ABSTRACT

Title of Document: SELENIUM COMPOUNDS CAN ACTIVATE EARLY BARRIERS OF TUMORIGENESIS

Min Wu, Doctor of Philosophy, 2011

Directed By: Assistant Professor Wen-Hsing Cheng, Department of Nutrition and Food Science

Selenium chemoprevention by apoptosis has been well studied, but it is not clear whether selenium can activate early barriers of tumorigenesis, namely senescence and DNA damage response. To test this hypothesis, we treated normal and cancerous cells with a gradient concentration of sodium selenite, methylseleninic acid and methylselenocysteine for 48 h, followed by a recovery of 1–7 days. Here we show that selenium compounds at doses of $\leq LD_{50}$ can induce cellular senescence, as evidenced by the expression of senescence-associated β-galactosidase and 5-bromo-2-deoxyuridine incorporation, in normal but not cancerous cells. In response to clastogens, the ataxia telangiectasia mutated (ATM) protein is rapidly activated, which in turn initiates a cascade of DNA damage response. We found that the ATM pathway is activated by the selenium compounds, and the kinase activity is required for the selenium induced senescence response. Pretreatment of the MRC-5 non-cancerous cells with the antioxidant *N*-acetylcysteine or 2,2,6,6-tetramethylpiperidine-1-oxyl suppresses the selenium induced ATM activation and senescence. Taken together, the results suggest a novel role of selenium in the activation of early tumorigenesis barriers specific in non-cancerous cells, whereby

selenium induces an ATM-dependent senescence response that depends on reactive oxygen species.

The tumor suppressor p53 is a substrate of the ATM kinase and plays an important role in senescence. To determine mechanism by which selenium induces the ATM-dependent senescence, we employed shRNA knockdown approach and other DNA damage assays to determine the role of p53 in the senescence response. Results from senescence-associated expression of β-galactosidase assay indicate that p53 shRNA MRC-5 cells did not show senescent phenotype with a series of concentrations of methylseleninic acid (0-10 μM) after 7-day recovery. However, loss of p53 renders MRC-5 cells more resistant to MSeA treatment and increased its genomic instability. We also observe that MSeA can cause increased irreversible G2/M arrest in scramble MRC-5 cells but treated p53 shRNA MRC-5 can recover back to non-treated status after 7-day recovery. Taken together, p53 is involved in the ATM-dependent senescence in the response of MRC-5 normal cells to selenium compounds.

SELENIUM COMPOUNDS ACTIVATE EARLY BARRIERS OF TUMORIGENESIS

By

Min Wu

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2011

Advisory Committee:
Assistant Professor Wen-Hsing Cheng, Chair
Professor David K. Y. Lei
Professor Liangli Yu
Dr. Thomas T.Y. Wang
Associate Professor Douglas A. Julin, Dean's Representative

Dedication

I dedicate this dissertation to my wonderful parents. Without their fully support, I cannot accomplish my Ph.D. in the United States.

Acknowledgements

First of all, I would like to thank all those who helped make this dissertation possible.

I would like to give my special thanks to my advisor, Dr. Wen-Hsing Cheng for all his guidance, encouragement, support, and patience. His sincere interests in science and life have been a great inspiration to me. Also, I would like to thank my committee members Dr. David Lei, Dr. Liangli Yu, Dr. Thomas Wang, and Dr. Douglas Julin for their very helpful insights, comments and suggestions as well as Dr. Robert Jackson for his input at my candidacy meeting. I would like to thank Dr. Norberta W. Schoene for helping me with my research work.

I would like to acknowledge all my current and former lab members: Ryan Wu, Alexandra Holmstrom, Mandy Kang, Tiffany Tzeng, Yongmei Qi, Caroline Rocourt, Shu Zhang, Junhao Ma, and Frederick M. Lartey. They have shared their knowledge, skills, caring and friendship with me. Especially, I would like to thank Ryan Wu and Alexandra Holmstrom, as they always gave very helpful suggestions about my research. I would also like to thank everyone in my department for their great patience and warm help.

I would like to give my special thanks to my boyfriend Tom Wu for his tremendous support to me and I have been having a wonderful life experience with him. I would like to thank Margaret Udahogora, as she is always willing to provide help to my research and life. I appreciate my friends and everyone I met in the University of Maryland, as they provided me their support in their way.

Table of Contents

List of Tables

List of Figures

CHAPTER 1: SELENIUM, GENOME MAINTENANCE, AND AGING

1.1 INTRODUCTION

Over the last century, advances in nutrition, medicine, and public health have contributed to a rapid growth of the elderly population. As such, the improvement of quality of life in the elderly is a critical social and healthcare issue. Although aging is an inevitable biological process, breakthroughs in the understanding of the longevity pathways in the last decades shed light on further extension of lifespan through dietary intervention approaches. In particular, the efficacy of calorie restriction has recently been confirmed in primates, in which lifespan is extended and age-related phenotypes and pathologies are attenuated (Youngman *et al.* 1992; Ingram *et al.* 2006). Moreover, results from animal studies support a role of resveratrol, a phytochemical rich in red wines, in the suppression of age-related pathologies in mice fed with a high fat diet (Baur *et al.* 2006). Furthermore, mid and old age mice administrated with rapamycin, an inhibitor of the TOR nutrient-sensing pathway, show extended lifespan (Harrison *et al.* 2009). These results point to the significance of nutritional control of aging intervention via bioactive food components.

Genome instability is a hallmark of aging. A number of aging theories center on defects in genome maintenance. Any damage on DNA, if not repaired, changes the genetic information and influences certain types of physiological and mental functions. In particular, telomere, a protein-DNA structure at the end of chromosomes, is shortened after each replication cycle in the majority of adult cells. In contrast, stem cells and some cancerous cells express the telomere-extending

enzyme, telomerase, to avoid the otherwise telomere erosion that eventually leads to replicative senescence (Allsopp *et al.* 1992). Replicative senescence occurs in cells of old individuals, and the onset of the process is accelerated in premature aging syndromes, including Werner syndrome and ataxia telangiectasia (Kudlow *et al.* 2007). Moreover, the WRN and ATM (ataxia telangiectasia mutated) protein, mutated in Werner syndrome and ataxia talengiectasia, respectively, play critical roles in the maintenance of telomere structure (de Lange 2002; Machwe *et al.* 2004). Restoration of telomerase rescues the replicative senescence in normal somatic cells (Weinrich *et al.* 1997), and fibroblasts from genome instability syndromes such as Dyskeratosis congenita (Westin *et al.* 2007) and Werner syndrome (Wyllie *et al.* 2000; Blasco 2005). These lines of evidence strongly support a critical role of genome and telomere stability in defending against aging.

Another popular theory of aging is the free radical theory of aging. Although proposed by Denham Harman in 1950s, this theory is still not yet definitively proven or disproven after intensive research in the last half century. Free radicals can be generated endogenously from normal metabolic processes including energy production in mitochondria and immune response in macrophases, as well as exogenously including radiation exposure, environmental pollutants, and the use of tobacco and alcohol. Free radicals contain unpaired electrons that are bioactive to attack electronically balanced molecules. When in access, free radicals oxidize DNA, proteins and lipids. There are antioxidant systems to reduce the amount of free radicals and DNA and protein repair proteins as a second line of defense once the macromolecules are damaged (Ames *et al.* 1993). Interestingly, the maximum

lifespan in vertebrates is negatively correlated with the rate of free radical production (Perez-Campo *et al.* 1998; Finkel and Holbrook 2000), and lipid peroxidation increases in elderly group (Kasapoglu and Ozben 2001). Of note, some components of these theories of aging may overlap. The aging theories of free radicals and DNA damage merge when one consider the accumulation of oxidative DNA damage during the aging process.

1.2 GENOME MAINTENANCE

The genome in the trillions of cells inside a human body constantly undergoes DNA damage events. DNA replication is especially vulnerable to DNA damage, resulting in mutations if left unrepaired. Performed by DNA repair and replication proteins, the fidelity of DNA replication ensures the survival of an organism and the descent of genetic information. Although mutations contribute to biological diversity and favor evolution, they jeopardize genome stability and contribute aging and age-related disorders. Moreover, it is also generally considered that DNA repair capacity decreases and DNA damage accumulates during the aging process. Although the etiology is still ambiquous, genome instability is generally believed to be associated with age-associated disorders such as Alzheimer's disease. The impact of mutations on genome stability is especially deleterious in post-mitotic cells such as neurons, where damaged cells cannot be replaced by proliferation. Interestingly, there seem to have mutation hotspots in age-associated disorders (Polymeropoulos *et al.* 1997; Zimprich *et al.* 2004; Coon *et al.* 2007; Munter *et al.* 2010). A genome-wide study revealed that the apolipoprotein E epsilon 4 gene is highly susceptible to mutations in

the late-onset of Alzheimer's disease (Coon *et al.* 2007). The GxxxG motif in the amyloid precursor protein can prevent Alzheimer's disease by decreasing the level of amyloid-beta peptides (Munter *et al.* 2010). Parkinson's disease, a degenerative disorder in central nervous system, is believed to correlate with mutations in alpha-synuclein (Polymeropoulos *et al.* 1997) and LRRK2 (Zimprich *et al.* 2004).

1.2.1 DNA Damage

The genome is constantly subjected to endogenous and exogenous DNA-damaging attacks. The various types of DNA damage include DNA base damage, DNA adducts, DNA strand breaks, DNA cross-links, DNA mismatch, and aneuploidy.

1.2.1.1 DNA Base Damage

DNA base damage is the most frequently occurred one. This type of damage can be generated by environmental sources such as exposure to ultraviolet (UV) light, ionizing radiation (IR), and alkylating agents, and by reactive oxygen species (ROS) that can be generated by normal metabolism in mitochondria and macrophases. ROS can directly cause DNA base deamination, depurination and depyrimidination. Moreover, IR can indirectly generate ROS, including the very potent hydroxyl radical (·OH). Results from the HPLC-MS/MS method showed several base damages in neoplastic monocytes induced by γ-rays, including thymine glycols, 5-formyluracil, 5-(hydroxymethyl) uracil, 8-oxo-7, 8-dihydroguanine, and 8-oxo-7,8-dihydroadenine (Cadet *et al.* 2004). The two ultraviolet lights, UVA and UVB, can cause multiple forms of base damages (Cadet *et al.* 1997; Ridley *et al.* 2009). Upon exposure to

UVB light, normal human epidermal keratinocytes developed 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-G) in a dose-dependent manner (Pelle *et al.* 2003). UVB can also trigger the intrastrand crosslink between two adjacent pyrimidines. UVA exposure can generate 8-oxo-G as well as cyclobutane pyrimidine dimers. However, UVA exposure to Chinese hamster cells generates not only cyclobutane pyrimidine dimers but a complex type of lesions (Biverstal *et al.* 2008). Bulky adducts can be induced by the exposure to benz(a)pyrene, which can be found in cigarette smoke. Rather than inducing one type of nucleotide base damage, it is always a complex of photoproducts that lead to mutagenesis after the exposure of mutagens.

1.2.1.2 DNA Strand Breaks

More severe DNA damages happen in the nucleotide backbones, resulting in DNA breaks. Single-strand breaks (SSBs) destroy the double helix structure of DNA and impose serious threat to genomic stability and cell survival. SSBs can be generated endogenously by ROS attack. It can also occur during the DNA repair pathways as an intermediates or interruption results (Hegde *et al.* 2008). SSBs can also arise as a result of defective DNA replication and transcription. DNA topoisomerase 1(TOP1) generates a transient nick in order to unwind DNA. Typically, this intermediate can be resealed rapidly, but collision with RNA or DNA polymerases, or in close proximity to other types of DNA lesion, can convert cleavage complexes into SSBs. SSBs can have an impact on cell fate in a number of ways if they are not repaired rapidly or appropriately. In replicating cells, unrepaired SSBs block or collapse DNA replication forks during S phase of the cell cycle, thus producing the more severe

double-strand breaks (DSBs) (Kuzminov 2001). In non-proliferating cells, such as neurons, cell death induced by SSBs might involve stalling of RNA polymerases during transcription. SSBs can block RNA polymerase II and stall transcription, particularly if they possess damaged termini (Zhou and Doetsch 1993; Kathe *et al.* 2004). Alternatively, SSBs can happen after exposure to environmental genotoxic agents. TOP1 inhibitors can induce 3'-phosphate and/or 5'-hydroxyl terminated single nucleotide gaps (Bernstein *et al.* 2008). If not repaired, DNA SSBs can be converted to the more toxic DSBs. The defects in repairing those breaks can be relevant to cellular dysfunctions and human diseases (Caldecott 2008), such as spinocerebellar ataxia with axonal neuropathy 1 (SCAN1) (Wilson 2007), ataxia-oculomotor apraxia 1(AOA1) and aging in neuronal cell population (Katyal and McKinnon 2008).

DSBs with defects on both strands in the double helix, are less frequent but more acute to the extent, compared to SSBs. DSBs are hazard because of their possible destiny to genomic rearrangements. However, DSBs are essential intermediates in recombination. DSBs can be triggered by the attack of deoxyribose and DNA bases by ROS and other electrophilic molecules. Unpaired SSBs and collapsed DNA replication forks can also develop DSBs. They are even created during the normal development of the immune system. In addition, DSBs can be generated exogenously by IR and other DNA-damaging agents such as campothecin, etoposide. Defects in repairing DSBs exhibit a complex pathology and can cause problems in immune systems, sensitivity to radiations, and cancer predisposition (McKinnon and Caldecott 2007).

1.2.1.3 DNA Cross-links and Mismatch

DNA cross-links can be induced by endogenous sources including malondialdehyde generated in lipid peroxidation, as well as exogenous sources such as formaldehyde, metals and some chemotherapeutic drugs. The cross-links can happen in the same strand, forming intrastrand cross-links or in the different strands resulting in interstrand cross-links. Covalent bonds can even occur between DNA and protein (Dizdaroglu 1992). The physical structures, stability and biological consequences of DNA cross-links can be dependent on the agents that induce them (Barker *et al.* 2005). If the cross-links cannot be repaired, the cell replication will be arrested and may lead to carcinogenesis. An examination in peripheral blood lymphocytes of 186 subjects exposed to formaldehyde showed a positive relationship between DNA protein cross-links and p53 mutation (Shaham *et al.* 2003). That may indicate the initial process of carcinogenesis in people exposure to formaldehyde. Mismatch sometimes happens during DNA synthesis. The common pairing is between adenine and thymine, guanine and cytosine. However, for example, uracil can be misincorporated into nascent DNA strand or by deamination of cytosine in the established strand. This type of DNA damage can also result from endogenous and exogenous attacks.

1.2.1.4 Aneuploidy

Aneuploidy is a type of chromosome abnormality, with an unusual number of chromosomes. During meiosis, the two germ cells should normally obtain half of the same number of chromosomes. But due to aging or other mutagenesis, the

segregation of paired chromosomes can be disturbed, resulting in one germ cell with an extra chromosome and another with a missing one. Causes of aneuploidy can be multifactorial. The incidence can be accelerated with aging. In female mammals, the cohesion level decreases with age and the centromeric cohesion protector Sgo 2 also declines, which is related to dysfunction of chromosome segregation (Lister *et al.* 2010). In mice, a mutation in the mitotic checkpoint kinase gene Bub1 generates aneuploidy (Leland *et al.* 2009). In most cases, the aneuploidy gametes cannot survive. The most common aneuploidy happens with extra copies of chromosomes 21, 18 and 13. For instance, the most common genetic disorder, Down syndrome affecting 1 in 800 births, obtains extra copy/portion of Chromosome 21. Besides birth defects, some cancer cells also have abnormal numbers of chromosomes (Duesberg and Rasnick 2000; Rajagopalan and Lengauer 2004). ROS can be responsible for the aneuploidy, and the incidence increases with the gastric cancer risk (Williams *et al.* 2009). The high expression of Bub1 can raise the possibility of DNA aneuploidy and progression of gastric cancer (Ando *et al.* 2010).

1.2.2 DNA Damage Repair Pathways

In all organisms, the maintenance of genome stability is essential for survival. However, the genome encounters constant assaults by ROS, genotoxic agents, nucleotide misincorporation during DNA replication, and the intrinsic biochemical instability of DNA itself. Failure to repair DNA lesions may result in genetic disorders, aging and cancer. In order to ensure the integrity of genome, all eukaryotic cells have developed a diverse response to counteract the potentially deleterious

effects of DNA damage. These damage repair pathways are well regulated to make sure faithful maintenance, replication and pass down of genetic information. Once DNA damages happen, they can be first sensed through different mechanisms, the most typical of which is dependent on the enzyme-substrate complex affinity and specificity (Cadet *et al.* 2004; Sung and Demple 2006; Margolin *et al.* 2008). This recognition mechanism involves photolyases and DNA glycosylases.

