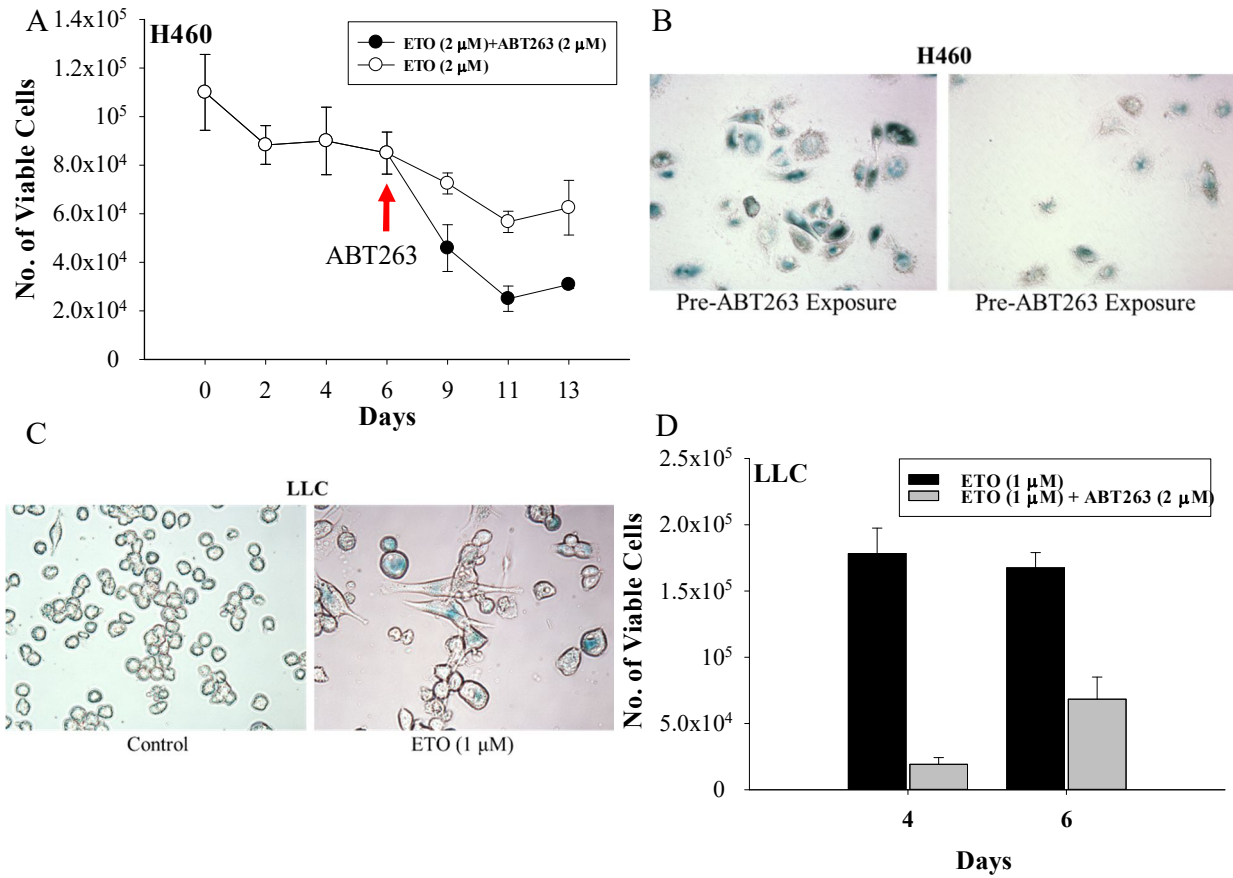
Figure 4.6. Effect of ABT263 on senescent H460 and LLC cells. A. Growth curves of H460 cells after induction of senescence by etoposide (2 μM) followed by ABT263 (48 h exposure). Red arrow indicates when ABT263 was added. **B.** SA-β-gal staining showing senescent H460 cells before and after ABT263 exposure. **C.** SA-β-gal staining showing the induction of senescence in LLC by etoposide (1 μM for 24 h). **D.** Cell viability of LLC cells induced to senescence by etoposide and then exposed to ABT263.

appear to be capable of resisting cell death and maintaining survival for an extended period of time (305). For example, senescent cells upregulate anti-apoptotic pathways, theoretically making them susceptible to cell death by interfering RNA or small molecules that target these pathways (243).

Recently, a novel approach to selectively eliminate senescent cells in mice has paved the way for a more efficient pharmacological clearance of such aging cells (287). This is based on the fact that many senescent cells up-regulate the expression of Bcl2 family proteins, which serves to attenuate or suppress apoptosis. Bcl2 inhibitors such as ABT737, ABT263 (oral version of ABT737) or histone deacetylase inhibitors that act to reduce expression of Bcl2 proteins have been shown to effectively target senescent cells (76,287). Additionally, several other compounds have been proposed to possess a senolytic activity (**Table 3**).

Since senescent cells are major components of malignant tumors, and retain the potential to recover after a period of dormancy, one could argue that combining these therapies with conventional pro-senescence cancer therapy might actually improve treatment outcomes. In doing so, treatment with senolytic agents could prevent (or significantly delay) proliferative recovery from therapy, and disease recurrence. We have demonstrated that the exposure of senescent tumor cells to ABT263 results in effective killing of these cells and a potential delay in proliferative recovery. Both preclinical studies and clinical trials are in progress combining ABT-263 and similar agents simultaneously with chemotherapy and for extended periods after chemotherapy. A primary element of the current work is that we are proposing to utilize ABT263 in sequence with chemotherapy to eliminate tumor cells that have entered a state of senescence, with the goal of not only enhancing the antitumor response but critically also preventing disease recurrence (**Figure**

Targeted Pathway	Method of Elimination
Bcl2 anti-apoptotic family	ABT263 (Navitoclax)(287) ABT-737 (76) A1331852 (306) A1155463 (306)
Cell surface glycoproteins, DPP4	Elimination by antibody-dependent cell-mediated cytotoxicity (ADCC) (187)
HSP90	17-DMAG (alvespimycin) (293)
PI3K/Akt survival pathway	Quercetin (243) Fisetin (306)
Tyrosine Kinase Receptors	Dasatinib (243)
Glucose and fatty acid metabolism, AMPK, autophagy	Phloretin (242) Cytochalasin B (242) Sodium oxamate (242) Etomoxir (242) Compound C (242)
Histone deacetylase inhibitors (HDACs)	Panobinostat (292)

Table 3. Senolytic agents. Summary of drugs that demonstrated the ability to clear senescent cells either *in vitro* or *in vivo*, indicative of possible targets for the elimination of senescent cells

4.1). Clinically, the ABT263 would be administered sequentially after exposure to the primary chemotherapy for short durations at times of peak TIS, thereby reducing overall patient toxicity. Furthermore, the patient could be treated with the primary form(s) of chemotherapy at lower, less toxic doses that induce primarily senescence when followed by the senolytic agent.

We have also shown that ABT263 exerts its senolytic activity via its target Bcl-X$_L$. Previously, Bcl-X$_L$ (as well as Bcl-w) has been shown to mediate the senolytic activity of ABT737 in eliminating senescent fibroblasts (76). It has been postulated that a Bcl-2 inhibitor that does not interfere with Bcl-X$_L$ will not exert a senolytic action. Although not very well understood, Bcl-X$_L$ appears to contribute to stabilizing the outer mitochondrial membrane in a different manner than Bcl-2. This difference is clearer in tumor cells (MCF7 breast tumor cells) exposed to senescence-inducing therapy such as doxorubicin, where Bcl-X$_L$ plays the main role in promoting the survival of these cells (307). Furthermore, we found that the selective Bcl-2 inhibitor ABT199 (venetoclax) has no effect on senescent H460 cells (data not shown). This suggests that Bcl-X$_L$ plays a primary role in the survival of senescent tumor cells especially in solid tumors. Accordingly, selective Bcl-X$_L$ inhibitors, such as the experimental compounds A1331852 and A1155463, have successfully eliminated several types of senescent cells including HUVEC, IMR90 and then to a lesser extent, primary human preadipocytes (306). Similar to ABT263, these compounds did not have the same effect on proliferating cells. Although ABT263 side effects can be controlled by careful dosing, thrombocytopenia remains a dose-limiting adversity, resulting from the dual inhibition of Bcl-2 and Bcl-X$_L$ in bone marrow cells (308). Therefore, future use of these more selective Bcl-X$_L$ inhibitors should provide a less toxic means to target senescent cells. Lastly, our data shows the upregulation of Mcl-1 while being exposed to etoposide and undergoing senescence. Mcl-1 is another member of the ABT263 family that plays a key role in regulating the mitochondrial

pathway of apoptosis (309). Its upregulation during senescence suggests it might be an additional target to induce killing in senescent tumor cells, especially after the development of S63845, a small molecule that selectively targets Mcl-1 (310).

While the use of ABT263 and other senolytics sounds appealing, several obstacles should be addressed. First, these drugs are associated with toxicities that might limit their combination with pro-senescence therapy. Therefore, a main purpose of this work is to propose a model where these drugs are used at lower doses that are less toxic, but can still effectively eliminate senescent tumor cells. Moreover, the treatment regimen must be *sequential*, avoiding the added toxicity resulting from conventional drug combination. The main purpose would be to use these drugs as clearance therapy to eliminate residual senescent cells. Second, if the effect of ABT263 on senescent tumor cells clinically will be equivalent to its effect *in vitro,* then the abrupt induction of cell death might result in tumor lysis syndrome. Of course, this issue will depend on the mass of the targetable senescent cells. Finally, a major concern is that ABT263 might eliminate beneficial non-tumor senescent cells that contribute to several homeostatic functions in the body such as wound healing (96). This concern should be weighed against the benefits of clearance therapy versus the medical condition of each patient in an individualized manner. Furthermore, it is logical to establish an approach whereby senolytic therapy can be administered following the confirmation of senescence induction in patients receiving pro-senescence chemotherapy. The impact of ABT263 on existing, or developing aging-related disorder in the presence of cancer has not been studied and multimorbidity should be taken into consideration. All together, ABT263 provides a promising means to eliminate senescent tumor cells; however, further characterization of this novel treatment concept is required.

4.5 Future studies

The successful demonstration of the ability of ABT263 to eliminate tumor cells invites for several follow up questions. First, the ability of ABT263 to exert the same effect must be tested *in vivo*. This requires the design of an experimental model where tumor xenografts are generated and exposed to senescence-inducing therapy. We were able to establish a model where low-dose etoposide can successfully induce senescence in A549 tumors implanted in NSG mice (**Figure 4.7**). Following the establishment of senescence in those tumors, the mice will receive low-dose ABT263 by oral gavage in cycles. A cycle consists of daily doses of ABT263 for five days followed by two, drug-free days to allow the animals to recover. In addition to measuring tumors volume and mass, toxicity profiles should be also generated to assess if this sequential treatment approach is advantageous in reducing the adverse effects that would be generated by simultaneous combination of both drugs.

Second, it is essential to demonstrate the ability of ABT263 to eliminate tumor cells induced into senescence by means other than DNA damaging agents in order to prove that the outcome is not a result of conventional pharmacological additive effect. Models where tumor cells can be induced into senescence using targeted therapy, such as palbociclib in breast tumor cells or androgen-deprivation therapy in prostate tumor cells, both of which are unlikely to be associated with robust DNA damage, should be used. The ability of ABT263 to eliminate senescent cells and interfere with proliferative recovery from senescence must be tested in these other models to validate (*i*) that its senolytic activity is consistent and (*ii*) that proliferative recovery is a consequence of senescence. For a proof of principle we have demonstrated that when tumor cells fail to undergo senescence due to the lack of $p21^{Cip1}$ fail to recover from acute exposure to chemotherapy (**Figure 4.8**). In addition, these cells responded to DNA damage with primarily apoptosis instead of

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senescence in cases where p21^{Cip1} function was present. This means that even without using ABT263, interfering with the senescent response to DNA damage decreases the survival potential of senescent cells.