1.2.2.1 Direct Reversal

The simplest DNA repair can be accomplished by direct reversal of mutations back to correct ones. The alkylation lesion O^6-methylguanine (O^6MeG) is derived from guanine residues in DNA after exposed to low levels of cellular catabolites. This mutation can be converted by O^6MeG DNA methyltransferase (MGMT) by direct transferring the alkyl group on guanine to a cysteine residue in the active site of MGMT. The intermediate product can be degraded in an ATP-dependent ubiquitin proteolytic pathway (Srivenugopal *et al.* 1996). The instant repair of O^6MeG indicates that this mutation is extremely detrimental to the cell. Recent research even implicates roles of MGMT in both processing O^6MeG damage and other repair pathways (Martin *et al.* 2009). DNA polymerases γ, δ and ε have the error correction ability. They can process mistakes in newly-synthesized DNA, so they all obtain the 3'-5' exonuclease activity. After base excision, the polymerase can re-insert the correct base to allow replication to resume (McCulloch and Kunkel 2008).

1.2.2.2 Base Excision Repair

Base excision repair (BER) can correct non-bulky damage after exposure to oxidative radicals, alkylating agents, X-rays or spontaneous loss of the DNA base itself (Hoeijmakers 2001). These can result in uracil, abasic sites, 8-oxo G, and SSBs. These alterations are at relatively high frequency, although in nature, they pose a significant threat to genome fidelity and stability.

There are two sub-pathways of BER, short-patch and long-patch BER, depending on the length of nucleotides needs to be repaired (Sung and Demple 2006) or tissue preference (Wei and Englander 2008). The short-patch BER comprises approximately 80-90% of all BER. They are activated by the cleavage of N-glycosidic bond at the damage sites by a DNA glycosylase. The cleavage generates an intermediate apyrimidinic/apurinic (AP) or abasic site. The AP endonuclease 1 (APE1) initiates the repair by cleaving the phosphodiester backbone generating a 3' hydroxyl and a 5'-deoxyribose-5-phosphate (5'-dRp) (Demple *et al.* 1991). The N-terminal of DNA polymerase β (Pol β) has the AP lyase activity, so it can remove the 5'-dRp moiety. Because Pol β also has polymerase activity, short-patch BER can synthesize nucleotides to fill the DNA gap. In a reconstituted *in vitro* experiment, uracil-DNA glycosylase, APE1, Pol β and DNA ligase III could form a simple short-patch BER (Kubota *et al.* 1996). Since the Pol β is the major polymerase involved in BER, the Pol β deficient mice have higher tendency to develop tumorigenesis (Cabelof *et al.* 2006). However, the role of Pol β may be partially substituted by Pol λ in mouse embryonic fibroblasts (Braithwaite *et al.* 2010).

Long-patch BER also needs Pol β, resulting in the adding of approximately 2-10 nucleotides. This BER shares the same upstream events with short-patch BER, including a DNA glycosylase, APE1 and DNA Pol β. Unlike short-patch BER, however, in long-patch repair, Pol β collaborates with proliferating cell nuclear antigen (PCNA) to replace several nucleotides to the repair gap. The resulting oligonucleotide overhang can be removed by the Flap endonuclease (FEN) 1 prior to sealing of the nick by a DNA ligase. In PCNA-independent long-patch BER pathway, Pol β plays a significant role in strand displacement DNA synthesis (Dianov *et al.* 1999).

1.2.2.3 Nucleotide Excision Repair

Nucleotide excision repair (NER) can be activated after the formation of bulky adducts on DNA by clastogens such as UV light, polycyclic aromatics and hydrocarbons (Hoeijmakers 2001). The DNA bulky adducts, such as pyrimidine dimers, bulky chemical adducts, and DNA intrastrand crosslinks, generate helical distortion of the DNA duplex and modifies DNA chemistry, thus being recognized by NER pathways. Compared to BER dealing with damages mainly arisen from endogenous sources, NER primarily targets DNA damage of exogenous origins.

NER can be categorized into two sub-pathways, global genomic NER (GGR) and transcription-coupled NER (TCR). While GGR concerns with the bulky DNA lesions occurred on chromosomes, TCR is responsible only for the DNA lesions after stalled transcription. Thus, it is believed that DNA repair by TCR is more efficient than GGR (Hanawalt 2002). The NER process includes the sequential steps of damage

recognition, DNA duplex opening, incision of damage sites on one strand, gap repair synthesis and ligation. The XPC/HR23B complex recognizes DNA damage in both GGR and TCR. TFIIH and XPG are recruited at the transcription stalled position by CSA (Cockayne syndrome A) and CSB (Costa *et al.* 2003). This is the only difference between GGR and TCR. Next, XPA and heterotrimeric replication protein A (RPA) are recruited to the damage sites that facilitate the loading of XPB, XPD and components of the multi-subunit transcription factor TFIIH to form a complex that unwinds DNA duplex around the lesion site (Costa *et al.* 2003). The endonucleases XPG and ERCC1/XPF then cleave one strand of approximately 30 bases of oligonucleotide containing the lesion 3' and 5' to the damage, respectively (Costa *et al.* 2003). Using the intact complementary strand, DNA Pol ε/δ and other replication factors synthesize the missing DNA in the gap. Finally, the nick in the repaired strand is sealed by a DNA ligase.

There are genetic disorders associated with deficiency in NER pathway, such as xeroderma pigmentosum (XP) and Cockayne's Syndrome (CS). XP proteins are responsible for damage recognition and DNA incision. The CS proteins (CSA and CSB) are required in TCR pathway for damage recognition. XP patients have mutations in one of the seven XP genes (*XPA* to *XPG*), while CS patients are suffering from mutations in *CSA* or *CSB*. Both XP and CSB patients are hypersensitive to UV irradiation.

1.2.2.4 Mismatch Repair

The DNA mismatch repair (MMR) is a post-replication pathway that recognizes and repairs errors such as A-G/T-C mismatches due to DNA polymerase misincorporation and insertion/deletion due to template slippage (Hoeijmakers 2001). DNA lesions are initially recognized by the MSH2/MSH6 (MutSα) and MSH2/MSH3 (MutSβ) heterodimers. MutSα is preferential for mismatch and single-base insertion/deletion recognition, and MutSβ is primarily involved in the larger insertion/deletion recognition (Tian *et al.* 2009). After the mismatch recognition, MutSα and MutSβ bind to ATP, pull the mutated DNA away and recruit additional MMR proteins to the lesions. The heterodimeric proteins hMLH1/hPMS2 (MutLα) and hMLH1/hPMS1 (MutLβ) form a protein complex with other replication factors and both MutS heterodimers. The replication machinery in the MMR pathway can discriminate newly synthesized DNA strand from the template that is attributed to the interaction between MutLα and PCNA (Clark *et al.* 2000; Iyer *et al.* 2008). Excision and re-synthesis of the nascent strand involves a number of DNA metabolism proteins including PCNA, RPA, RFC, exonuclease I, DNA Pol δ/ε, and endonuclease FEN1.

Cells with defective MMR fail to repair replication errors and insertion/deletion, leading to elevated frequencies in spontaneous mutations and increased microsatellite instability (MSI). This form of genomic instability dramatically predisposes individuals to colorectal cancers, including hereditary non-polyposis colorectal carcinoma (HNPCC) (Ma *et al.* 2000; Chen *et al.* 2005). Likewise, mutations in several human MMR genes are associated with a variety of sporadic tumors.

1.2.2.5 Double-strand Break Repair

Double-strand breaks are highly toxic DNA lesions because they affect both strands of DNA. DSBs can be caused by a variety of sources including IR and certain genotoxic chemicals and ROS (Hoeijmakers 2001). Failure to repair these DNA lesions can result in problems for transcription, replication, and chromosome segregation. To counteract the detrimental consequences, cells have evolved two distinct pathways of DSB repair: homologous recombination (HR) and non-homologous end joining (NHEJ). The application of repair pathway can influenced by stage within the cell cycle at the time of damage acquisition (Mao *et al.* 2008) or the type of DSBs (Takashima *et al.* 2009).

HR pathway is a highly coordinated repair process restricted to late S and G2 phases of the cell cycle. This is an error-free approach as it applies an undamaged sister chromatid as a template. Once DSBs are sensed by MRN (MRE11-RAD50-NBS1) heterotrimeric complex that activates the ATM kinase or by the ATR (ATM and Rad3-related)-interacting protein that activates ATR (Falck *et al.* 2005). The coordinated action of C-terminal binding protein interacting protein (CtIP), the MRN complex, and exonuclease 1 processes and generates the 3' single-stranded DNA (ssDNA) overhangs (Limbo *et al.* 2007). The HR-signaling 3’ tail is bound with RAD51, together with RPA, RAD52, RAD54, BRCA1 (breast cancer susceptibility gene 1) and BRCA2, to assemble nucleoprotein filament (Nowacka-Zawisza and Krajewska 2009). The RAD51 nucleoprotein filament then searches the undamaged DNA on the sister chromatid for a homologous repair template, followed by DNA strand exchanges (Arnaudeau *et al.* 2001). The damaged DNA strand invades the undamaged DNA duplex, so DNA polymerase can extend the 3' end of damaged

strand. Finally, the Holliday junction can be removed by resolvases and gaps are ligated by DNA Ligase I. In contrast to HR, NHEJ does not require a homologous template and is considered error-prone. In this pathway, DSBs are sensed by the Ku70/Ku80 heterodimeric protein and then activates DNA-dependent protein kinase (DNA-PK). It then phosphorylates a subset of downstream targets including XRCC4 to facilitate the repair of DSBs. The final ligation step is accomplished by DNA Ligase IV complex (Ma *et al.* 2005).

Defects in DSB repair pathway can result in genetic diseases. For example, the genome instability syndromes Nijmegen breakage syndrome, ataxia telangiectasia and ataxia telangiectasia-like disorder are characterized by NBS1, ATM and MRE11 mutations, respectively. Defects in BRCA1 and BRCA2 are strongly associated with breast cancer (Lee *et al.* 2006).

1.3 DNA DAMAGE CHECKPOINTS

After DNA damage, checkpoint responses are activated, which delay or arrest cell cycle progression and facilitate DNA repair. Although the DNA damage checkpoints occur in all stages of the cell cycle (Sancar *et al.* 2004), different checkpoint pathways are activated at different stages of the cell cycle.

1.3.1 G1 Phase Checkpoint

The G1 cell cycle checkpoint prevents damaged DNA from being replicated and is the best understood checkpoint in mammalian cells. Central to this checkpoint is the accumulation and activation of p53, a critical tumor suppressor. In normal cells, p53

levels are low due to its nuclear export and rapid cytoplasmic degradation. After IR exposure (ATM and ATR), UV irradiation (ATR) and replication fork stall (ATR), p53 is phosphorylated at Ser-15 by ATM or DNA-PKcs (Shieh *et al.* 1997; Canman *et al.* 1998). This phosphorylation stabilizes p53 by disassociation with MDM2 and enhances p53 transcription transactivation activity. p53 acts as a transactivator for up-regulation of many tumor suppressor genes. One of the p53 targets is p21, which promotes G1 arrest by the inhibition of the cyclin-dependent kinase 2 (Cdk2) required for G1/S transition. There are other phosphorylation sites on p53, including Ser-20 whose phosphorylation suppresses MDM2-mediated p53 degradation. ATM can also phosphorylate MDM2 on Ser-395 to reduce its affinity to p53, thus blocking the degradation of p53 in the cytoplasm (Maya *et al.* 2001; Balass *et al.* 2002).

The G1 phase cell cycle arrest can also be performed in a p53-independent manner. Ceramide induces dephosphorylation of Rb (retinoblastoma) protein, leading to increased p21 protein level, inhibition of Cdk2 kinase activity, and G1 arrest (Kim *et al.* 2000). Actin cytoskeleton ablation slows p21 degradation without affecting its expression level, and causes an Rb-dependent but p53-independent G1 phase arrest (Lee *et al.* 2009).

1.3.2 S Phase Checkpoint

The S phase checkpoint monitors cell cycle progression and suppresses DNA synthesis with damaged DNA. Analyses of cells from individuals with A-T (ataxia telangiectasia) and Nijmegen breakage syndrome implicate the involvement of ATM and NBS1 in the S phase checkpoint response. After IR exposure, DSBs trigger ATM

phosphorylation of Chk2 on Thr-68, which in turn phosphorylates the Cdc25A phosphatase on Ser-123. This event destabilizes Cdc25A through nuclear exclusion and ubiquitin-mediated proteolytic degradation and prevents DNA synthesis. Another pathway of IR-induced S phase checkpoint is Cdc25A-independent. Upon IR damage, ATM phosphorylates NBS1 on Ser-343, BRCA1 on Ser-1387, and SMC1 (structural maintenance of chromosome protein 1) on Ser-957 and Ser-966 (Kim *et al.* 2002; Yazdi *et al.* 2002). Mutations in any of these proteins or the indicated phosphorylation sites result in attenuated S-phase checkpoint activation. Interestingly, the NBS1 and BRCA1 proteins are required for optimal phosphorylation of SMC1 upon IR (Kim *et al.* 2002; Yazdi *et al.* 2002). Other proteins can also affect the ATM-dependent S phase checkpoint. In Ku80-deficient cells, IR-induced S phase checkpoint response is stimulated, probably due to an enhancing ATM kinase activity (Zhou *et al.* 2002).

ATR is another critical S-phase checkpoint kinase. Upon cellular exposure to UV irradiation, MRN facilitates ATR activation by stalled replication forks; subsequently, ATR phosphorylates NBS1 on Ser-343, inhibits DNA replication, and activates an S phase checkpoint response (Olson *et al.* 2007). It is also shown that ATR exhibits overlapping functions with ATM after cellular exposure to IR. ATR initiates a "slow" S phase checkpoint response by phosphorylating Chk1 on Ser-317 and Ser-345 (Zhou *et al.* 2002), which in turn destabilizes Cdc25A as ATM does. In addition, SMC1 is phosphorylated on Ser-957 and Ser-966 upon cellular exposure to UV irradiation or hydroxyurea in an ATM-independent manner (Kim *et al.* 2002). Interestingly, blocking ATR activity in colon cancer cells can trigger a prolonged S-phase arrest,

namely S-phase stasis, so it may indicate the possible application in cancer treatment by promoting their radiosensitization (Hurley *et al.* 2007).

1.3.3 G2 Phase Checkpoint

The G2 phase checkpoint prevents S-phase cells with DNA damage from chromosome segregation. Cdc2 is a key G2 checkpoint protein that regulates the transition from G2 phase to mitosis. ATM and ATR indirectly modulate the phosphorylation status of these sites in response to DNA damage. In the response of G2 cells to IR, UV light and stalled replication fork, ATR plays a major role while ATM plays a supporting role in the checkpoint response (Graves *et al.* 2000). It is reported that, upon DNA damage, the downstream kinases Chk1 and Chk2 phosphorylate the dual specificity phosphatase Cdc25C on Ser-216, a phosphorylation event considered to be a prerequisite for the G2/M checkpoint (Kaneko *et al.* 1999). Phosphorylation of this residue stimulates the translocation of the 14-3-3/Cdc25C protein complexes from the nucleus to the cytoplasm, thereby preventing Cdc25C from activating Cdc2. This results in the maintenance of the Cdc2/Cyclin B1 complex in its inactive state and blockage of mitosis entry.

1.4 SENESCENCE

Multicellular organisms contain two major types of cells: postmitotic cells and mitotic cells. The advantage of mitotic cells over postmitotic cells is that they can repair, renew and even regenerate damaged cells. However, this replication capability has limitations. In 1961, Hayflick discovered that there was a finite number a normal cell can divide (Hayflick and Moorhead 1961). Once the cells enter the nondividing

state, known as cellular senescence, they lose the proliferate potential yet maintain cellular metabolism. After decades' investigation, it is clear that this type of cellular senescence, known as replicative stress, is caused by telomere attrition after each cycle of chromosome replication (Bodnar *et al.* 1998). Telomeres are the repetitive DNA sequences at each chromosome end and are protected by telomere-associating proteins and the formation of telomere sequence T-loops. However, the so-called end of replication problem gradually shortens telomere length. When telomeres reach a critically short stage, the protective T-loops cannot be formed and the telomere-protein structure cannot be maintained, resulting in replicative senescence. The exposed chromosome ends are recognized by the normal DNA damage response pathways, accelerating genome instability. The discovery of telomerase shed light on the understanding of how to maintain telomere length (Greider and Blackburn 1985). In tumor cells and early embryogenesis, telomerase is expressed and telomere homeostasis is maintained. This enzyme is composed of two telomerase reverse transcriptase (TERT), telomerase RNA (TERC) and dyskerin (DKC1) (Cohen *et al.* 2007). Reconstitution of telomerase in normal human fibroblast cells prevents telomere shortening and replicative senescence, thus expanding their replication lifespan (Vaziri and Benchimol 1998; Hiyama and Hiyama 2007).