Lastly, it has been shown that simultaneous treatment of cisplatin and ABT-263 enhances apoptosis in both p53 wild-type and mutant non-senescent NSCLC cells (311). However, it is not known whether p53 is involved in apoptosis induced by ABT-263 in cells induced into senescence by chemotherapy. To address this question, we started by knockout and knocking down p53 in H460 and A549, respectively (**Figure 4.9**). Next, the effect of p53 deletion in chemotherapy treatment and also subsequent treatment with senolytic agents for overall cell death (PI/Annexin V on apoptosis and LDH release) must be determined. Reciprocally, doxycycline-inducible expression of p53 in H1299 cells needs to be established where the effect of p53 overexpression and ABT-263-induced apoptosis upon promotion of senescence cells can be determined. From these studies the contribution of p53 to the apoptotic switch precipitated by ABT-263 in TIS cells should be inferred to provide deeper mechanistic insight on the connection between senescence and apoptosis.
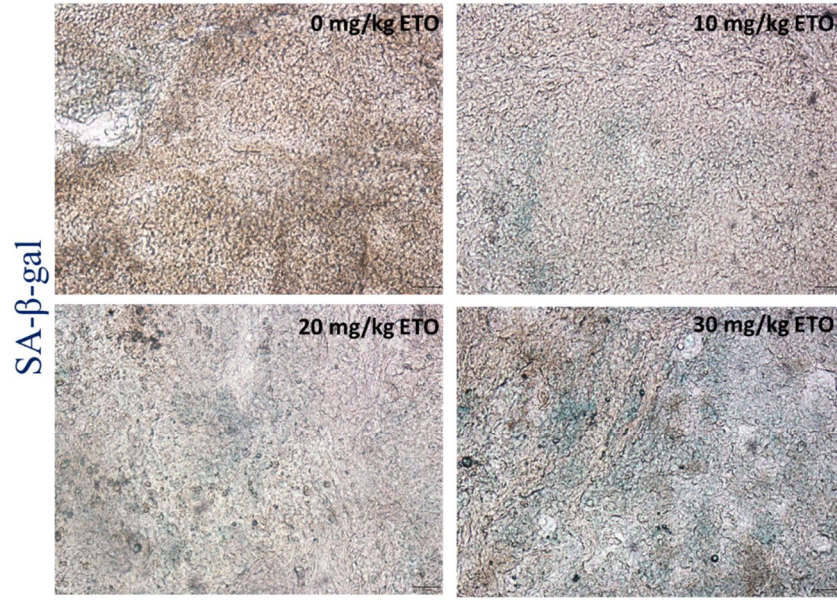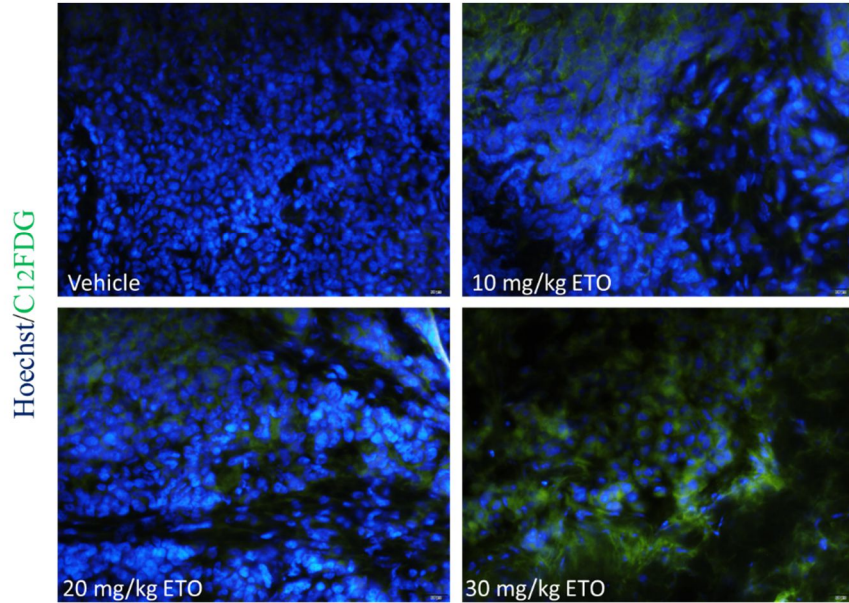
Figure 4.7. Detection of SA-β-gal activity in lung tumors after chemotherapy. Palpable A549 tumors in NSG mice were treated with either vehicle or the indicated doses of etoposide in 5 injections over a week. After the last dose the animals were euthanized, tumors collected and frozen sectioned. Sections were then stained for SA-β-gal both histochemically (X-Gal - upper panel) and using fluorescent microscopy (C_{12}-FDG - lower panel). Increase in blue (upper) or fluorescent green (lower) SA-β-gal staining with etoposide exposure.

HCT116 WT ETO (1 µM)   HCT116 p21 (-/-) ETO (1 µM)

SA-β-gal

C$_{12}$FDG

TUNEL

HCT116 p21 (-/-)

No. of Cells

- ● Control
- ○ Etoposide 1 µM
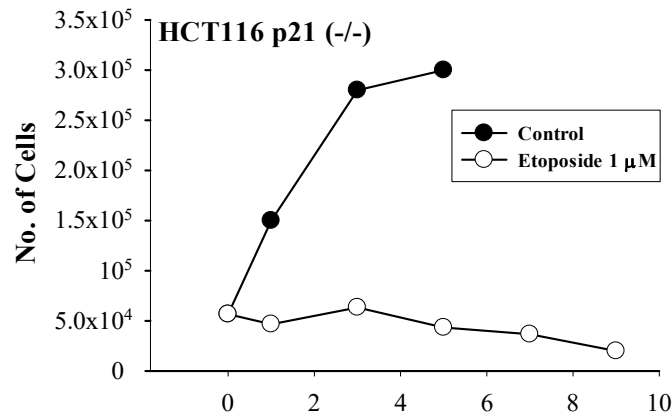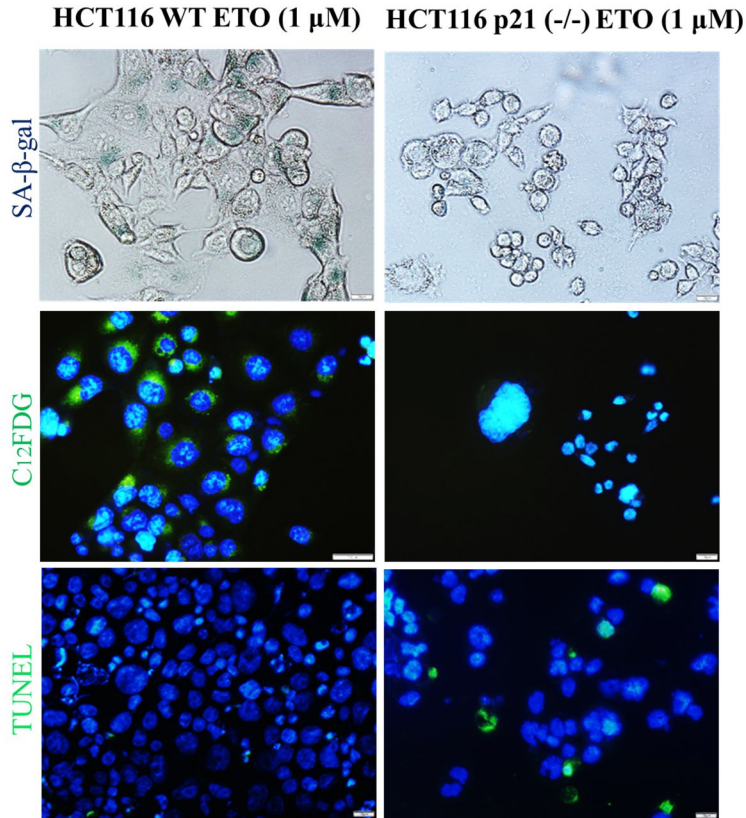
Figure 4.8. Cells that fail to senesce in response to chemotherapy do not recover. A. Histochemical SA-β-gal staining, C$_{12}$FDG staining, and TUNEL assay comparing the response of HCT116 WT and p21^{Cip1} (-/-) cells to etoposide (1 µM) exposure for 24 h. All assays were performed 48 hours following drug removal **B**. Temporal growth assessment of both HCT116 WT and p21^{Cip1} (-/-) after etoposide (1 µM) exposure for 24 h.

121
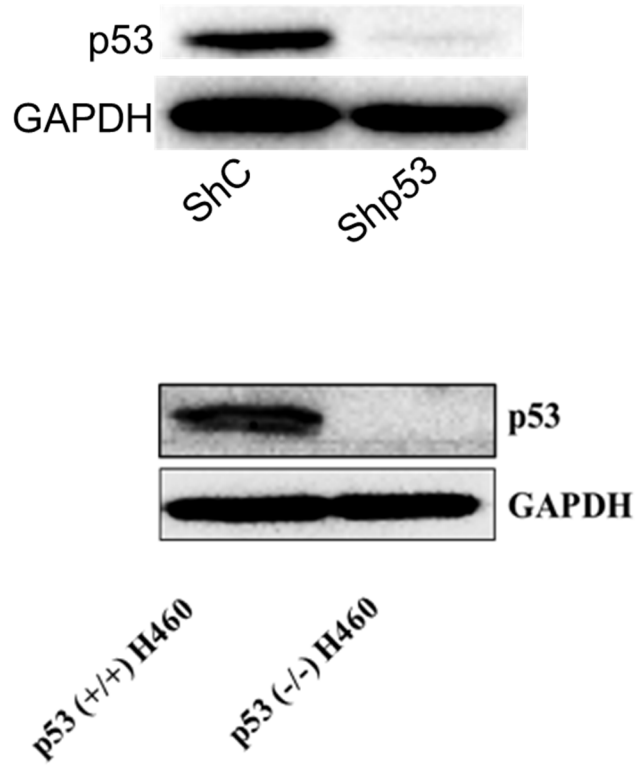
Figure 4.9. Knockout of p53 in A549 cells using shRNA and H460 cells using CRISPR/Cas9.

Chapter Five: Etoposide-induced senescence and proliferative recovery occur independently of autophagy in H460 lung cancer cells

5.1 Introduction

Autophagy is a homeostatic process that involves lysosomal-dependent intracellular degradation of organelles and damaged proteins (312). At basal levels, autophagy contributes to several physiological processes and can be further induced in response to a variety of cellular stresses including nutritional deprivation, oxidative stress, accumulation of misfolded proteins and endoplasmic reticulum (ER) stress and DNA damage (313–315). Once the autophagic response is triggered, double-layered membranous vesicles, called *autophagosomes*, start sequestering cytoplasmic structures directed for degradation. Several autophagy-related (*ATG*) genes have been described which play diverse roles in cargo recognition, sequestration and delivery to the lysosomes (316). Autophagy is further regulated by a network of signaling pathways that involve PI3K/mTOR, AMPK, and the Ras/PKA pathway amongst others (317). Autophagy has been extensively studied as a component of several pathologies including cancer (316), and similar to senescence, has been established as a primary response to cancer therapy in tumor cells (318).

Several observations have closely linked senescence and autophagy together. First, as in the case of senescence, autophagy plays a powerful tumor suppressive function as many ATG genes are

frequently mutated or deleted in transforming cells, resulting in the accumulation of free radicals and further genomic instability (319). Furthermore, autophagy contributes to forms of tumor dormancy where it facilitates the survival of tumor cells (320). More importantly, therapy-induced senescence in tumor cells is almost always accompanied by a robust autophagic vacuolation (62,220). However, the connection between autophagy and senescence remains enigmatic (321) in that while autophagy is believed to facilitate the transition into senescence (322–325), other evidence suggests that autophagy inhibition can either promote or prevent senescence (326,327). Both senescence and autophagy contribute to tumor cell survival and cell fate, but the putative nature of their interaction has not been fully resolved.

As we have established that therapy-induced senescence in tumor cells is not necessarily a terminal process, in this chapter we continue using etoposide to induce senescence in lung cancer cells in efforts to investigate whether autophagy blockade interferes with the ability of the cells to maintain their senescent state and to recover proliferative capacity. Utilization of pharmacological and genetic approaches to inhibit autophagy determined that autophagy was functionally *non-cytoprotective* is disagreement with its historical role as a survival mechanism. Furthermore, autophagy inhibition did not affect sensitivity of the tumor cells to etoposide, their capacity to undergo and maintain senescence or to recover proliferative capacity.

5.2 Experimental procedures

5.2.1 Cell lines

The wild-type (WT) TP53 H460 cell lung cancer cell line was generously provided by Dr. Richard Moran and Dr. Charles Chalfant, respectively, at Virginia Commonwealth University. The ATG5-knocked down H460 variant was generated as follows. Mission shRNA bacterial stocks for ATG5

were purchased from Sigma-Aldrich (TRCN00151963) and lentivirus generation was conducted in the HEK 293TN cells. Cotransfection was performed using lipofectamine (Invitrogen, 11668–019) with a packaging mixture of psPAX2 and pMD2.G constructs (Addgene, 12260, 12259). After 48 h, viruses shed into the media were collected and used to infect H460 cells under ultrasonic centrifugation for 2 h. Selection was performed in Puromycin (Sigma-Aldrich, P8833) (1-2 µg/ml). All cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (Thermo Scientific, SH30066.03), 100 U/ml penicillin G sodium (Invitrogen, 15140–122), and 100 µg/ml streptomycin sulfate (Invitrogen, 15140–122). Cells were maintained at 37°C under a humidified, 5% CO2 atmosphere at subconfluent densities. At all etoposide concentration (Sigma-Aldrich, E1383) utilized, cells were exposed to the drug-containing medium for 24 h, followed by replacement with fresh medium. Incubation with Chloroquine (CQ, 10 µM) or Bafilomycin A1 (Baf, 5 nM) was utilized to interfere with lysosomal acidification and autophagosome/lysosome fusion, respectively. Cells were treated with the autophagy inhibitors for 3 h prior to the subsequent exposure to both etoposide and the autophagy inhibitor for an additional 24 h to ensure blockade of autophagy. Drugs were protected from light during handling.

5.2.2 Cell Viability and Clonogenic Survival

Growth curves were generated based on cell viability as assessed by Trypan blue exclusion. Cells were seeded, treated (on day 0), and counted at the indicated time points following the removal of the drug from the medium. For clonogenic assays, cells were seeded, pre-treated with CQ (10 µM) or Baf (5 nM) for 3 h, then treated with etoposide (0.25, 0.5, 1.0, or 5 µM) alone or in combination with CQ or Baf; drugs were removed and replaced with fresh media after 24 h. Cells were

incubated for 7 days, then fixed with methanol, stained with crystal violet, and counted (ColCount, Discovery Technology International).

5.2.3 Analysis of senescence and autophagy by flow cytometry and microscopy

All of the flow cytometry analyses were performed using BD FACSCanto II and BD FACSDiva software at the Virginia Commonwealth University Flow Cytometry Core Facility. For C_{12}FDG (Life Technologies, D2893) and acridine orange analyses, 10,000 cells per replicate within the gated region were analyzed. Three replicates for each condition were analyzed in each independent experiment. Labeling procedures, gating, and analysis followed our previously published protocols with minor adjustment for the tested cell line (62,220,253). For β-galactosidase and C12FDG staining β-galactosidase labeling was performed as previously described by Dimri et al(80) and in our previous publications(62,220,253). Phase contrast images were taken using an Olympus inverted microscope (20X objective, Q-Color3™ Olympus Camera; Olympus, Tokyo, Japan). The C_{12}FDG staining protocol was adopted from Debacq-Chainiaux et al (185).