Another form of senescence is stress-induced senescence that can be initiated by oncogene activation and other stimuli such as oxidative stress and DNA damages. Oncogene activation can promote premature senescence in many normal cells, serving as an early barrier that prevents neoplastic cells from proliferation. It is likely that the overexpression of oncogenes promote mitogenic signals, thus eliciting the

senescence response. However, the overexpression of RAS mutation can stimulate cell proliferation and transformation (Sarkisian *et al.* 2007). In addition, chromatin modification can drive cells to senesce. For example, inactivation of c-Myc gene in various tumor tissues causes senescence that is associated with reduced histone H4 acetylation and increased histone H3 lysine9 methylation (Wu *et al.* 2007). The chromatin structure changes thus can alter the expression of proto-oncogenes or tumor suppressor genes, which frequently occurs among malignant tumors. Finally, cells can respond to senesce due to elevated ROS levels from endogenous energy production or exogenous agents. For example, in MRC-5 cells exposed to selenium compounds, the elevated ROS level leads to the ATM-dependent senescence (Wu *et al.* 2010). Therefore, stress-induced senescence can serve as an early barrier that halts precancerous cells from proliferation and prevents tumorigenesis before it turns malignant.

Although a senescence response can be induced by various stimuli, there are two well recognized pathways involved in establishing and maintaining the senescence growth arrest. The two distinct pathways are governed by the gatekeeper tumor suppressor proteins p53 and Rb (Bringold and Serrano 2000; Campisi 2001; Itahana *et al.* 2004). These two proteins can mediate senescence by acting as transcription factors. They play pivotal role in both pathways and their activation greatly influences their downstream cascades. However, the detailed mechanisms of how these two pathways involved in initiating and sustaining senescence is not clear. These two pathways function independently or in a coordinated manner. Interestingly, replicative senescence can be reversed when cells express low p16 and p53 (Beausejour *et al.*

2003). Therefore, a consolidated, if not yet comprehensive, picture of senescence is emerging.

1.4.1 p53 Pathway

p53 is a crucial mediator of cellular senescence induced by a myriad of stimuli, including telomere dysfunction (Lechel *et al.* 2005; Cosme-Blanco *et al.* 2007), DNA damage (Seluanov *et al.* 2001), and oncogene activation (Ferbeyre *et al.* 2002). p53 has a very short lifetime of 20-30 min due to the constitutive ubiquitin modification by MDM2. As discussed above, cellular stress responses elicit phosphorylation of p53 on Ser-15 and Ser-20 by ATM, ATR, and DNA-PK_{cs} and uncouple the MDM2-mediated ubiquitination from p53 (Unger *et al.* 1999; Turenne *et al.* 2001; Boehme *et al.* 2008). Treatment of human umbilical vascular endothelial cells with interferons that induce oxidative stress and senescence, and this stress response depends on p53 and ATM (Kim *et al.* 2009). Likewise, p53-dependent senescence can be triggered in dysfunctional telomeres that are reminiscent of DNA breaks containing γH2AX at telomeres (Hao *et al.* 2004; Deng *et al.* 2008). The p53-dependent senescence can also be induced by overexpressing oncogenes. Cells with RAS activation display mitochondrial dysfunction and an increased ROS level, leading to senescence in a p53-dependent manner (Moiseeva *et al.* 2009). Alternatively, the p16-Rb pathway can also be activated to provide a second barrier of proliferation in oncogenic cells (Kotake *et al.* 2009).

p53 plays a critical in the maintenance of replicative senescence. Inactivation of p53 in senescent, telomere-shortened human cells restores cell proliferation (Beausejour *et*

al. 2003). However, the enforced re-entry of cell cycle results in a state of acute genomic instability, known as crisis that is independent on telomere length (Beausejour *et al.* 2003). Interestingly, p53 transactivates p21 expression, but not all the p21-mediated senescence is p53-dependent. In primary murine fibroblasts, p53 activation together with RAS activation displays senescence phenotypes in a p21-independent manner (Castro *et al.* 2004). Likewise, p21 is known to induce replicative senescence in EJ human bladder carcinoma cells independent of p53 (Fang *et al.* 1999). It is possible that p53 develops a mechanism that can selectively activate its downstream targets, which relies on the nature of DNA damage (Hill *et al.* 2008). At least in some cells, the induction of senescence by DNA damage, telomere dysfunction, and oncogene activation converges on the p53 pathway, which is both necessary and sufficient to establish and maintain the senescence.

1.4.2 Rb Pathway

Although p53 inactivation reverses the senescence arrest in some cells, it fails to explain the p21-depdenent senescence in p53-inactivated cells. Alternatively, the p16-Rb pathway can work partially in parallel to the p53-dependent senescence induction. Both p16 and Rb are tumor suppressors, of which p16 activates Rb. Activated Rb binds to and inhibits E2F protein, halting cells from entering S phase. The p16-Rb pathway can be induced by overexpression of oncogenes and genotoxic stress. Extended culture of human keratinocytes on fibroblast feeder cells result in loss of p16 function due to CpG methylation of p16 promoter (Darbro *et al.* 2006). In the cells, the senescence response to DNA damage or dysfunctional telomeres depends

primarily on the p53 pathway. In vitro, p16 can compensate the role of p53 in cellular senescence induction under p53 deficient background (Beausejour *et al.* 2003) and is required for RAS-induced senescence (Michaloglou *et al.* 2005; Li *et al.* 2010). Thus, the p16-Rb pathway provides a complementary barrier, but this pathway cannot be overcome by loss of p53 function.

Because p16-Rb can arrest cell proliferation at G1 phase, it is possible that chromatin changes can induce cellular senescence. Cells undergoing replicative senescence develop dense foci of heterochromatin which coincide with Rb-dependent heterochromatic repression of genes encoding cyclins and other positive cell cycle regulators; in particular, many of the repressed genes are targets of E2F transcription factors (Narita *et al.* 2003). Association of Rb with some of these genes can reverse their transcriptional properties. However, in the p16-Rb pathway, once it is activated, particularly by p16, the senescence cannot be reversed by subsequent inactivation of p53, silencing of p16, or inactivation of Rb (Beausejour *et al.* 2003). Therefore, Rb and p16 is likely to involve only in initiating repressive chromatin at E2F target genes, but not necessarily involve in maintenance of the heterochromatic domains. These findings may indicate that the p16-Rb pathway appears to be particularly important for ensuring the irreversibility of the senescence and refractory to subsequent inactivation of p53, Rb, or both.

Although the Rb pathway is essential for the transcriptional repression of loci in senescent cells, the mechanism by which Rb regulates gene expression in senescent cells is largely unknown. Because Rb/E2F complexes are usually silent, they most

likely indirectly regulate the genes that are highly expressed by senescent cells. Alternatively, the Rb pathway may not be necessary for senescence-associated increases in gene expression, despite its importance for the senescence maintenance. However, it is of great interest that many of the genes that are up-regulated in senescent cells are physically clustered (Zhang *et al.* 2005). This finding suggests that chromatin reorganization may also be responsible for up-regulating gene expression in senescent cells where chromatin structures are in an unpacked state.

1.5 SELENIUM

Selenium is non-metal and belongs to the chalcogen group together with oxygen and sulfur. This essential trace mineral maintains a spectrum of biological and physiological functions. Current recommended daily allowance (RDA) of selenium in the US is 55 μg/day for the general healthy adults and 60-70 μg/day during pregnancy and lactation. Selenium is widely distributed in inorganic forms in the soil and in organic forms in certain foods such as Brazil nuts, seafood, and dairy products. Wide variations have been found in selenium status in different parts of the world. Although selenium deficiency is rare in the US, mammals who consume foods grown on selenium-deficient land are prone to dietary selenium deficiency. The reported symptoms include inflammation and injury of the muscles, degeneration of the pancreas, and abnormal coloration. The two well-known selenium-deficient diseases are Keshan disease and Kashin-Beck disease. Keshan disease is named after the selenium deficiency area where it was first discovered and predominantly affects children and young women. It is a cardiovascular disease, the etiology of which is the

increased susceptibility of heart muscle to Coxsackievirus B3 due to selenium deficiency (Beck *et al.* 1994). Kashin-Beck disease is a chronic osteoarthropathy of young children due to dietary deficiency in both selenium and iodine (Moreno-Reyes *et al.* 1998). Based on a mouse model engineered to carry selenoprotein deficiency in osteo-chondroprogenitors, it is convincing that selenoproteins play critical roles to prevent the bone pathologies (Downey *et al.* 2009). The upper limit intake of selenium is set at 400 μg/day, because free selenium may substitute sulfur in Cys or selenium could play as a pro-oxidant. Different selenium compounds enter its metabolic pathway at distinct stages. It is the key metabolite, hydrogen selenide, which generates ROS thus giving selenium the pro-oxidative characters (Figure 1.1). Generally, inorganic forms of selenium are more toxic. Organic selenium compounds have high bioavailability, but are also toxic in large doses. Nano-size selenium has equal efficacy, but much lower toxicity (Wang *et al.* 2007).

Selenium can regulate metabolic functions through selenium-containing proteins. The immune response and maintenance of tissue homeostasis can possibly be influenced by the selenium status (Carlson *et al.* 2009; Carlson *et al.* 2010). The interaction between selenium and heavy metals may help protect the body from the poisonous effects of heavy metals and other harmful substances. Selenium may boost fertility, especially among men. This mineral has been shown to improve the production of sperm and sperm movement. There is also possible role of selenium on cardiovascular diseases. Over the long-term epidemiological studies, there is a reverse relationship between selenium intake and cancer risk. The Nutritional Prevention of Cancer Trial conducted in the United States concluded that daily selenium intake at a

supranutritional level significantly decreases risks of cancer, and the prevention is most successful for prostate, lung, and colon cancers (Clark *et al.* 1996; Lippman *et al.* 2005). Furthermore, a role of selenium in preventing cancer in patients with prostatic intraepithelial neoplasia has been inferred (Marshall *et al.* 2006; Thompson 2007). Nonetheless, the recent termination of Selenium and Vitamin E Cancer Prevention Trial was due to no prevention effect on prostate cancer by selenium alone or combining with vitamin E (Lippman *et al.* 2009).

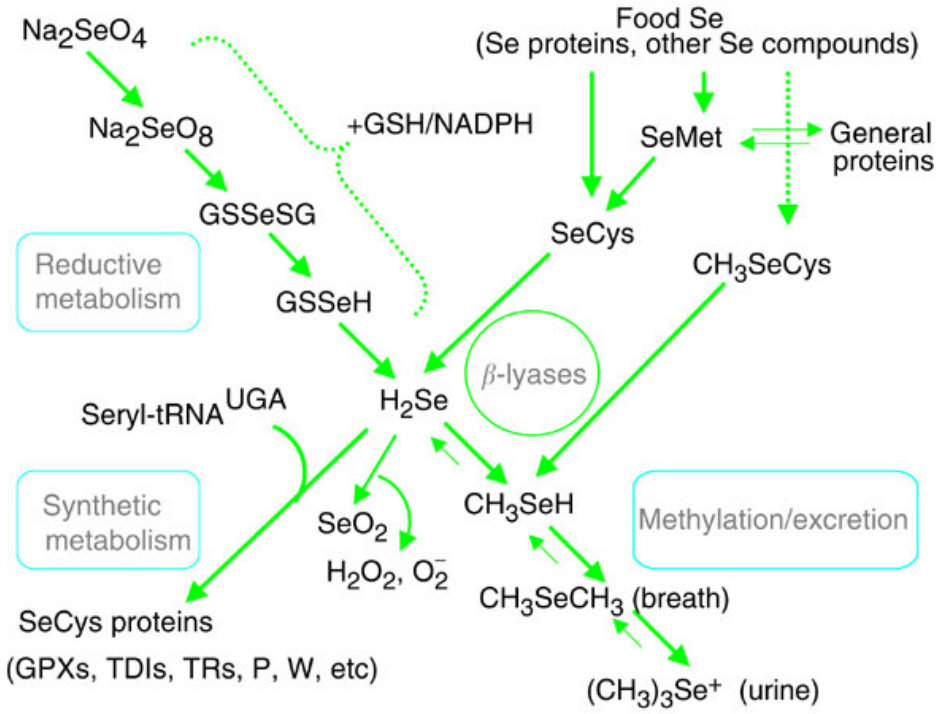


Figure 1.1 The metabolic pathway of selenium (adapted from (Combs 2004)**.** We obtain selenium compounds from food mainly as organic selenium. Selenium compounds can enter it metabolic pathway at different stages. It is believed that H_2Se plays a central role in the anti-carcinogenesis. Its overexpression can generate superoxide (O_2^-) and hydrogen peroxide (H_2O_2) to induce apoptosis. The downstream methyl-metabolites are also involved in anti-carcinogenesis by contributing to cell cycle arrest, induction of apoptosis, cell proliferation inhibition. SeMet, selenomethionine; SeCys, selenocysteine; GSSeSG, selenodiglutathione; GSSeH, selenoglutathione; GPXs, glutathione peroxidases; TDIs, iodothyronine 50-deiodinases; TRs, thioredoxin reductases, P, selenoprotein P; W, selenoprotein W.

1.5.1 Selenoproteins

Selenium substitutes sulfur in Cys, forming the 21st amino acid, selenocysteine (Sec). During the translation process, Sec is incorporated into selenoproteins. Although selenium and sulfur share similar chemical properties, Sec is more active than Cys because of the lower pK_a value and stronger nucleophilicity. Interestingly, there is no free pool of Sec. Selenium compounds including Sec are hyperactive, resulting in rapid NADPH oxidation and accumulation of ROS, so there is no free pool of Sec (Papp *et al.* 2007).

In general, metal ions serve as cofactors when they are protein-associated. However, selenium is co-translationally incorporated into selenoproteins as Sec (Papp *et al.* 2007). The UGA codon can be decoded by Sec-tRNA^{Sec} that contains anticodon of UGA and the 21st amino acid Sec, thus resulting in Sec incorporation during the translation process of selenoproteins (Squires and Berry 2008). Mammalian cells can use both organic and inorganic forms of selenium for Sec incorporation. The key metabolite selenide can be generated by reducing selenite in the glutathione-glutaredoxin and thioredoxin systems (Bjornstedt *et al.* 1992). Dietary selenomethionine and Sec can be lyased to provide selenide for Sec biosynthesis. Selenophosphate synthetase 2 (SPS2) converts selenide into monoselenophosphate, which is the active selenium donor for the formation of Sec-tRNA^{Sec}. This transformation of tRNA^{Sec} needs Sec synthase. Selenoprotein mRNA contains in-frame UGS codon for Sec incorporation, and a Sec insertion sequence (SECIS) that forms a stem-loop structure in the 3-untranslated region. The SECIS of selenoprotein

mRNA is associated with Sec-specific elongation factor and SECIS-binding protein 2 (SBP2). This protein-mRNA complex works in concert with Sec-tRNA^{Sec} to avoid UGA being recognized as the otherwise stop signal, and enables the incorporation of Sec into a nascent polypeptide (Squires and Berry 2008).

Selenoproteins do not exist in all species. There are selenoproteins in archaea, bacteria, and most eukaryotes, but not in yeast and higher plants. Based on computational sequence analyses of the entire human genome, it is concluded that there is a total of 25 and 26 selenoproteins in humans and mice, respectively (Kryukov *et al.* 2003). The majority of selenoproteins play direct or indirect roles in redox regulation. The selenium-dependent glutathione peroxidases (GPX1-5) and thioredoxin reductases (TrxR1-3) directly suppress oxidative stress. Moreover, the expression of GPX2 can be up-regulated through redox-sensitive transcription factor Nrf2/Keap1 system (Banning *et al.* 2005), and selenoprotein H together with selenoprotein W has been proposed as a sensor of nuclear oxidative stress (Panee *et al.* 2007; Han *et al.* 2008). Selenoproteins have been implicated in many metabolic and functional pathways such as aging, cancer, and virus infection (Lu and Holmgren 2009; Zhang *et al.* 2010), but many of the selenoproteins, especially the newly identified ones through genomic analysis (Kryukov *et al.* 2003), do not have known functions.

The deficit of selenium can dramatically affect the expression of selenoproteins to various degrees and in a tissue-specific manner. For example, GPX1 is more sensitive than other selenoproteins to body selenium fluctuation, and dietary selenium

deficiency suppresses GPX1 expression to a greater extent in liver and heart than in testis in a rat study (Thompson *et al.* 1998; Brigelius-Flohe 1999).