5.2.4 Western blotting and immunofluorescence

Western blotting was performed as previously described (62,220,253). Primary antibodies were used at a 1: 1000 dilution except for GAPDH (1:8000 dilution). For immunofluorescence cells were fixed with methanol, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin (BSA). Cells were exposed to a 1:100 dilution of the p21 antibody and incubated overnight at 4°C, followed by exposure to the secondary antibody for 1 h at room temperature. After incubation, cells were mounted with DAPI and imaging was performed with an Olympus

inverted microscope (20X objective, Q-Color3™ Olympus Camera, 555 filter and UV light, Olympus, Tokyo, Japan). Primary antibodies: SQSTM1/p62 (BD Biosciences, 610497), ATG5 (Cell Signaling Technology, 2630), LC3B (Cell Signaling Technology, 3868), p21^{Cip1} (BD, 610234).

5.2.5 TUNEL assay

Quantification of apoptotic cells via flow cytometry was achieved per the manufacturer's instructions (AnnexinV-FITC apoptosis detection kit, BD Biosciences, 556547). For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, cells were harvested and centrifuged at 10,000 rpm for 5 min onto slides (Shandon Cytospin 4, Thermal Electron Corp) 48 h after etoposide removal. Slides were fixed with 4% formaldehyde for 10 min and then washed with PBS for 5 min at room temperature. The cells were then fixed with a 1:2 dilution of acetic acid and ethanol for 5 min, followed by staining with a 1:1,000 dilution of DAPI at room temperature. Coverslips were sealed using clear nail polish and apoptosis was assessed by evaluating three fields per condition with an Olympus inverted microscope (20X objective, Q-Color3™ Olympus Camera, 488 filter and UV light, Olympus, Tokyo, Japan).

5.3 Results

5.3.1 Senescence and autophagy are collaterally induced in H460 NSCLC cells in response to etoposide

As we have demonstrated before, as early as 3 days after the initiation of exposure to etoposide, H460 cells exhibit numerous features collectively indicative of senescence, specifically a flattened and enlarged appearance with abundant granulation, and histochemical staining for SA-β-gal

activity. As would have been anticipated based on the fact that etoposide was previously shown to promote autophagy in A549 and U1810 NSCLC cell lines (328), autophagy was also evident in the H460 cells exposed to etoposide, as indicated by increased formation of acridine orange-stained acidic vesicular organelles in the H460 cells (**Figures 5.1A and B**).

LC3B is soluble protein that targets damaged cellular components for sequestration in the autophagosome. During autophagosomal maturation, LC3B becomes lipidated by conjugating to phosphatidylethanolamine (conversion of LC3 from form I to form II) (329). LC3BII is also subject to lysosomal degradation when autophagy goes to completion; however, measuring the localization and lipidation of LC3B is used as a marker for autophagy induction and formation of autophagosomes. On the other hand, p62/SQSTM1 is an adapter protein that binds to LC3B and serves as a receptor for ubiquitinated proteins targeted for degradation (330). p62/SQSTM1 degradation is frequently measured as a sign of autophagy completion. Accordingly, the induction of autophagy in H460 cells exposed to etoposide was confirmed based on the increased formation of GFP-LC3 puncta indicative of autophagosomes formation (**Figure 5.1C**). Furthermore, etoposide exposure resulted in increased lipidation of LC3 and degradation of p62/SQTSM1 (**Figure 5.1D**), indicating that the autophagic process is progressing to completion (i.e. autophagic
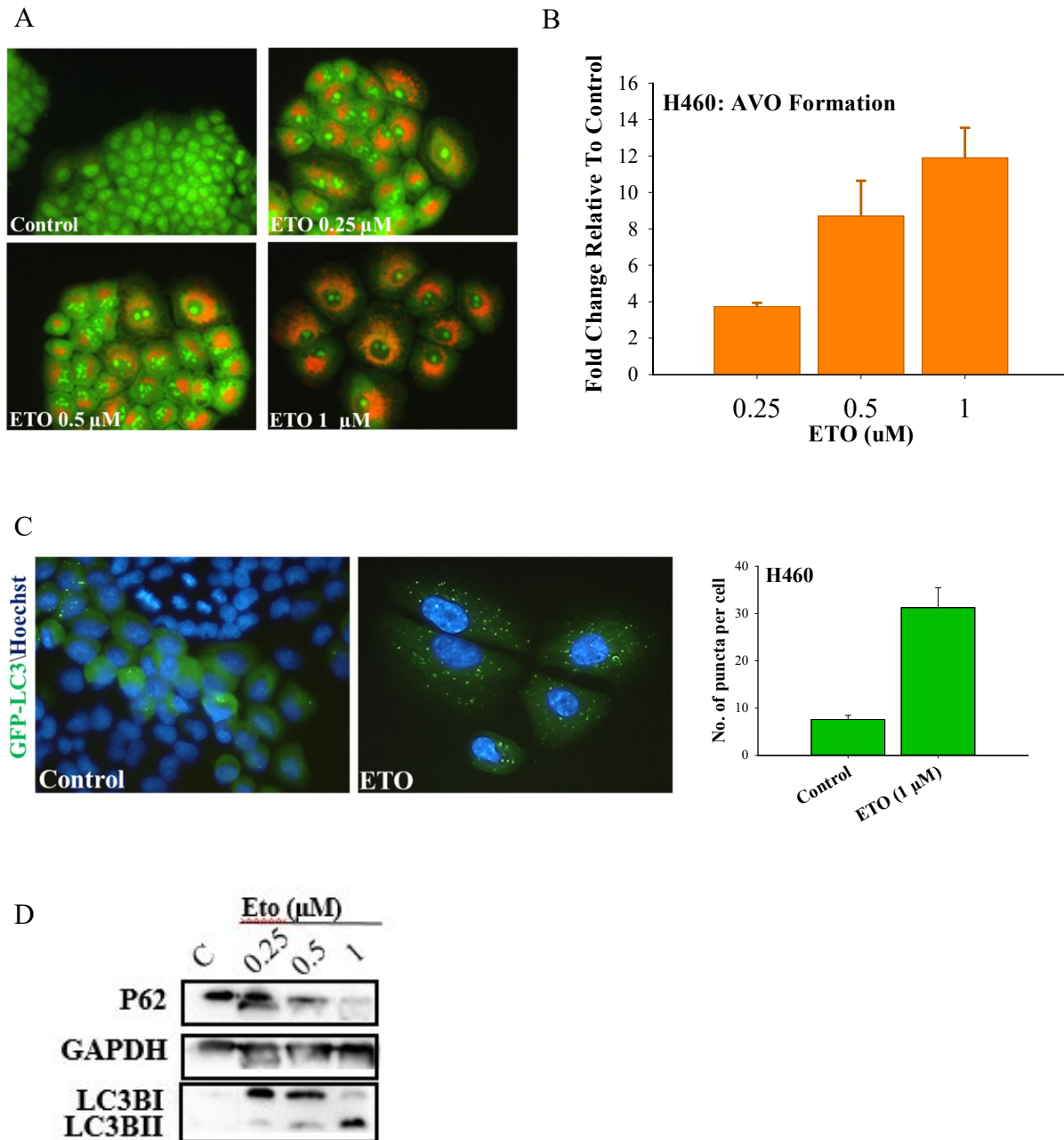
Figure 5.1. Autophagy as a primary response to etoposide in H460 NSCLC cells. A. Fluorescence microscopy showing concentration-dependent increase in acridine orange-stained vacuoles induced by 0.25, 0.5, and 1 µM etoposide **B.** Quantification of acidic vesicular organelles (AVOs) by FACS analysis in response to increasing concentrations of etoposide. **C.** Fluorescence microscopy showing increased GFP-LC3 puncta in response to etoposide (1µM) exposure. Imaging performed 48 h after drug removal. (20x objective). The number of LC3B puncta formed per cell is provided in the bar graph.