1.5.1.1 Glutathione Peroxidases (GPX)

There are total 5 selenium-dependent GPXs in humans: the ubiquitous GPX1, the gastrointestinal GPX2, the plasma GPX3, the ubiquitous phospholipid hydroperoxide GPX4, and the olfactory epithelium- and embryonic tissue-specific GPX6 (Di Leonardo *et al.* 1994; Arthur 2000; Pappas *et al.* 2008). GPX1–3 are involved in the reduction of hydrogen peroxide and organic hydroperoxides, forming tetrameric protein complexes (Chu *et al.* 1993; Yan and Chen 2006; Olson *et al.* 2010). GPX1, the most abundant selenoprotein, expresses in all types of cells and is a major antioxidant against acute oxidative stress (Cheng *et al.* 1998; Cheng *et al.* 2003). Unlike GPX1, the expression of GPX3 is tissue-specific, the most abundant of which includes liver, kidney, lung and heart (Schwaab *et al.* 1998). The excreted, extracellular GPX3 resides in plasma to reduce hydrogen peroxide and lipid hydroperoxides. GPX1 mRNA and protein levels fluctuate to dietary selenium status to a greater extent than other selenoproteins; together with plasma GPX3, they are indicators of body selenium status (Rayman 2009; Akbaraly *et al.* 2010). Expression of the gastrointestinal GPX2 prevents intestinal mucosa inflammation (Esworthy *et al.* 2005). GPX4 is a monomeric enzyme, and prefers to reduce phospholipid and cholesterol hydroperoxides instead of hydrogen peroxide. There are three isoforms of GPX4 according to their subcellular localization: cytosol, mitochondria and nuclear. Only cytosolic GPX4 is essential for embryonic development and cell survival.

Mitochondrial and nuclear GPX4 are involved in sperm maturation and male fertility because it has been found to be a main structural component of the sperm mitochondrial capsule in mature spermatozoa (Ursini *et al.* 1999; Imai *et al.* 2009; Schneider *et al.* 2009).

The relevance of GPX proteins to certain diseases is evidenced. In Alzheimer's disease patients, the erythrocyte GPX1 level is altered, which may account for the occurrence of the disease, as the neurons are subject to oxidative stress (Vural *et al.* 2010). Another age-related disease, Parkinson's disease, is also inversely correlated with total GPX activity, and the decreased anti-oxidant capacity in peripheral blood can result in increasing 8-oxo-G level and neurondegeneration (Chen *et al.* 2009). Moreover, the GPX1 Pro1981Leu polymorphism is associated with lung cancer (Knight *et al.* 2004). In many cancer cells, GPX1 and GPX4 are down-regulated (Lindahl 1993; Rayman 2002; Bakkenist and Kastan 2003; Boitani and Puglisi 2008; Cheng *et al.* 2008), and overexpression of GPX3 can inhibit tumor growth (Shargorodsky *et al.* 2010). In contrast, GPX2 has been reported to promote cancerous cell growth by an unknown mechanism (Brigelius-Flohe and Kipp 2009).

1.5.1.2 Thioredoxin Reductases (TrxRs)

There are three TrxRs in mammals: the cytosolic TrxR1 (Gladyshev *et al.* 1996), the mitochondrial TrxR2 (Gasdaska *et al.* 1999), and the tissue-specific thioredoxin-glutaredoxin reductase (TrxR3), especially in testis (Sun *et al.* 2001). Interestingly, TrxR1 exhibits a high expression level in neuronal tissues (Soerensen *et al.* 2008). Under dietary selenium deficiency, the expression of TrxRs is prioritized in the brain,

suggesting a critical role of TrxRs in the brain (Schomburg *et al.* 2003). Mice with null deletion of TrxR1 or TrxR2 are embryonic lethal (Conrad *et al.* 2004), while the essentiality of TrxR3 is unknown.

TrxRs use thioredoxin nucleoside diphosphate as a substrate, and the enzymatic activity is regulated by dietary selenium. Decreased enzymatic activity of TrxRs is associated with ROS accumulation and age-related neurodegeneration such as Alzheimer's disease and Parkinson's disease (Andersen 2004; Seyfried and Wullner 2007). As such, TrxRs are proposed as potential targets for ROS-associated neurodegenerative diseases. On the other hand, since tumor cells may take electrons from the Trx system, TrxRs have emerged as new targets for anti-cancer drug development (Felix *et al.* 2004). TrxRs may optimize malignant cell growth during tumorigenesis, and have been found to be overexpressed in many aggressive tumors (Park *et al.* 2006).

1.5.1.3 Iodothyronine Deiodinases (DIOs)

The DIO family includes DIO1-3, which are involved in the regulation of thyroid hormones thyroxine (T4), 3,5,3'-triodothyronine (T3) and reverse triiodothyronine (rT3) (Bianco and Kim 2006). The expression and function of DIOs are tissue-specific (St Germain *et al.* 2005). DIO1 regulates T3 production in the thyroid glands and circulates T4 level. DIO1 knockout mice display abnormal concentrations of thyroid hormones and their metabolities (Galton *et al.* 2009). DIO2 is expressed in the thyroid, central nervous system, pituitary, and skeletal muscle, where this selenoprotein regulates T3 circulation and production. DIO2 knockout mice exhibit

abnormalities in auditory functions, thermogenesis, and brain development. DIO3 are expressed in fetal tissues, placenta, neonatal brain and skin to control local deiodination process. DIO3 knockout mice show reduced viability, growth retardation, impaired fertility, reduced T3 and increased T4 levels (St Germain *et al.* 2005).

There are a few human diseases associated with defective expression of DIOs and thyroid hormone metabolism. Individuals with Graves' hyperthyroidism show increased T3 and DIO1 levels (Bianco *et al.* 2002). The inhibition of DIO1 provides a feasible treatment for this disease. SBP2 is a rate-limiting factor mediating Sec incorporation. A truncated SBP2 leads to features of abnormal thyroid hormone metabolism, which is related to DIO1 dysfunction (Azevedo *et al.* 2010). Combined selenium and iodine deficiency leads to the condition of myxedematous cretinism showing increased oxidative damage and altered thyroid hormone metabolism (Kohrle *et al.* 2005). The link between DIOs dysregulation and cancer is evidenced. For example, the expression of DIO1 is reduced or lost in breast cancer (Garcia-Solis and Aceves 2003), and dysfunction of DIO1 and DIO2 is associated with papillary thyroid cancer (Ambroziak *et al.* 2005).

1.5.1.4 Selenophosphate Synthetases (SPSs)

In eukaryotes, SPS catalyzes the formation of selenophosphate, the selenium donor, from ATP and selenide during the process of Sec biosynthesis. Interestingly, SPS2 is a selenoprotein and plays essential role in the generation of selenophosphate (Xu *et al.* 2007). In contrast, SPS1 is not a selenoprotein and does not involve in

monoselenophosphate synthesis (Xu *et al.* 2007). Although SPS1 is an essential gene for the regulation of glutamate level and mitochondrial function in drosophila (Shim *et al.* 2009), its role in mammals remains unclear.

1.5.1.5 Selenoprotein 15 (Sep15)

The expression of Sep15 is high in the thyroid, parathyroid and prostate (Gladyshev *et al.* 1998). The major cellular location of Sep15 is endoplasmic reticulum, suggesting a role of this selenoprotein in protein folding and disulfide bond formation (Fomenko and Gladyshev 2003). There are a few identified Sep15 single nucleotide polymorphysm, including 811 (C/T) and 1125 (A/G) in the 3' UTR and 1125 (A/G) in the SECIS recognition region (Gladyshev *et al.* 1998). The 1125A polymorphic Sep15 variant could possibly determine the Sec incorporation efficiency during translation (Apostolou *et al.* 2004). Although Sep15 polymorphisms may not be associated with the prostate cancer risk, they are highly relevant to the prostate cancer mortality (Penney *et al.* 2010). A recent report implicates Sep15 in the promotion of tumorigenesis (Irons *et al.* 2010). Irons et al. injected mouse colon cancer CT26 cells with or without Sep15 knockdown to BALC/c mice, and found that the pulmonary metastasis is significantly reduced in mice injected with Sep15-deficient CT26 cells. As Sep15 knockdown can affect genes involved in cellular growth, proliferation and G2-M cell cycle arrest, it could explain the anticarcinogenesis benefits by the downregulation of Sep15 (Irons *et al.* 2010).

1.5.1.6 Selenoprotein P (SelP)

SelP is the second selenoprotein discovered in mammals. SelP is considered to be critical in the maintenance of selenium homoeostasis as it bears ten Sec residues and two SECIS in the 3'-UTR (Burk and Hill 2005). SelP is the dominate selenoprotein that stores selenium in plasma, but plasma SelP level decreases only in the response to severe dietary selenium deficiency (Perssonmoschos *et al.* 1995). SelP is mainly synthesized as a glycoprotein in the liver and secreted into body fluids. A solid body of evidence suggests that SelP facilitates selenium delivery from the liver to the peripheral tissue, especially the brain (Burk *et al.* 2003; Chen and Berry 2003). SelP knockout mice exhibited overall decreased mRNA expression of selenoprotein mRNA in the brain and testis, including SelW, GPX1 and GPX4, consistent with a role of this selenoprotein as a selenium transporter (Hoffmann *et al.* 2007). Moreover, SelP has been reported to reduce phospholipid hydroperoxide and protect neuronal cells from oxidative stress (Saito *et al.* 1999; Steinbrenner *et al.* 2006). The research conducted by using postmortem tissues from Alzheimer's brain revealed a co-localization of a high SelP level in amyloid-β plaques and neurofibrillary tangles (Bellinger *et al.* 2008). Furthermore, reduced expression of SelP is linked to certain cancers. For instance, single nucleotide polymorphisms in SelP enhance the incidence of prostate cancer risk in Sweden men, probably due to an increased oxidative stress and the resultant tumor progression promotion (Cooper *et al.* 2008).

1.5.1.7 Other Selenoproteins

Selenoprotein W (SelW) is a 10 kDa selenoprotein, expressed in muscle, spleen, testis and brain in rats (Iliakis *et al.* 2003). Its status in muscle can be influenced by

selenium intake, probably through regulating the mRNA level (Li *et al.* 2008). SelW contains a redox motif and can bind glutathione as the GPXs (Gundimeda *et al.* 2008). Although the exact metabolic function of SelW is still under investigation, there are indications about its potential functions. Like the other selenoproteins, SelW is considered as an antioxidant enzyme, as its overexpression in CHO cells and H1299 human lung cancer cells resulted in less sensitivity of both cell lines to H_2O_2 exposure (Wang *et al.* 2009). It is postulated that SelW serves as a H_2O_2 signal transducer, so regulatory disulfide bonds in specific target proteins can react to the oxidative stress (Beilstein *et al.* 1996). Moreover, because the SelW knockdown mouse embryonic cerebral cortex cells exhibited more sensitivity to H_2O_2, it implies the essential role of SelW in protecting neuron development against oxidative stress (Davis *et al.* 2002). In breast and prostate epithelial cells, SelW mRNA was the only selenoprotein mRNA which could be increased by supplemented sodium selenite and high-selenium serum (Edes *et al.* 2010). The knockdown of SelW could result in G1-phase arrest in these cells, which indicates the possible physiological targets of selenium (Edes *et al.* 2010).

Selenoprotein H (SelH) is a recently discovered 14-kDa selenoprotein, with its functions largely unknown. From sequence and structural analyses, there is a conserved thioredoxin-like CXXU motif in SelH (Schnabel *et al.* 2005), indicating its role as an antioxidant enzyme. It is localized in the nucleolus (Schnabel *et al.* 2005), with a DNA-binding domain, suggesting that it may also act as a transcription factor (Panee *et al.* 2007). SelH increased glutathione levels and GPX activity, and up-regulated some other selenoproteins in response to stress in murine hippocampal

HT22 cells (Panee *et al.* 2007). The overexpression of SelH in HT22 cells rescued cells from UVB-induced damage by reducing the superoxide formation (Blankenberg *et al.* 2003). SelH could also protect neurons from UVB exposure by inhibiting apoptosis and mitochondrial depolarization (Smolkova *et al.* 2004). SelH may also be important in the embryonic development as its mRNA level increased in the early stages of embryos (Schnabel *et al.* 2005). The expression level of SelH was elevated in LNCap and mouse lung cancer LCC1cells (Schnabel *et al.* 2005). It suggests that SelH can maintain genome stability by reducing oxidative stress thus preventing cancerous incidence.

Selenoprotein M (SelM) is another recently identified small selenoprotein, localized in the endoplasmic reticulum (Davis and Uthus 2003). Its expression can be found in various tissues, especially high in the brain (Davis and Uthus 2003). The transgenic pCMV/GFP-hSelM mice had decreased H_2O_2 concentration but high activity of antioxidant enzymes after 2,2'-azobiz injection, which supported the antioxidant function of SelH (Hwang *et al.* 2007). The overexpression of SelM in several neuron cell lines revealed decreased oxidative stress and apoptotic cell death in response to H_2O_2 (Bartkova *et al.* 2006). The transgenic mice brain with human mutant presenilin-2 overexpression showed suppression of SelM transcriptional products, indicating the possible protective functions of SelM in the pathology of patients with the Alzheimer's disease (Burma *et al.* 2001). In fact, SelM may protect the brain by activating the ERK pathway to inhibit the α/γ-secretase activity in the proteolysis and Tau phosphorylation (Gorgoulis *et al.* 2005). Recent study revealed that, besides the role in redox regulation, SelM may also influent the cytosolic calcium, as the

knockdown of SelM in cortical cultures resulted in increased baseline level of cytosolic calcium (Bartkova *et al.* 2006).

1.5.2 Selenium Regulating Age-related Diseases through Genome Maintenance

Chronic diseases are the leading cause of mortality in the world, accounting for 60% of all deaths. Chronic diseases progress slowly and gradually, including cardiovascular diseases, cancer and diabetes. The incidence of chronic diseases increases with age and genome instability, the cause of which includes malnutrition, tobacco use, alcohol abuse and sedentary life style. Recent research collectively implicates the benefit of consuming nutrients higher than the nutrition needs or bioactive food components in the prevention of chronic diseases. This review focuses on selenium and selenoproteins. Selenium and selenoproteins play important roles in the defense against oxidative stress (Arbogast and Ferreiro 2010), the regulation of thyroid hormone activity (Ambroziak *et al.* 2005; Kohrle *et al.* 2005; Bianco and Kim 2006) and age-related chronic diseases including cancer, cardiovascular disease, and diabetes. However, the indiscriminant use of selenium supplements should be approached with caution as safety is not guaranteed for the effects of such supplementation at extremely high doses, especially on a long-term basis.

1.5.2.1 Selenium and Tumorigenesis

Several decades after establishing the nutritional essentiality of selenium, a solid body of recent evidence indicates a role of selenium in various stages of tumorigenesis. For

example, the Nutritional Prevention of Cancer Trial conducted in the United States provided the evidence of a reverse relationship between selenium and the incidence of cancer at a supranutritional level (Clark *et al.* 1996). Moreover, in a study conducted by Schrauzer and colleagues using subjected from 27 countries, dietary intake of selenium was concluded to be inversely correlated with total age-adjusted cancer mortality (Schrauzer *et al.* 1977). Animal studies have indicated a role of selenium in the suppression of tumorigenesis, and the efficacy of which depends on the formulation of selenium species (Lu *et al.* 1995; Spallholz *et al.* 2001; Fang *et al.* 2010). In Muc2/p21 double mutant mice, sodium selenite could reduce intestinal tumor formation through promoting apoptosis and inhibiting cell proliferation (Fang *et al.* 2010). The transgenic adenocarcinoma prostate mice which were fed with methylseleninic acid (MSeA) and methylselenocysteine (MSeC) showed decreased prostate tumor volume and increased survival age (Spallholz *et al.* 2001).

Recent epidemiological studies in general lend support for selenium chemoprevention. The Nutritional Prevention of Cancer Trial, carried out by Clark and co-workers in the USA, was the first double-blind, placebo-controlled intervention trial in a western population, designed to test the hypothesis that selenium supplementation could reduce the risk of prostate, colon and lung cancer (Clark *et al.* 1996). The 1312 subjects were randomized to placebo or 200 g selenium per day in the form of selenized yeast, which includes selenomethionine and 20 other species of selenium compounds. Several intervention studies constructed in Linxian, China, suggested that selenium could contribute to reducing the esophagea/gastric cardia cancer (Stewart *et al.* 1999). In contrast, the Selenium and Vitamin E cancer

prevention trial failed to prove a role of selenomethionine in the suppression of prostate cancer in healthy men (Klein *et al.* 2000; Lippman *et al.* 2009). The inconsistent and controversy results obtained from different studies may be explained by distinct selenium formulation and serum selenium baselines (Waters *et al.* 2005; Marshall *et al.* 2006; Thompson 2007; Lippman *et al.* 2009). Moreover, selenium may interact with other food components for chemoprevention (Bartkova *et al.* 2005). Nonetheless, the general perception is that selenium intake higher than the nutrition needs in the form of organic compounds suppresses tumorigenesis.

Selenium in principle can also participate in the process of tumorigenesis through antioxidative selenoproteins. Analysis of mRNA levels in paired lung sample specimens of 33 non-small cell lung cancer patients showed down-regulation of SelP, and this suppression may cause the enhancement of ROS (Gresner *et al.* 2009). Knockdown of TrxR1 in RKO cells exhibited enhanced cytotoxicity, promoting apoptosis independent on regulating the thioredoxin redox status but dependent on nitric oxide (Shen *et al.* 2001). The immunocytochemical examination of several primary human cancers including breast cancer, prostate cancer and colorectal carcinoma revealed increased TrxR levels in them (Zhuo *et al.* 2004). It strongly suggests the involvement of TrxR in the process of carcinogenesis and also provides potential targets for cancer treatment.