flux). Collectively, these data indicate that etoposide induces a senescent growth arrest in H460 cells accompanied by robust autophagy.

5.3.2 Autophagy plays a non-cytoprotective role in response to etoposide in H460 cells

Autophagy has historically been considered a survival response under conditions of nutrient deprivation or hypoxia, as well as a mechanism of resistance to therapy (331–335). To determine whether autophagy might be interfering with the antiproliferative and/or potential cytotoxic effects of etoposide in the H460 cells, autophagy was suppressed using both pharmacological and genetic strategies, and the impact on sensitivity to etoposide was monitored. Cells were pretreated for 3 hours with the autophagy inhibitors chloroquine (CQ, 10 μM) or bafilomycin A1 (Baf, 5 nM) followed by 24 hours of exposure to etoposide in the presence of the CQ or Baf. The presence of CQ and Baf resulted in failure of lysosomal acidification, which is reflected by the yellow staining of vacuoles by acridine orange (**Figure 5.2A**); autophagy inhibition was confirmed by decreased degradation of p62/SQTSM1 in the presence of CQ or Baf (**Figure 5.2B**). Inhibition of autophagy did not alter sensitivity to etoposide as determined by clonogenic survival assays (**Figure 5.2C**) (except moderately with Baf at 1 μM etoposide).

Autophagy was also blocked genetically using shRNA-meditated knockdown of *Atg5*. Confirmation of gene silencing is shown by immunoblotting where p62/SQTSM1 levels are increased and LC3BI to LC3BII conversion is reduced (**Figure 5.3A**). Furthermore, Atg5 knockdown was maintained throughout a time course of 7 days marked by failure of p62/SQTSM1 degradation (**Figure 5.3B**) Silencing of *Atg5* did not sensitize the H460 NSCLC cells to etoposide
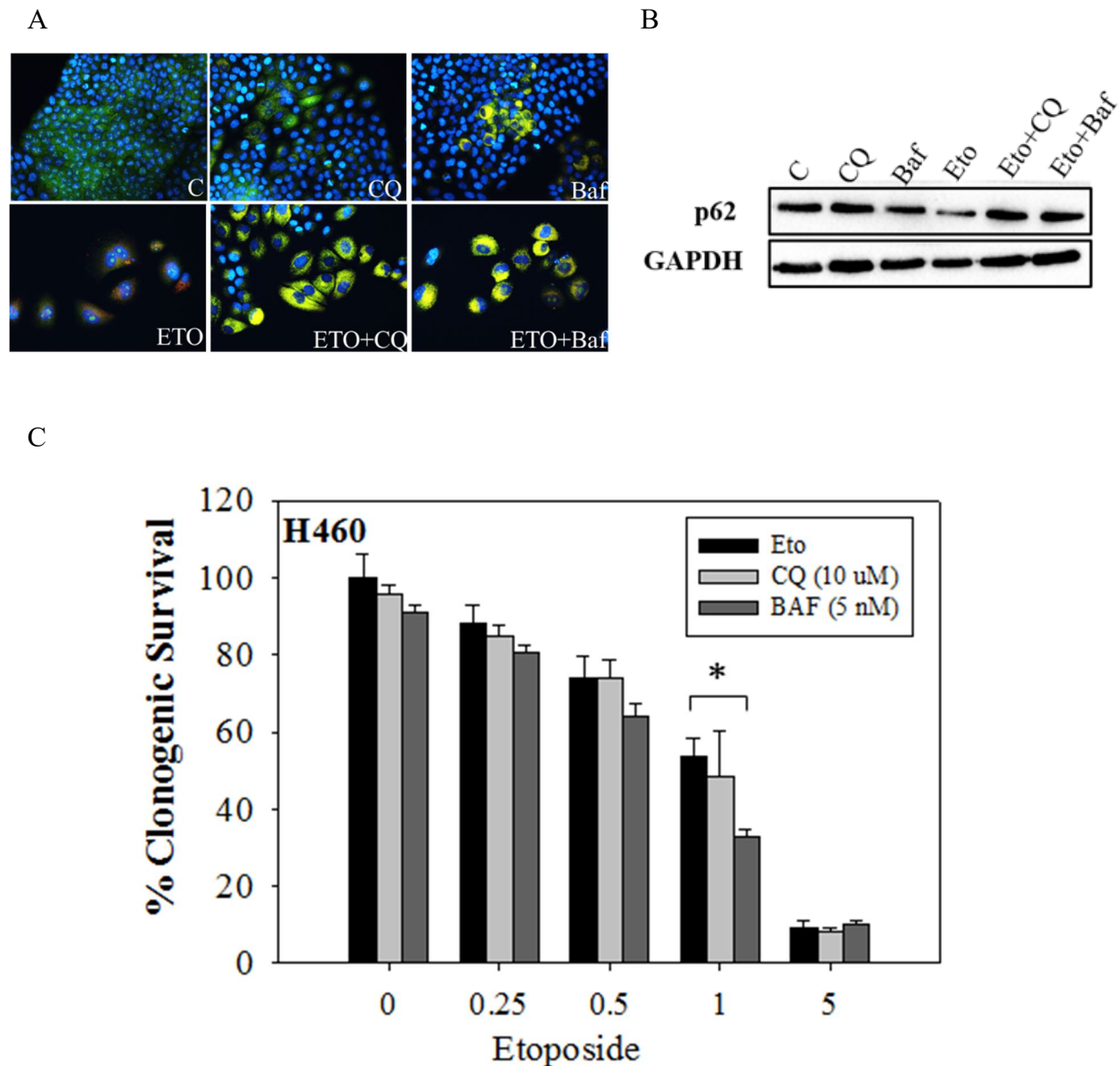
Figure 5.2. Pharmacological inhibition of autophagy does not affect the survival of etoposide-induced senescent H460 cells. A. Fluorescence microscopy showing failure of lysosomal acidification following CQ (10 μM) or Baf (5 nM) co-treatment with etoposide (1 μM). Cells were pretreated with CQ and Baf followed by an additional 24 h with etoposide. Images were taken 48 h after drug removal. Nuclei stained with Hoechst 33342 (20x objective). **B.** Western blot showing autophagy blockade by CQ (10 μM) and Baf (5 nM) based on levels of p62/SQSTM1 **C.** Clonogenic survival assay showing influence of CQ (10 μM) or Baf (5 nM) on sensitivity of H460 cells to etoposide. Cells were pretreated with CQ or Baf for 3 h followed by co-treatment with etoposide for 24 h. Colonies were counted 7 days following removal of drugs and replacement with fresh medium. Bars represent mean survival ± SD relative to untreated controls ($\alpha=0.05/3$, * $p<0.016$).

A

B

C
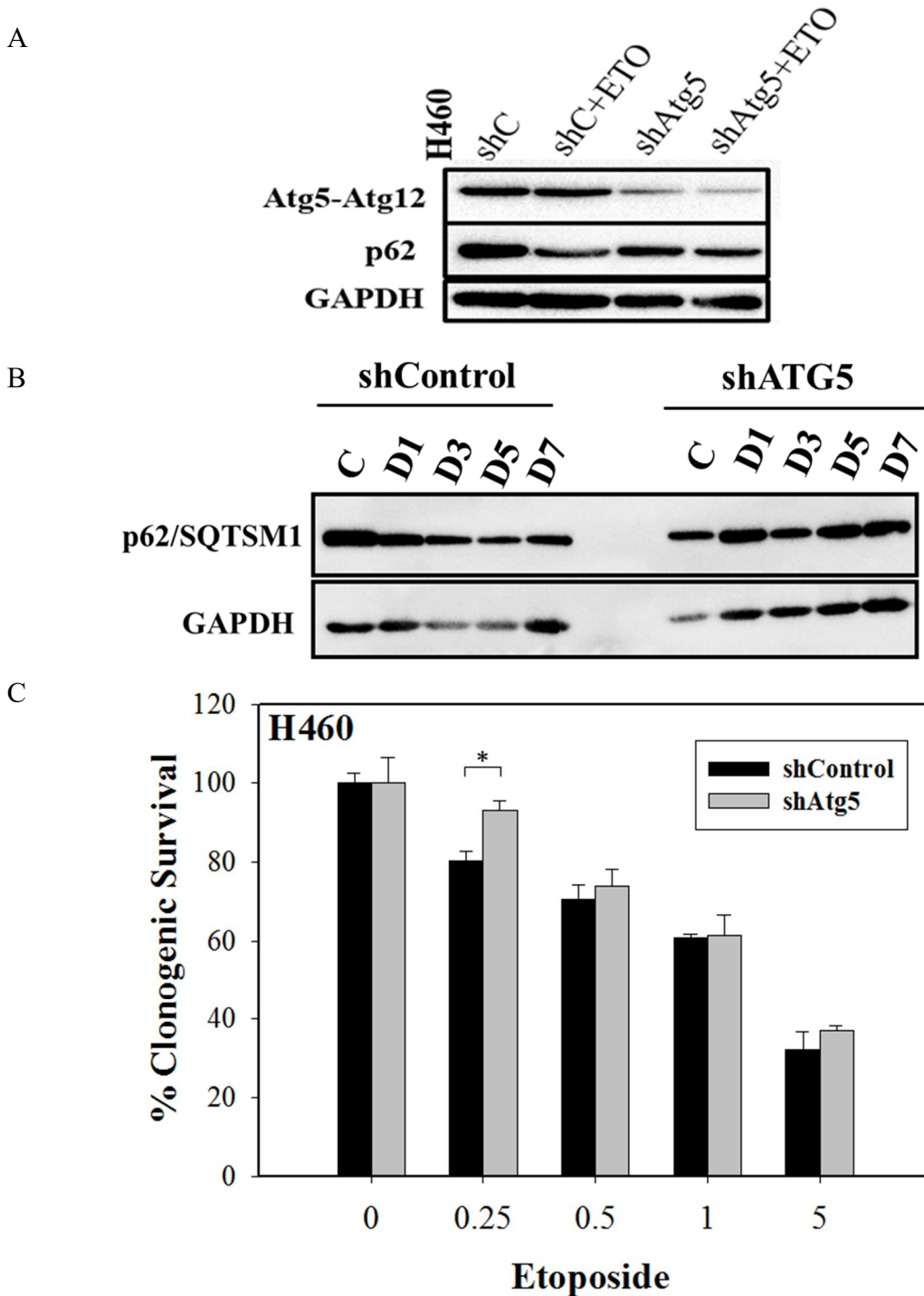
Figure 5.3. Genetic inhibition of autophagy does not affect the survival of etoposide-induced senescent H460 cells. A. Western blot following shRNA-mediated knockdown of Atg5. **B.** Western blot showing p62/SQSTM1 degradation over time in autophagy-proficient cells after etoposide (1 μM) treatment and p62/SQSTM1 accumulation in Atg5-silenced autophagy-deficient cells after etoposide (1 μM) treatment indicating that genetic silencing of Atg5 using shRNA results in autophagy inhibition sustained for at least 7 days after drug removal. **C.** Clonogenic survival assay comparing sensitivity of shControl and shAtg5 H460 cells in response to multiple etoposide concentrations. Bars represent mean survival ± SD relative to untreated controls (α=0.05/3, * p<0.016).

(except for a small effect at the 0.25 μM concentration)(**Figure 5.3C).** These studies indicate that autophagy inhibition does not alter the drug sensitivity of H460 tumor cells as they undergo senescence in response to etoposide, indicating that etoposide-induced autophagy is *non-cytoprotective* (336,337).

5.3.3 Autophagy inhibition does not interfere with the ability of H460 cells to senescence or to recover proliferative capacity after senescence induction

In previous studies of radiation-induced autophagy and senescence in HCT-116 colon carcinoma cells, we demonstrated a direct correspondence between the extent of autophagy and senescence, where both responses were related to the extent of DNA damage (220). However, in this work, as well as studies of doxorubicin-induced autophagy and senescence in breast tumor cells (62), we reported that the two responses are clearly dissociable. Our studies further demonstrate that the senescence induced by etoposide in the H460 cells is not affected by autophagy, as genetic silencing of autophagy failed to influence the promotion of senescence by etoposide (**Figure 5.4A and B**).