Selenium chemoprevention may be executed by its pro-oxidative, rather than anti-oxidative properties (Drake 2006). Consistent with this notion, our recent reports suggest that selenium compounds can act as a pro-oxidant and mitigate tumorigenesis

(Qi *et al.* 2010; Wu *et al.* 2010). MSeA exhibits high anti-carcinogenic potential in cells and in mice (Hu *et al.* 2006; Zhao *et al.* 2006; Li *et al.* 2007) through its metabolite, methyl selenol. ROS are generated during the catabolism process of selenium compounds, which can subsequently damage DNA and result in senescence or apoptosis (Li *et al.* 2007; Wu *et al.* 2010). Apoptosis is a genetically controlled format of cell death, providing a non-inflammatory mechanism for eliminating multicellular organisms of dangerous or unneeded cells. Sodium selenite can trigger p53-dependent apoptosis in LNCap prostate cancer cells by induction of superoxide (Li *et al.* 2007). Selenium compounds at doses $\leq LD_{50}$ induces an ATM- and ROS-dependent DNA damage and senescence response in MRC-5 and CCD 841 normal fibroblasts but not in two lines of cancer cells (Wu *et al.* 2010). At lethal doses, selenium compounds target the hMLH1 protein of the MMR pathway for an ATM-dependent G2/M checkpoint and DNA damage responses, and induce apoptotic response in colorectal cancer cells in a manner depending on ATM and ROS (Qi *et al.* 2010). Lack of hMLH1 may explain why MMR-deficient colorectal cancer cells are resistant to selenium-induced cell death.

1.5.2.2 Selenium and Cardiovascular Diseases

Enhanced oxidative stress in cardiac and vascular myocytes is causative to cardiovascular disease (Dhalla *et al.* 2000). Oxygen free radicals can oxidize phospholipids and proteins, disrupting membrane permeability and the integrity of subcellular organelles (Valko *et al.* 2007). It has been proposed that the antioxidative selenoproteins may attenuate atherosclerosis and protect against cardiovascular

diseases (Navas-Acien *et al.* 2008). Results from 25 studies from 1966 to 2005 have pointed to an inverse association between body selenium status and coronary heart disease (Flores-Mateo *et al.* 2006). However, there are two reports that do not support a role of selenium in the prevention of cardiovascular diseases (Stranges *et al.* 2006; Hawkes and Laslett 2009).

The antioxidative activity of GPX1 and GPX4 may account for much of the selenium protection against cardiovascular diseases. Because GPX4 can reduce phospholipid hydroperoxides and oxidized cholesteryl esters, it may prevent the accumulation of oxidized low-density lipoproteins in the artery wall (Kong *et al.* 2003). Under selenium deficiency, a build-up of hydroperoxides inhibits the enzyme prostacyclin synthetase that is responsible for the production of vasodilatory prostacyclin in the endothelium, which is associated with vasoconstriction and platelet aggregation (Meydani 1992; Vitoux *et al.* 1996). Thus, the balance is therefore tipped towards the pro-aggregatory state. In men with coronary artery disease, platelet aggregate is inversely related to selenium status (Salonen *et al.* 1991). Epidemiologic studies provide sufficient evidence for the association of mild hyperhomocysteinemia, high blood homocystein, with cardiovascular diseases (Allan *et al.* 1999; Macip *et al.* 2002; Macip *et al.* 2003). Homocystein can enhance ROS level (Ward and Chen 2001), partially through inhibiting antioxidant enzymes such as GPX1. Rather than altering the GPX1 transcriptional level, but it affects the GPX1 protein synthesis (Stiff *et al.* 2004). Considering the proposed reverse relationship between GPX1 activity and risk of cardiovascular events, it may be applied to predict populations with high risk of cardiovascular diseases (Lee *et al.* 1999). In fact, there is possible

interaction between GPX1 and homocystein, as patients with below-median GPX1 value but above-median homocystein level, tended to have a three-fold chance to develop cardiovascular diseases (Guijarro *et al.* 2007). Therefore, simultaneous assessment of GPX1 and homocystein is an appropriate way for interpretation (Guijarro *et al.* 2007).

The homocystein is biosynthesized from Met, not directly obtained from food. High level of homocystein can result in elevated S-adenosylhomocystein concentration, but this can be reversed by folic acid and vitamin B12 towards the formation of S-adenosylmethionine. Since these two S-adenosyl derivatives are the only methyl providers for all methylation reactions, folic acids and vitamin B12 can increase the methylation potentials (Noguchi *et al.* 2008). Thus folate deficiency could possibly exaggerate the risk of cardiovascular diseases by silencing the antioxidant enzymes in the antioxidant defense system (Cheng 2009). Besides, the folate-deficient erythroblasts cultured in folate-deficient medium revealed increased uracil misincorporation into DNA, thus continuously accumulating p53 and p21 (Hurst and Fairweather-Tait 2009). However, the results from selenite-fed and folate-fed Weanling.Fischer-344 rats revealed that, folate-deficiency can be partially rescued by selenium through inducing the conversion of homocystein to glutathione (Rayman *et al.* 2009).

1.5.2.3 Selenium and Diabetes

Although taking selenium above the nutritional level can provide many health benefits, body selenium concentrations is known to be higher in diabetes than in

normal individuals. A cross-sectional study involving 8876 subjects (Bleys *et al.* 2007) and a cohort study conducted in Northern Italy (Stranges *et al.* 2010) collectively suggest a positive association between high serum selenium and increased risk of diabetes. However, the SELECT study does support the association between high selenium intake and type 2 diabetes (Lippman *et al.* 2009). Cardiac dysfunction occurs during both type 1 and type 2 diabetes may be attributed to redox imbalance. In contrast, feeding diabetic rats with 5 micro mole/kg body weight/day sodium selenite prevents the antioxidant system defects induced by diabetes (Ayaz and Turan 2006).

In fact, selenium can function as insulin-like molecule due to its easy absorption. Comparing the diabetic and non-diabetic non-obese diabetic mice, the results confirmed the insulin-like effect of selenium in regulating serum glucose level and less liver damage (Zhou *et al.* 2003). In the same study, it also showed that selenium could turn on the insulin signaling pathway by activating Akt and PI3K kinases. Moreover, selenoproteins can also participate in the glucose regulation. For example, increased SelP concentration could result in the dysfunction of the insulin signaling pathway, thus impairing glucose homeostasis (di Fagagna *et al.* 2004). This is likely achieved by silencing adenosine monophosphate-activated protein kinase. Considering their involvement in the glucose metabolism, selenium and selenoproteins can serve as potential targets in treating type 2 diabetes.

1.5.2.4 Selenium and Other Age-related Diseases

In addition to the important role in mitigating carcinogenesis and cardiovascular diseases, selenium is also involved in the suppression of other age-related diseases. Available lines of evidence suggest a role of selenium in the attenuation of aging and age-related pathologies. There is a tendency of plasma selenium levels to decrease with age, leading to a high incidence of age-related diseases (Shamberg.Rj and Frost 1969; Reid *et al.* 2002). Interestingly, serum selenium in centenarians are maintained at nutritionally adequate level, suggesting that there is a positive association between selenoprotein expression and longevity (Anne-Marie and Tasnime 2007). Although the distribution of selenium in human body is broad, the level of selenium in the brain is always well maintained to ensure its functions (Zhang *et al.* 2010). The dysfunction of brain cells will lead to certain diseases. Alzheimer's disease is a brain disease that causes problems in memory, thinking and behavior. Parkinson's disease happens because of the degeneration of the central nervous system that impairs motor skills, cognitive processes, and other functions. Elevated ROS levels contributed to the pathologies of Alzheimer's disease and Parkinson's disease, which can be suppressed by antioxidative selenoproteins (Zhang *et al.* 2010). Since SelP is responsible for selenium transport, it is essential for neuronal normal functions. The postmortem tissue from the patients with the Alzheimer’s disease showed association of SelP co-localization with the amyloid-beta plaques, indicating the link between SelP and Alzheimer’s pathology (Bellinger *et al.* 2008). Also, reducing SelP expression in neuronal N2A cells resulted in increased apoptosis through aggregated amyloid beta-induced toxicity (Heiss *et al.* 2007). In addition, GPX3, the plasma GPX, is primarily

synthesized in the kidney, and plasma GPX activity is decreased in chronic renal failure (Zachara *et al.* 2003).

1.6 RESEARCH BACKGROUND

Tumorigenesis is a developing process, composed of initiation, promotion and progression. The initiation and promotion are considered as the early stages of tumorigenesis, as they have the restricted location. Gradual cellular and genetic changes at each stage eventually result in uncontrolled cell proliferation. Under normal conditions, cell division is tightly controlled, but once cells bearing detrimental damaged DNA escaped from the control or important pathways or genes involved in regulations dysfunction, they will lead to the cancer development. Regarding the development of tumorigenesis, apoptosis, senescence together with DNA damage response have been proposed as three barriers against carcinogenesis (Symonds *et al.* 1994; Bartkova *et al.* 2005; Gorgoulis *et al.* 2005; Bartkova *et al.* 2006). Apoptosis has been already well studied in cancerous cells, while the other two barriers in early tumorigenesis are now of great interest in cancer research. Precancerous lesions are considered to represent the early stages of tumorigenesis, as they are less aggressive and bearing less DNA damage (Hanahan and Weinberg 2000), due to the effective tumorigenesis barriers (Figure 1.2). Interestingly, considering the nature of each tumorigenesis stage, we should neither expect each barrier performs constantly through the process nor the barriers have equivalent effect on tumorigenesis. Also, the effect of these barriers can be tissue-specific. Overall, these three barriers in the normal tissues are negligible, and the proliferation is only

high in normal colon cells. However, in the precancerous lung bronchial epithelial cells, apoptosis is induced while the proliferation is also high. In contrast, in colon adenoma, senescence significantly decreases the proliferation index. In fact, further progression to carcinoma requires cells bearing DNA lesions escape from apoptosis, senescence and DNA damage response (Figure 1.2).

Though controversial research results were obtained, it is still believed that selenium can act against tumorigenesis (Clark *et al.* 1996; Lippman *et al.* 2005; Marshall *et al.* 2006; Thompson 2007; Lippman *et al.* 2009). The discrepancies among studies may be explained by different forms of selenium compounds and different cell lines or tissues applied in the study (Lu *et al.* 1995; Spallholz *et al.* 2001; Fang *et al.* 2010). The anti-carcinogenesis function of selenium can be partially related to the antioxidative selenoproteins. For example, low plasma SelP level was positively correlated with the increased risk of respiratory and digestive tract cancer in middle-aged Sweden men (Persson-Moschos *et al.* 2000). An enhanced TrxR level was found in breast cancer, prostate cancer and colorectal cancer through the immunocytochemical assay (Zhuo *et al.* 2004). However, the chemoprevention effect of selenium cannot only be explained by its antioxidant property. Specific inhibition of tumor cell growth by selenium metabolites (Drake 2006), modulations of cell cycle and apoptosis, and effect on DNA repair have all been proposed. Therefore, in order to contribute to the understanding of how selenium counteracts with tumorigenesis, my research examined the response of the normal cells and cancerous cells towards selenium treatments. Especially, my study focuses on the two tumorigenesis barriers, senescence and DNA damage response.

p53 pathway plays a central role in apoptosis, senescence and DNA damage checkpoint in various cell and animal models (Kastan and Bartek 2004). Thereafter, the time of defects in the p53 pathway and the importance of maintaining normal functional p53 pathway are investigated during cancer progression. Since apoptosis or senescence, and both of these are suppressed during cancer progression (Bartkova *et al.* 2005; Gorgoulis *et al.* 2005), chemicals that can restore these barriers during tumorigenesis should be promising in the cancer therapy. In fact, Na_2SeO_3 can trigger p53-dependent apoptosis in LNCap prostate cancer cells by inducing superoxide (Li *et al.* 2007). In murine fibroblasts, activation of oncogenic Ras induced the mitogen-activated protein kinase cascades, causing a p53-dependent senescence (Ferbeyre *et al.* 2002). After UV irradiation or neocarzinostatin treatment, p53 activated p53-inducible gene 3, which was essential in the DNA damage response pathways and participated in transmitting DNA damage signals to intra-S and G2/M checkpoint machinery (Lee *et al.* 2010). Therefore, it is worth to investigate the involvement of the p53 pathway in the early tumorigenesis barriers in response to selenium treatments.

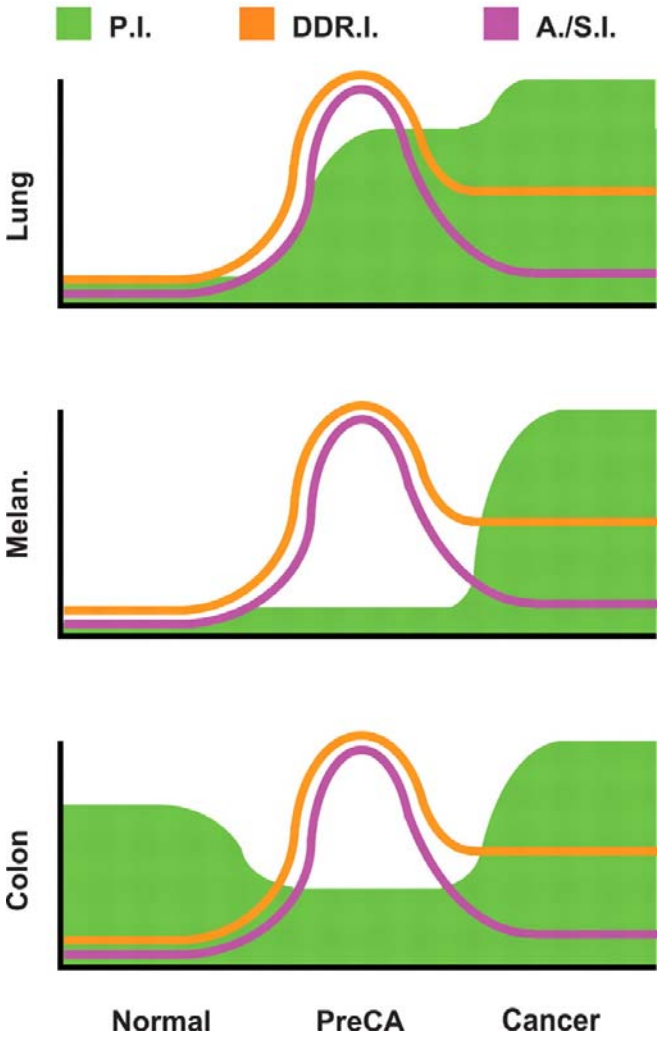


Figure 1.2 Three barriers inhibit tumorigenesis at different stages (adapted from (Halazonetis *et al.* 2008)**.** The normal cells, precancerous cells and cancerous cells represent distinct stages of tumorigenesis: initiation, promotion and progression. Apoptosis, senescence and DNA damage response have been proposed as three barriers that can block tumorigenesis. The effect of each barrier on tumorigenesis is shown in the figure (green for P.I., orange for DDR.I., and purple for A./S.I.). In precancerous lesions, three barriers are activated and prevent the cells with damaged DNA from proliferation. The exception in precancerous lung lesions may result from already high ROS levels inside. However, the inhibition effect of these barriers may vary dependent on cell types and damage types. P.I., proliferation index; DDR.I., DNA damage response index; A./S.I., apoptosis/senescence index; PreCA, precancerous; melan.,melanocytes

CHAPTER 2: MATERIALS AND METHODS

2.1 CELLS AND CULTURE CONDITIONS

The MRC-5 normal lung fibroblasts (Coriell Institute, Camden, NJ), CCD 841 normal colon fibroblasts and PC-3 prostate cancer cells (ATCC, Manassas, VA) were maintained in Minimum Essential Medium eagle (MEM, Mediatech Inc, Herndon, VA) supplemented with 15% heat-inactivated fetal bovine serum, 1 ng/ml essential amino acid, 1 ng/ml nonessential amino acid, 1 ng/ml vitamins, and 100 U/ml penicillin and streptomycin at 37°C in a 5% CO_2 incubator. The HCT 116 colon cancer cells (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech Inc, Manassas, VA) added with 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin and streptomycin. Sodium selenite (Na_2SeO_3), methylseleninic acid (MSeA), methylselenocysteine (MSeC), N-acetylcysteine (NAC) and 2, 2, 6, 6-tetramethyl-piperidine-1-oxyl (Tempo) were obtained from Sigma-Aldrich (St. Louis, MO), and were dissolved in phosphate-buffered saline (PBS). NAC is a free radical scavenger mainly responsible for H_2O_2 and Tempo is mainly targeting superoxide (O_2^-). KU55933 and NU 7026 were purchased from Tocris (Ellisville, MO), and were dissolved in DMSO. KU55933 is an ATM kinase inhibitor and NU 7026 is a DNA-PKcs kinase inhibitor. The cells were treated with the selenium compounds for 48 h, washed once with PBS, followed by a 1-7 days recovery.

2.2 SENESCENCE ASSAYS

Replicative senescence is defined by the limited proliferation cycles of somatic cells. Since proposed by Dimri et al. in 1995 (Dimri *et al.* 1995), Senescence-associated β-galactosidase (SA-β-gal) is a widely biomarker to directly identify the senescent cells. SA-β-gal is located in the lysosome and at the suboptimal pH level (pH=6.0), its activity enables detection of senescence. SA-β-gal is an enzyme involved in catalyzing the hydrolysis of β-galactosidase into monosaccharides, which is only in senescent cells. It can cleave natural and artificial β-linked terminal galactosyl residues. In the application of this senescent biomarker, X-gal (bromo-chloro-indolyl-galactopyranoside) can be cleaved into galactose and 5-bromo-4-chloro-3-hydroxyindole, which can be oxidized into an insoluble blue product. However, a question was raised after the application of the SA-β-gal, whether this enzyme was distinctly active at suboptimal pH or differently expressed in the senescent cells. After studying the effect of pH values on SA-β-gal activity level in young and senescent human umbilical vein endothelial cells, researchers confirmed that increased lysosomal content in senescent cells resulted in the expression of SA-β-gal lysosomal activity (Kurz *et al.* 2000). However, fibroblasts taken from patients with defective lysosomal β-galactosidase could still undergo senescence (Lee *et al.* 2006). Also, the late passage of normal fibroblasts expressing GLB1 shRNA which preventing the expression of SA-β-gal activity, exhibited a senescent status (Lee *et al.* 2006). Therefore, SA-β-gal activity is not a prerequisite for the happening of senescence but a detectable result, at least partially due to increased expression of the lysosomal β-galactosidase protein.

I detected SA-β-gal by using a Senescence Detection Kit (MBL Co. Ltd., Woburn, MA) according to the manufacturer's instructions. Cells were seeded onto 24-well plates with a density of 5,000 cells/well and treated with Na_2SeO_3 (0.1-10 μM), MSeA (0.1-10 μM), and MSeC (20-500 μM)) for 48 h, followed by a 7 days recovery. The cells were washed once in PBS, fixed with the provided fixative buffer at room temperature for 15 min, washed three times in PBS, and stained in the staining solution containing X-gal at a concentration of 1 mg/mL for 8 h. Cells were then overlaid with 70% glycerol and observed under a light microscope.

I also performed a BrdU (5-bromo-2'-deoxyuridine) incorporation assay to indicate the status of DNA replication. DNA replication is semi-conservative. If DNA replication happens, BrdU, a nucleotide analog can be taken by the newly synthesized DNA strand. By using the immunofluorescent approach, this analog can be detected. Sterilized coverslips were first put in each well of 6-well plates. Cells were then cultured on coverslips and incubated with Na_2SeO_3 (1 μM), MSeA (1 μM) and MSeC (50 μM) for 48 h followed by a 7 days recovery. Cells were pulse-labeled with BrdU (10 μM) for 1 h, followed by three times washing in PBS and fixation in 4% paraformaldehyde (in PBS) for 30 min. The fixed cells were then permeabilized in HCl (0.1 N) containing pepsin (100 μg/ml) for 30 min at 37^{o}C. DNA was denatured by HCl (1.5 N) for 15 min and then by sodium borate (0.1 M) for 5 min. After washing three times in PBS, the cells were incubated in an anti-BrdU antibody conjugated with FITC for 1 h at room temperature according to the manufacturer's instruction (BD Pharmingen, San Diego, CA). A drop of ProLong® Gold antifade reagent with DAPI (Invitrogen) was added to a slide and then coverslips were

mounted on the slides. I employed the filter cube set 49 (excitation, 365 nm; filter, 395 nm; emission, 445 nm) and set 38 (excitation, 470 nm; filter, 495 nm; emission, 525 nm) for visualization of DAPI and FITC, respectively, through a Zeiss AxioObserver 100 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). All the photomicrographs were taken using the same magnification scale (200X) and exposure time (400 ms) within 10 min to avoid auto-fading of the fluorescence signal.

2.3 IMMUNOFLUORESCENCE

Sterilized coverslips were first put in each well of 6-well plates. Cells were seeded onto coverslips and incubated with Na_2SeO_3 (1-2 μM), MSeA (1-2 μM) and MSeC (50-100 μM) for 2 days, followed by a 0-7 days recovery. The cells were first washed three times in PBS, fixed in 4% paraformaldehyde (in PBS) for 15 min, permeabilized in 90% methanol for 10 min at -20^{o}C, and washed three times in PBS (5 min per wash). The cells were then incubated in 0.3% Triton-X for 15 min and washed three times in PBS. Cells were blocked in 3% BSA in PBS for 30 min, followed by overnight incubation at 4^{o}C with antibodies against H2AX (1:100; Abcam, Cambridge, MA), γH2AX (phospho-H2AX on S139, 1:100, Abcam), ATM (1:100; Epitomics, Burlingame, CA), and anti-phospho-ATM on S1981 (1:100; Rockland, Gilbertsville, PA). After washing in PBS with 0.1% Tween-20 for 5 times (5 min per wash), the cells were incubated with secondary antibodies conjugated with fluorescence dyes (Alexa 488 goat anti-rabbit lgG and Alexa 594 goat anti-mouse lgG, Invitrogen) for 1.5 h at room temperature in dark. The coverslips were washed again in PBS with 0.1% Tween-20 for 5 times (5 min per wash). At last, a drop of

ProLong® Gold antifade reagent containing DAPI (Invitrogen) was added to a slide and the coverslips were mounted onto the slide. The slides were then placed under a Zeiss AxioObserver 100 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) for image acquisition. The fluorescence signals were visualized by using the filter cube set 49 for DAPI (excitation 365 nm, filter 395 nm, and emission 445 nm), filter cube set 38 for GFP (excitation 470 nm, filter 495 nm, and emission 525 nm) and filter cube set 43 for DsRed (excitation 550 nm, filter 570 nm, and emission 605 nm). All the photos were taken using the same magnification scale (630X), followed by deconvolution according to the manufacturer's instruction. The pATM S1981 and γH2AX focus-positive cells are defined as those containing at least five foci (Camphausen *et al.* 2004).

2.4 FLOW CYTOMETRY ASSAY

Flow cytometry assay is widely used to identify the proportions of cells in different stages of cell cycle (G0/G1, S, and G2/M phase) by measuring the amount of DNA in cells. This method relies on a single time point, revealing cell population in different stages of cell cycle but not cell cycle kinetics. The duration of each phase, however, can be estimated from the percentage of cells in this phase if the length of the cell cycle is known. Propidium iodide (PI) is a commonly used nuclei fluorescent dye and requires blue light as the excitation source (e.g., 488 nm argon ion laser). PI can bind to the DNA in the nuclei. However, it can also stain double-stranded RNA, so RNase A is added to the staining solution. The intensity and brightness of PI staining should be proportional to the amount of DNA in the cell. Because cells contain different

amounts of DNA depending on where they are in the cell cycle, it can thus be determined what percentage of cells are in the different parts of the cell cycle based on the intensity of fluorescence of the nuclei.

Cells were cultured in 10 cm^2 dishes and treated with 1-2 μM Na_2SeO_3, 1-2 μM MSeA and 50-100 μM MSeC for 6-48 h, followed by a 1-7 days recovery. The culturing medium was collected before rinsing the cell monolayers with PBS. Then cells were incubated in trypsin/EDTA for at least 15 min to ensure complete detachment and maximum separation as single cells. Cells were then resuspended in ice-cold PBS, and centrifuged at 4°C with a speed of 500×g for 5 min. Cells were washed again with cold PBS and resuspended by pipetting into100 μl PBS. 5 mL of -20°C 70% ethanol was added dropwise while vortexing to each tube for fixation and samples were stored in at -20°C. Prior to the analysis, samples were centrifuged at 4°C at a speed of 500×g for 5 min. Fixative ethanol was aspirated, and cells were washed with 5 mL PBS with 0.15% Triton X-100. Then cells were washed again with PBS at 4°C at a speed of 500×g for 5 min, and supernatant was taken out. Cells were vortexed and resuspended in 1 mL propidium iodide solution (25-50 μg/mL) containing RNaseA (100 μg/mL) for 30 min at room temperature in dark. The DNA was then analyzed by a FACScalibur cytometer with CELLQuest program (Becton Dickinson, San Jose, CA). ModFit LT (Version 3.0, Verity Software House, Topsham, ME) was applied for cell cycle analysis on overlaid histograms.

2.5 CELL SURVIVAL ASSAY

Cells were seeded at a concentration of 10,000 MRC-5 cells, 5,000 PC-3 cells, and 5,000 HCT 116 cells per 6-plate well, incubated with Na_2SeO_3 (1-10 μM), MSeA (1-10 μM), and MSeC (20-500 μM)) for 48 h, followed by a 7 days recovery. On the 7th day, the cells were washed once with PBS and then trypsinized with 200 μl/well at 37°C for 5 min. After that, normal MEM or DMEM medium was added to neutralize the effect of trypsin. The final volume for each well was 1 mL. Cells were well pipetted up and down to ensure the separation of cells. Then, according to the manufacturer's instruction, 10 μl cells of each treatment were transferred to the INCYTO C-Chip disposable hemacytometers (SKC Inc., Eighty Four, PA), and the cell number was counted under a light microscope.

2.6 MTT ASSAY

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazole. It can be reduced by mitochondrial reductases to a purple dye, formazan, which can be dissolved in DMSO. Because the reduction only occurs when enzymes are active, the MTT assay is a colorimetric assay and used to assess the viability and the proliferation of cells (Mosmann 1983). Depending on the solvent to dissolve the formazan, the colored solution can usually be measured by a spectrophotometer at a wavelength between 500 nm and 600 nm. Because chemicals can affect cell viability and growth, MTT assay can be applied to determine the cytotoxicity of chemicals. Since agents can impose stress on cells thus causing cell death and metabolic changes, by comparing treated cells and untreated cells, this assay can give a dose-

response curve. However, there is a major limit regarding to this assay. MTT assay reflects the mitochondrial enzymatic activity, so if cell number is constant but cellular metabolism dramatically increases, it can no longer be used as a measure for cell viability or growth.

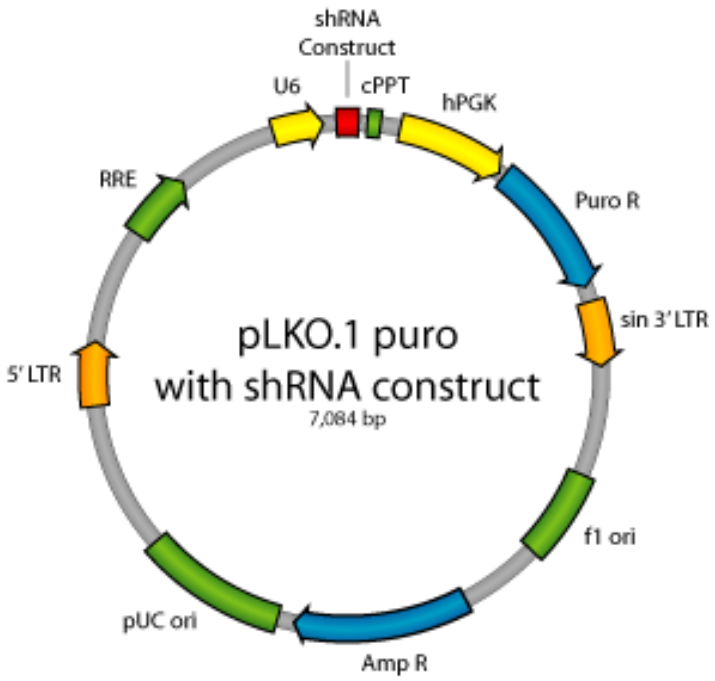
HCT 116 cells and HCT 116 complemented with hMLH1 cells were seeded at a concentration of 5,000 cells per 24-well plate. They were treated with MSeA (1-10 μM) for 48 h, followed by a 7 day recovery. On the 7^{th} day, 200 mg/ml MTT solution was freshly made, dissolved in serum-free MEM medium. First the medium was removed from the 24-well plate, and then a final concentration of 1 mg/ml MTT in serum-free medium was added, 1 mL per well. The plates were incubated at 37ºC for 2 h. After that, MTT medium was removed and plates were sat on the bench for dry. 100 μl of DMSO (Sigma-Aldrich, MO) was added to each well and the plates were shook for 5 min for crystal dissolving. Then plates were put into the 37ºC incubator for another 5 minutes to dissolve air bubbles. The absorbance readings were taken by the plate reader (BMG LabTech, Cary, NC) at 594nm.

2.7 shRNA KNOCKDOWN ASSAY

Small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) are designed for being successfully delivered into mammalian cells to interfere with RNA translation. The limit of cytosolic delivering siRNA oligonucleotides into cells depends on the cellular transfection capability and is primarily utilized during transient in vitro studies. The introduction of shRNA into mammalian cells through infection with viral vectors allows for stable integration of shRNA and long-term knockdown of the

targeted gene. Real-time PCR or other functional assays are used to detect the knockdown efficacies. Usually, the infection of more than two types of shRNA is suggested, as to provide a level of control and to verify dose dependency of functional effects.

The p53 shRNA lentivirus (Figure 2.1) was a generous gift from Dr. Yan Dong at the Tulane University. MRC-5, HCT 116 and HCT 116+hMLH1 cells were transfected with 1:100 p53 shRNA lentivirus. After transfection, MRC-5 cells were cultured in MEM medium supplemented with 5 μg/mL puromycin and the HCT 116 and HCT 116+hMLH1 cells were cultured in DMEM medium supplemented with 8 μg/mL puromycin. The successfully transfected cells were selected using 10 mm Pyrex® cloning cylinders (Corning Incorporated, Corning, NY). The selected transfected cells were seeded in 6-wells plates till cells were 80-90% confluent. Then cells were washed with RNA-free PBS once and 1 mL TRIzol® reagent (Invitrogen) was added to each well to harvest samples. These cells samples were used to examine the knockdown efficiency by quantitative real-time PCR.



Scramble:
5'-
CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTT
TTT-3'
E3 clone:
5'-
CACCATCCACTACAACTACATCTCGAGATGTAGTTGTAGTGGATGGTGTTT
TT-3'

Figure 2.1 Scheme of p53 shRNA lentivirus.
Note: Red color indicates the shRNA sequence. Green color indicates loop region. E3 clone targets p53 gene 14017-14037 (amino acid 231-237)

2.8 IMMUNOBLOTTING ANALYSIS

MRC-5 cells and MRC-5 p53 shRNA cells were cultured in 15 cm^2 petri dishes and treated with 1 μM MSeA for 0-48 h, followed by a 1-7 days recovery. Cells were washed twice with ice-cold PBS, scripted from petri dishes and centrifuged at 4^oC with a speed of 3000 rpm for 5 min. Cells were lysed in RIPA lysis buffer (Rockland, PA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, inhibit serine proteinase), Roche protease inhibitor cocktail (X50) and phosphatase inhibitor (cocktail I, 1:100, Sigma) with vortexing and then sat on ice for at least 30 min. After that, cells were centrifuged at 20,000 X g for 30 min at 4^oC to separate supernatants from the pellets, and then supernatants were transferred to a new tube. The total protein concentration was measured using the BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL) and the readings were taken at 594 nm. 30 μg of the whole cell extract was heated for denaturation and then loaded for separation by gel electrophoresis using 4-20% Tris-HCl Criterion^{TM} precast gels (Bio-Rad, Hercules, CA). The proteins were transferred onto a PVDF membrane at a voltage of 100 V for 1h on ice. After that the membrane was blocked with either 5% milk or 5% BSA for phosphorylated proteins in TBS with 0.1% Tween 20 (TBS-T) for 1h at room temperature. The membrane was briefly washed with TBS-T followed by overnight incubation at 4^oC with antibodies against mouse p53 (1:500; Cell Signaling Technology, Danvers, MA), mouse phospho-p53 Ser15 (1:1000, Cell Signaling Technology), rabbit p21 (1:5000; Abcam) and mouse p16 (1:500, Abcam). After washing in TBS-T for 3 times (10 min per wash), the membrane was incubated with HRP-conjugated secondary antibodies: Immonopure® goat anti-mouse lgG (H+L)

(Thermo Scientific, 1:10,000) and Immonopure® goat anti-rabbit lgG (H+L) (Thermo Scientific, 1:10,000) for 1h at room temperature. After washing again in TBS-T for 3 times (10 min per wash), the membrane was incubated with the chemiluminescent reagent SuperSignal® ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific) for development. The exposure time for the film was 1 min.

2.9 STATISTICAL ANALYSIS

The data were analyzed by using the SAS 9.0 software (SAS Institute Inc., Cary, NC). Two-tailed student's t-test was applied to determine statistical significance between the treatments and the control. The linear regression was also computed to confirm the Se dose-dependent senescence response in Figure 2 ($p < 0.0001$).