H460 human NSCLC cells undergo growth arrest followed by proliferative recovery upon exposure to etoposide, a mainstay chemotherapeutic agent. To examine whether recovery might be dependent on maintenance of autophagy, recovery was evaluated in the presence of the pharmacological autophagy inhibitors as well as with ATG5 silencing.
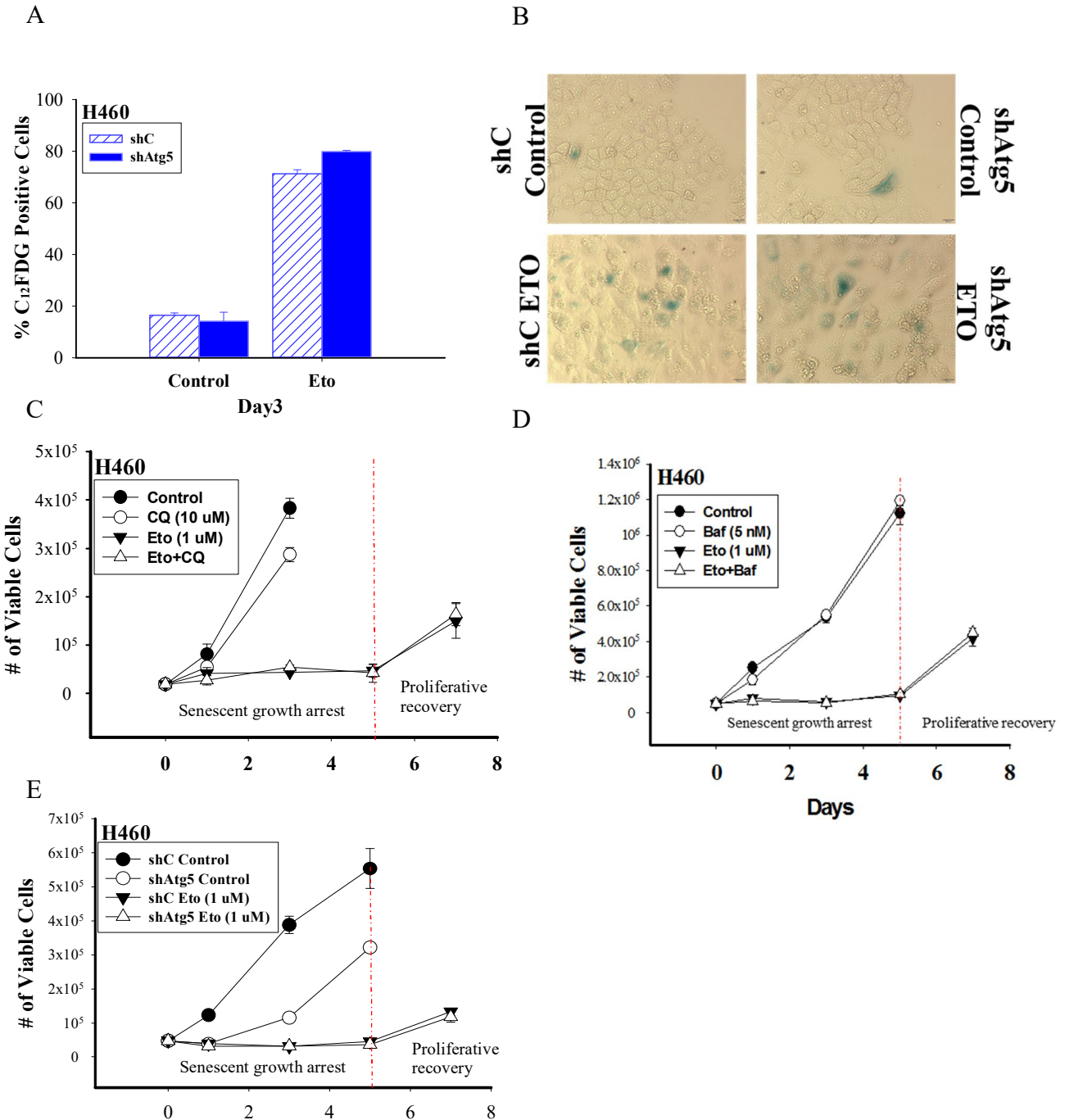
Figure 5.4. Autophagy inhibition does not interfere with the ability of cells to senesce or to recover proliferative capacity after senescence induction. A. Percent senescence based on $C_{12}FDG$ staining at day 3 post-etoposide exposure in shControl cells and shAtg5 cells. **B.** Etoposide-induced senescence in both autophagy-proficient and autophagy-deficient H460 cells by staining for SA-β-gal activity (20x objective). **C. and D.** Temporal responses to etoposide in H460 cells after pharmacological autophagy inhibition. Viable H460 cell number was determined at the indicated days following etoposide exposure in combination with 10 μM CQ (left panel) or 5 nM Baf (right panel). **E.** Temporal response to etoposide in shControl H460 cells and H460 cells with knockdown of Atg5.

The temporal response studies in **Figures 5.4C and D** indicate that neither CQ nor Baf was able to alter the profile of growth arrest; furthermore, proliferative recovery occurred in the cells treated with etoposide both in the absence and presence of CQ or Baf . Furthermore, growth arrest and proliferative recovery profiles were virtually identical in H460 cells where *Atg5* was silenced as in the autophagy-competent cells (**Figure 5.4E)**.

5.4. Discussion

Both senescence and autophagy are established responses to stress resulting from DNA damage and oxidative injury (47,159,312).When apoptosis is not the predominant response to therapy, senescence represents a major determinant of cell fate, where cells remain in a growth-abrogated state while maintaining their metabolic activity (181,203). Senescence could represent one basis for tumor cells remaining dormant for prolonged periods of time (140). It has been suggested that the regulatory pathways of both processes are intertwined (338–340) and it is clear that senescent cells develop abundant acidic vacuoles (341). However, the relationship of the autophagic response to the induction and maintenance of senescence does not appear to be consistent across the types of stimuli that promote these responses or the cell lines in which they have been studied (327).

While autophagy might have been anticipated to contribute to maintenance of the metabolic integrity of the senescent tumor cells, inhibition of autophagy did not appear to affect the survival of senescent cells. Furthermore, inhibition of autophagy failed to prevent the recovering population from resuming growth. Consequently, while autophagy may represent an intrinsic component of the senescent response elicited by cancer therapy, the current studies indicate that autophagy plays a minor, if any, role in facilitating proliferative recovery in this system or interfering with the fate

of the senescent cells. This may be related to the fact that the etoposide-induced autophagy is nonprotective in function, as discussed below.

Although the bulk of the literature has focused on the cytoprotective function(s) of autophagy (342,343) we and others have shown in a number of studies that interference with autophagy failed to alter drug or radiation sensitivity or to promote apoptosis (220,336,337). While the non-cytoprotective function of radiation-induced autophagy was previously shown to be dependent on the cells being mutant or null in p53 (337), it is clear in the current work that autophagy induced by etoposide in the p53 wild-type H460 cells is *non-cytoprotective.* These findings are consistent with a recent report by Eng et al (344) demonstrating non-cytoprotective autophagy induced by more than 30 chemotherapeutic drugs in the A549 NSCLC cell line. Furthermore, previous studies addressing the involvement of autophagy in promoting an effective antitumor immune response following chemotherapy also identified the nonprotective form of autophagy (although this terminology had not been established at that time) (345). Taken together, these data suggest that the function of autophagy should be further investigated in other cancer models, with particular attention to its relationship with cellular senescence (58,89,93,209).

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Vita

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