CHAPTER 3: SELENIUM-INDUCED SENESCENCE REQUIRES ATM ACTIVATION AND ROS GENERATION IN NON-CANCEROUS BUT NOT IN CANCEROUS CELLS

(Published in Wu et al., 2010, *Journal of Biological Chemistry* **285**(16): 12055-12062)

Genomic instability is a hallmark of carcinogenesis. Recent advances suggest that major barriers of human tumorigenesis at the early stage include DNA damage response and senescence (Bartkova *et al.* 2005; Gorgoulis *et al.* 2005; Bartkova *et al.* 2006), both of which involve ATM activation. Heritable mutations in ATM cause ataxia-telangiectasia, a genomic instability syndrome characterized by cancer predisposition, neurodegeneration, and premature aging. In response to DNA damage, the ATM kinase is rapidly activated and mediates multiple downstream pathways, resulting in DNA damage checkpoint response and repair. ATM kinase activation in humans requires ATM autophosphorylation at Ser-1981 (Bakkenist and Kastan 2003). On the other hand, cellular senescence, a form of cell cycle withdrawal, can limit the proliferation of cells with persistent genomic instability. In the MRC-5 diploid fibroblasts expressing *mos*, the oncogene-induced senescence can be suppressed by inhibition of ATM, suggesting that ATM plays a major role linking pathways of senescence and DNA damage response during early tumorigenesis (Bartkova *et al.* 2006).

Selenium is an essential trace mineral widely distributed in inorganic forms in soil and in organic forms in certain foods. The Nutritional Prevention of Cancer Trial conducted in the U.S. concluded that daily selenium intake at a supranutritional level significantly decreases risks of cancer, and the prevention is most successful for

prostate, lung and colon cancers (Clark *et al.* 1996; Lippman *et al.* 2005). Furthermore, a role of selenium in preventing cancer in patients with prostatic intraepithelial neoplasia has been inferred (Marshall *et al.* 2006; Thompson 2007). However, the recent Selenium and Vitamin E Cancer Prevention Trial concluded that selenium supplementation alone or in combination with vitamin E does not prevent prostate cancer risks in general healthy men (Lippman *et al.* 2009). Differences in selenium formulation and the body selenium status prior to entering the trials may explain the seeming discrepancies between these two cohorts of clinical studies (Clark *et al.* 1996; Lippman *et al.* 2005; Waters *et al.* 2005; Lippman *et al.* 2009). Nonetheless, a consensus drawn from the two trials is that selenium supplementation prevents cancer risks only in men entering the trial with suboptimal levels of body selenium. Thus, it is necessary to elucidate the mechanism of tumorigenesis suppression offered by selenium.

Metabolites of selenium compounds have been shown to induce ROS, which in turn can induce oxidative modifications and breaks on DNA. Previous studies focus on selenium-induced stress responses in various cultured cancer cells, from which it is suggested that much of the role of selenium in cancer prevention is attributed to ROS-induced apoptosis or cell cycle arrest in cancer cells (Stewart *et al.* 1999; Spallholz *et al.* 2001; Li *et al.* 2007). Consistent with this notion, it has been shown that selenium-induced apoptosis in cancer cells can be suppressed by antioxidants (Shen *et al.* 2001) and is p53-dependent (Zhao *et al.* 2006). Furthermore, selenium can sensitize cancer cells to other apoptotic inducers including TRAIL and doxorubicin (Hu *et al.* 2006; Li *et al.* 2007).

Available evidence from the literatures has not provided a full understanding of selenium in tumorigenesis, or the linkage of selenium metabolites to genomic maintenance (Cheng 2009). It is known that selenium-induced oxidative stress or DNA damage can lead to apoptosis in some cancer cells (Zhou *et al.* 2003); however, the roles of selenium in cellular senescence have not been studied. To investigate additional barriers of tumorigenesis elicited by selenium, we treated the MRC-5 normal lung fibroblasts, the CRL-1790 normal colon fibroblasts, and the PC-3 prostate and HCT 116 colon cancer cells with selenium compounds of organic and inorganic origins in a series of studies. We employed a number of senescence markers, including the measurement of SA-β-gal and levels of the pulse-labeled BrdU, together with investigating the ATM-mediated DNA damage response pathway, to explore a role of selenium in the activation of early tumorigenesis barriers. Our results indicate that selenium induces an ATM-dependent senescence response via redox regulation in non-cancerous but not in cancerous cells, suggesting a novel mechanism of selenium in counteracting tumorigenesis.

3.1 SENESCENCE WAS INDUCED BY SELENIUM COMPOUNDS IN MRC-5 AND CCD 841 NON-CANCEROUS CELLS BUT NOT IN PC-3 OR HCT 116 CANCEROUS CELLS

To determine whether or not selenium can counteract tumorigenesis through cancer barriers other than the well-studied apoptosis, we first assessed senescence phenotypes after cellular exposure to the selenium compounds. MRC-5, CCD 841, PC-3 and HCT 116 cells were treated with Na_2SeO_3 (0.1-10 μM), MSeA (0.1-10 μM), and MSeC (5-500 μM) for two days, followed by a 7 days recovery in normal

medium in the absence of the selenium treatment. The cells were then subjected to the detection of SA-β-gal, a hallmark of cellular senescence. The selenium treatment resulted in the expression of SA-β-gal in the non-cancerous MRC-5 and CCD 841 cells in a dose-dependent manner (by regression analysis, $p < 0.0001$), from a concentration as low as 0.1 μM (Na_2SeO_3 or MSeA) and 5 μM (MSeC) (Figures 3.1A-C, Figures 3.2 and 3.3). Strikingly, there was no detectable SA-β-gal in the cancerous PC-3 and HCT 116 cells (Figures 3.1A-C, Figures 3.4 and 3.5) treated with selenium at doses that result in the majority of the MRC-5 cells senesce (2 μM for Na_2SeO_3 and MSeA; 100 μM for MSeC), or even higher doses. To confirm the selenium-induced senescence, we measured the rate of BrdU incorporation, an indicator of DNA replication. The selenium-treated cells were pulse-labeled with BrdU, which can be incorporated into the newly-synthesized DNA during S phase of the cell cycle. Compared to the MRC-5 cells without selenium treatment, cellular exposure to Na_2SeO_3, MSeA, or MSeC resulted in a 4-, 6.6-, and 2.3-fold reduction in BrdU incorporation, respectively (Figure 3.1D and Figure 3.6). Thus, treatment of MRC-5 cells with the selenium compounds resulted in DNA replication suppression, a feature of cellular senescence. Taken together, selenium compounds induce cellular senescence in the non-cancerous, but not in cancerous cells.

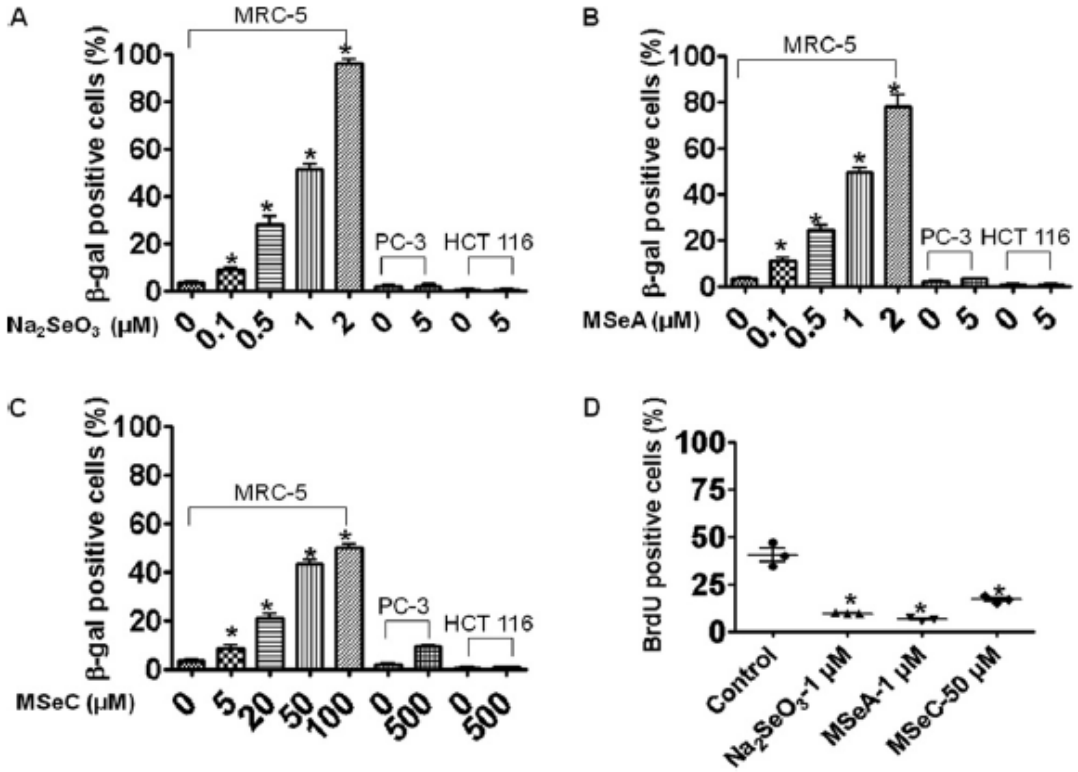


Figure 3.1 Senescence is induced in selenium-treated MRC-5 cells but not in PC-3 or HCT116 cells. Non-cancerous MRC-5 and cancerous PC-3 and HCT 116 cells (5,000/well) were seeded onto 24-well plates and treated with the selenium compounds for 48 h, followed by a 7-day recovery for SA-β-galactosidase detection (*A*–*C*). SA-β-galactosidase-positive cells were presented with their respective S.D. values (n=3). *D*, detection of pulse-labeled BrdUrd. MRC-5 cells were cultured on coverslips and incubated with the selenium compounds at the indicated concentrations for 48 h, followed by a 7-day recovery. BrdUrd-positive cells are presented with their respective S.D. values (*, p <0.01 compared with cells treated with selenium only).

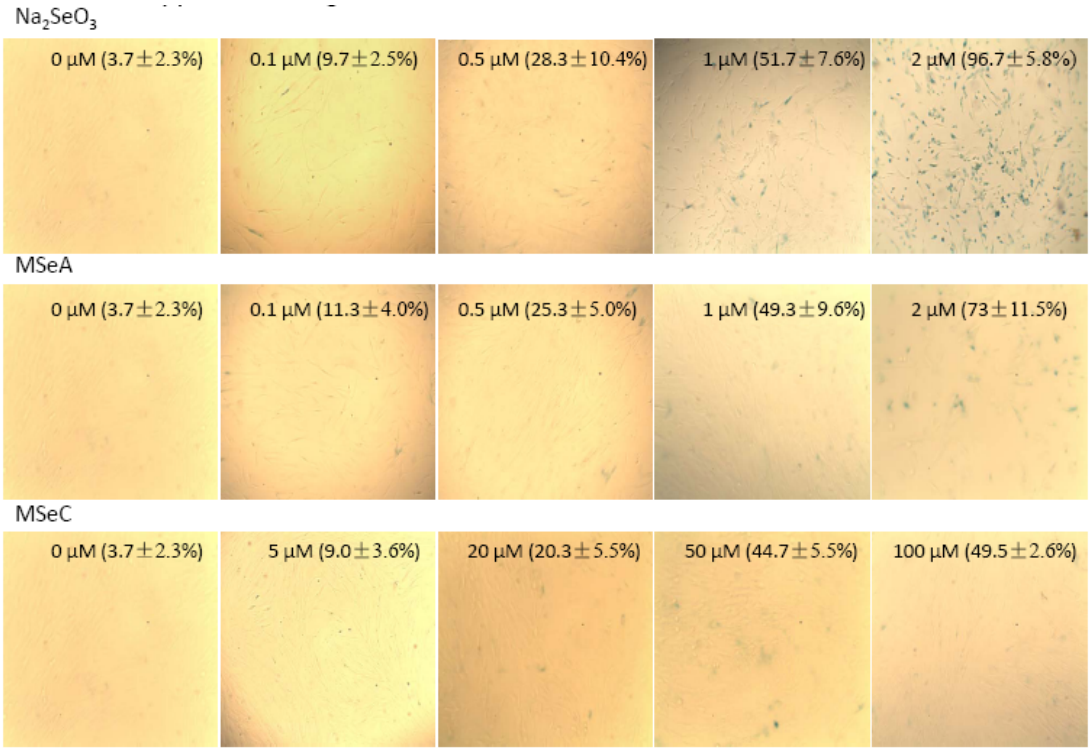


Figure 3.2 Senescence is induced in selenium-treated MRC-5 cells. Cells (5,000/well) were seeded onto 24-well plates treated with the selenium compounds for 48 h, followed by a 7 days recovery. SA-β-gal was determined as described in Figure 3.1.

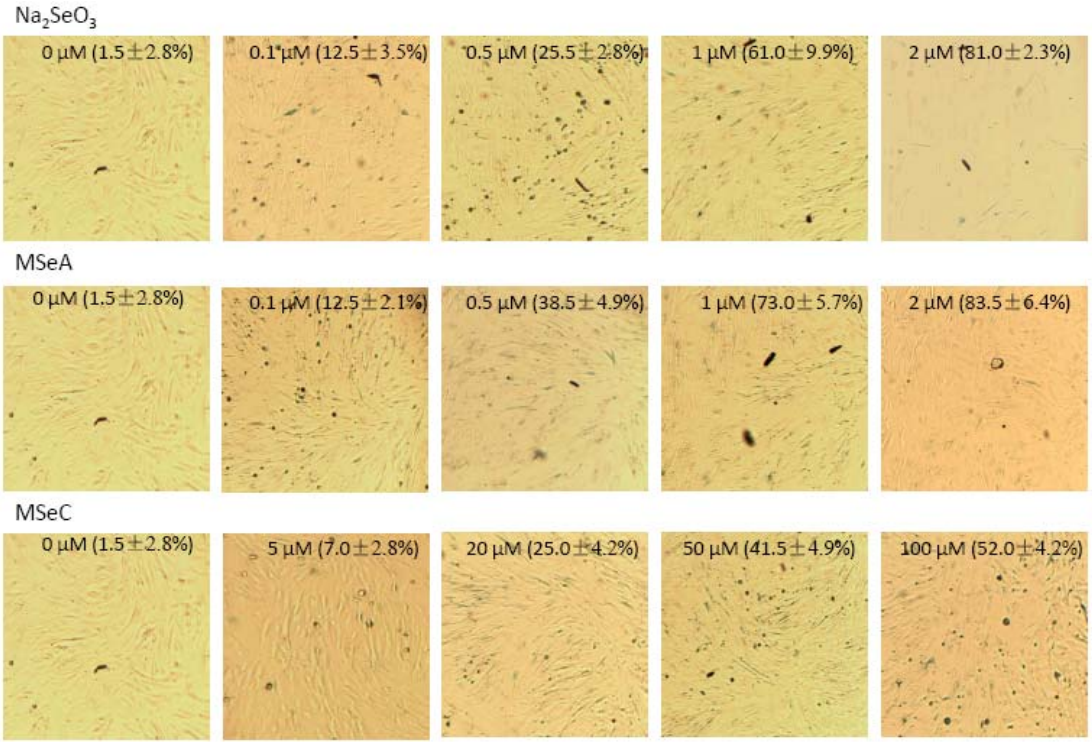


Figure 3.3 Senescence is induced in selenium-treated CCD 841 normal colon cells. Cells (5,000/well) were seeded onto 24-well plates treated with the selenium compounds for 48 h, followed by a 7days recovery. SA-β-gal was determined as described in Figure 3.1.

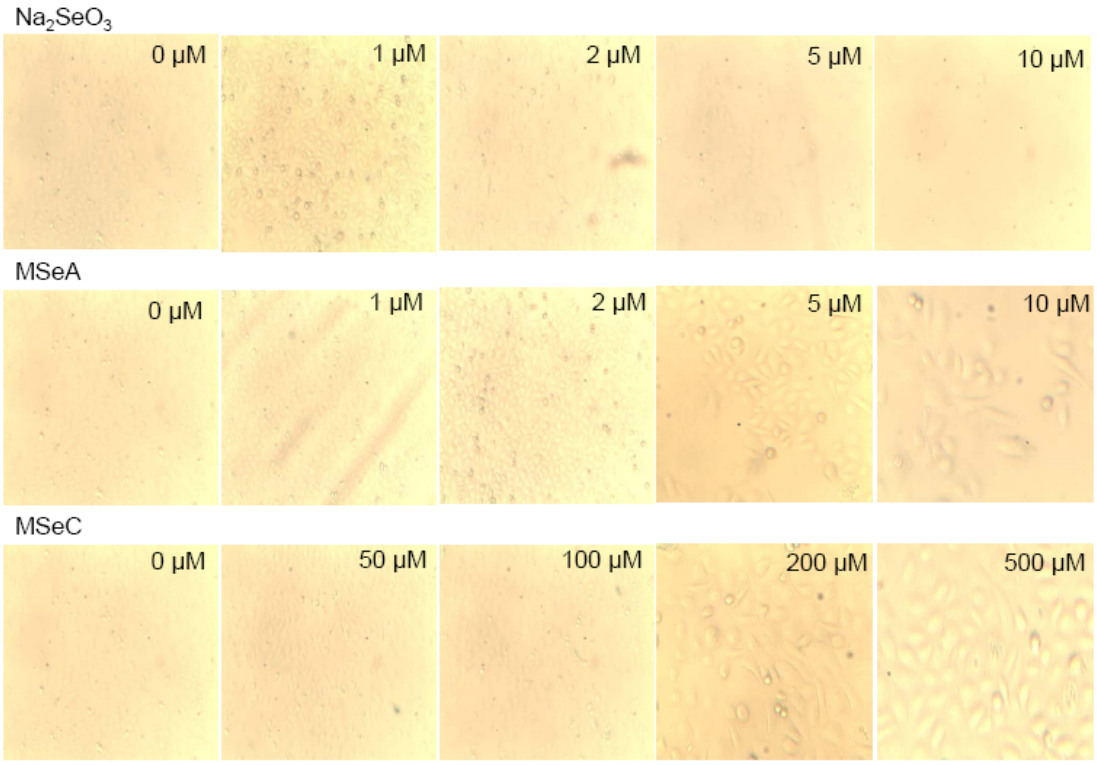


Figure 3.4 Senescence is not induced in selenium-treated PC-3 cells. Cells (5,000/well) were seeded onto 24-well plates treated with the selenium compounds for 48 h, followed by a 7 days recovery. SA-β-gal was determined as described in Figure 3.1.

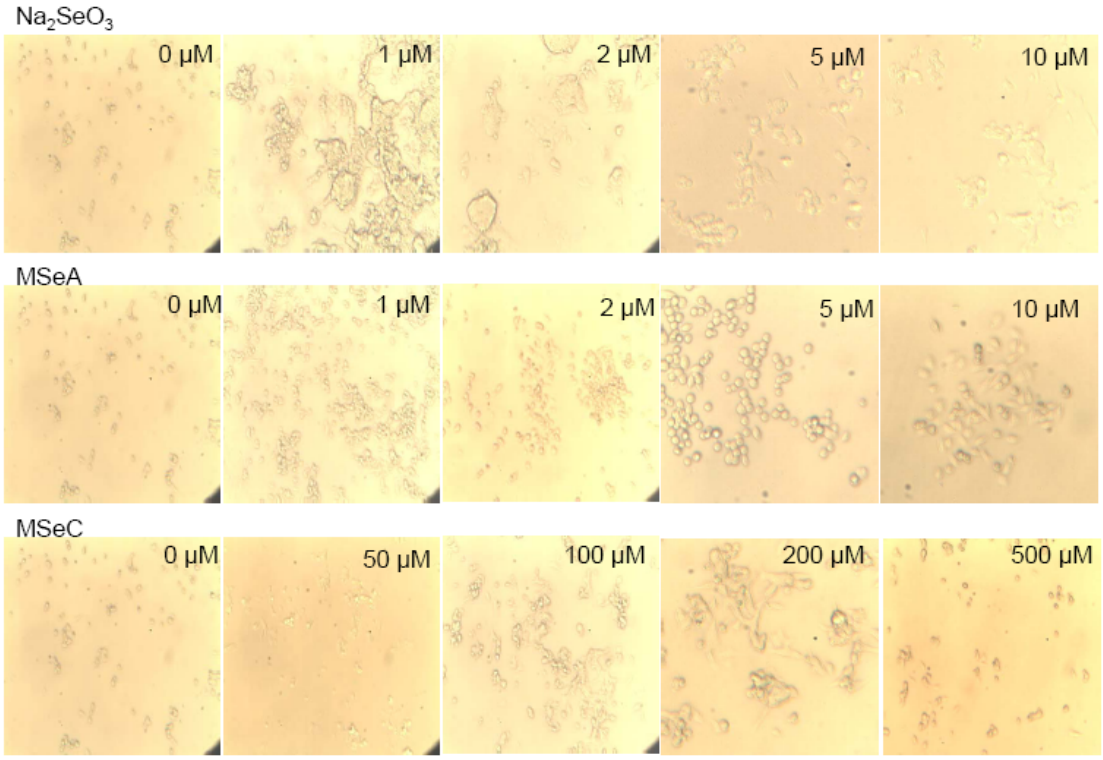


Figure 3.5 Senescence is not induced in selenium-treated HCT 116 cells. Cells (5,000/well) were seeded onto 24-well plates treated with the selenium compounds for 48 h, followed by a 7 days recovery. SA-β-gal was determined as described in Figure 3.1.

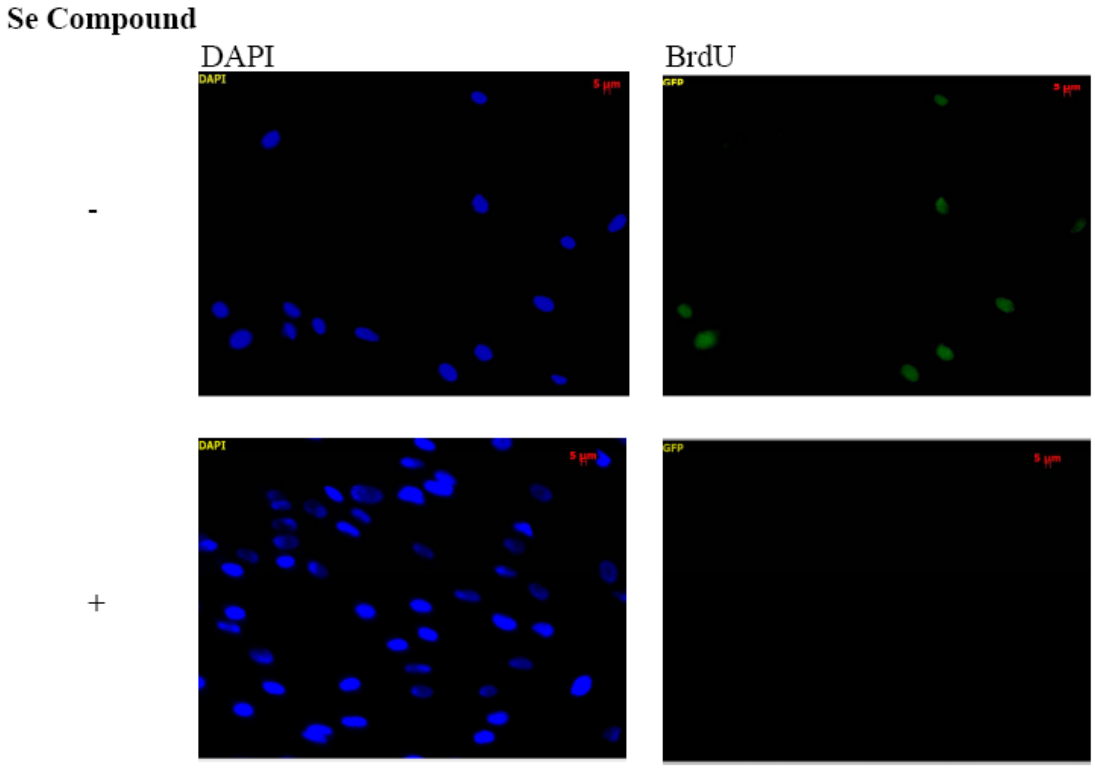


Figure 3.6 Representative pictures of BrdU assays. MRC-5 cells were seeded onto coverslips treated with the indicated selenium compounds and concentration for 48 h, followed by a 7 days recovery. Blue, DAPI; green, BrdU.

We next performed survival assays to determine the cellular sensitivity to the selenium compounds and estimate the individual LD_{50}. Results from the cell proliferation analysis showed that PC-3 and HCT 116 cancerous cells are more resistant than MRC-5 cells to treatment with Na_2SeO_3 (Figure 3.7A), MSeA (Figure 3.7B), and MSeC (Figure 3.7C) at day 7. When the two cancerous cells were treated with doses of selenium equivalent to their respective LD_{50} range, there was no SA-β-gal detected (Figures 3.1A-C and Figures 3.4 and 3.5). In contrast, treatment of MRC-5 cells with selenium at doses comparable to their LD_{50} resulted in significant SA-β-gal expression. Moreover, there was no increase in sub-G1 cell population 3 days after cellular exposure to the selenium compounds (Table 3.1), suggesting that an apoptotic cell death pathway is not activated. The results further support the observation that the selenium-induced senescence occurs specifically in non-cancerous cells; cancerous cells are deficient in selenium-induced senescence.

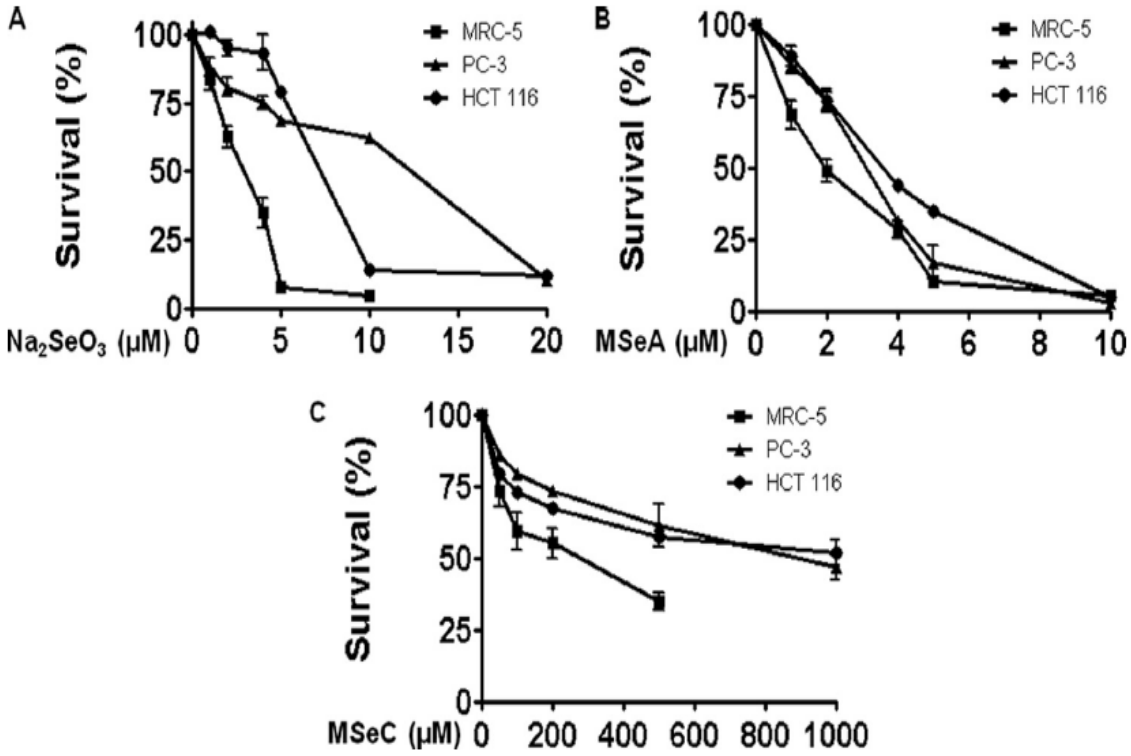


Figure 3.7 Sensitivity of MRC-5, PC-3, and HCT 116 cells to the selenium compounds. Cells were cultured in 6-well plates and treated with 1–20 μM Na_2SeO_3 (*A*), 1–10 μM MSeA (*B*), and 50–1000 μM MSeC (*C*) for 48 h, followed by a 7-day recovery (n =3). The number of cells was counted, and the number in the condition without selenium treatment was set as 100%.

Cell line	Treatment	Sub-G1 cells %
HCT 116	Control	3.0
	Day 3- 1 μM Na_2SeO_3	4.0±1.0
	Day 3- 1 μM MSeA	3.3±0.6
	Day 3- 1 μM MSeC	3.6±0.6
PC-3	Control	2.3±0.6
	Day 3- 1 μM Na_2SeO_3	1.7±0.6
	Day 3- 1 μM MSeA	2.7±0.6
	Day 3- 1 μM MSeC	3.7±1.2
CRL-1790	Control	7.3±1.5
	Day 3- 1 μM Na_2SeO_3	6.0±1.7
	Day 3- 1 μM MSeA	8.0±1.0
	Day 3- 1 μM MSeC	7.0±1.0
MRC-5	Control	9.0±2.0
	Day 3- 1 μM Na_2SeO_3	0
	Day 3- 1 μM MSeA	0
	Day 3- 1 μM MSeC	2.7±4.6

Table 3.1 Effect of selenium compounds on DNA fragmentation in HCT 116, PC-3, CCD 841 and MRC-5 cells. Cells were treated with 1 μM Na_2SeO_3, 1 μM MSeA, or 50 μM MSeC for 48 h and followed by a 3 days recovery. Cells were stained with propidium iodide for cell cycle analysis. The table represented the percentage of DNA fragments in the phase of G0/G1 phase as their respective mean ±S.D. values.

3.2 SELENIUM TREATMENT RESULTED IN ATM KINASE ACTIVATION AND γH2AX FORMATION IN NON-CANCEROUS CELLS

ATM activation and DNA damage response occur in senescent cells at early stage of tumorigenesis (Zhou *et al.* 2003; Bartkova *et al.* 2005; Gorgoulis *et al.* 2005; Bartkova *et al.* 2006). Since ATM autophosphorylation at Ser-1981 (pATM S1981) is known as a marker of ATM pathway activation in response to DNA breaks and other type of damage (Bakkenist and Kastan 2003), we tested whether selenium can induce pATM S1981 formation in MRC-5 cells by immunofluorescence studies. Treatment of MRC-5 cells with Na_2SeO_3 (0-2 μM, Figure 3.8A), MSeA (0-2 μM, Figure 3.8B), and MSeC (0-100 μM, Figure 3.8C) resulted in a significant increase in pATM S1981 focus-positive cells in a dose- and time-dependent manner. The ATM protein was localized in the nucleus at a comparable level in either the presence or absence of the selenium treatment (Figure 3.8G). Co-treating MRC-5 cells with the selenium compounds and the ATM kinase inhibitor, KU55933 (Cheng *et al.* 2008), suppressed the selenium-induced pATM S1981 focus formation (Figure 3.9), providing proof of evidence for the assay.

H2AX phosphorylation at Ser-139 (known as γH2AX) is a marker of DNA breaks and is known as a substrate of several kinases, including the PI3K family members ATM and DNA-PK_{cs} (Ward and Chen 2001; Zhou *et al.* 2003; Stiff *et al.* 2004). Thus, we determined whether the selenium compounds can induce γH2AX formation. Analysis of the immunofluorescent results indicated that treatment of MRC-5 cells with Na_2SeO_3 (1-2 μM, Figure 3.8D), MSeA (1-2 μM, Figure 3.8E), and MSeC (50-100 μM, Figure 3.8F) resulted in significant increases in the population of cells

expressing γH2AX foci, the extent of which is comparable between day 3 and day 7 post-treatment. To determine whether ATM kinase activity attributes to the increased γH2AX focus formation, we co-treated the MRC-5 cells with the selenium compounds and KU55933 (Cheng *et al.* 2008). Immunofluorescent analyses of γH2AX foci showed that KU55933 marginally suppresses γH2AX focus formation in the selenium-treated MRC-5 cells (Figures 3.8H-J and Figure 3.10), suggesting that ATM is not the major kinase attributable to the selenium-induced γH2AX focus formation. We next co-treated the MRC-5 cells with the selenium compounds and NU 7026, a kinase inhibitor of DNA-PK_{cs}. Compared to KU55933, treatment of the cells with NU 7026 resulted in a more robust suppression in the selenium -induced γH2AX focus formation (Figures 3.8H-J). Interestingly, the effect of KU55933 and NU 7026 on the inhibition of selenium-induced γH2AX focus formation appears to be additive. Thus, ATM is not the major kinase that involves in the selenium-induced γH2AX focus formation in MRC-5 cells.

Cancer cells are characterized by genomic instability and increased oxidative stress (Hartwell 1992; Duensing and Munger 2004; Valko *et al.* 2006; Fruehauf and Meyskens 2007). Thus, we assessed pATM S1981 and γH2AX expression in PC-3 cells, which is negative in selenium-induced senescence (Figures 3.1A-C and Figure 3.4). Compared to the non-cancerous MRC-5 cells, there were significantly greater PC-3 cell populations exhibiting intrinsic pATM S1981 (58.4 vs. 4.7%) and γH2AX (85.0 vs. 33.3%) foci (Figures 3.8A, 3.8D, 3.8K, 3.8L) in the absence of selenium treatment. Although seven days post-treatment of Na_2SeO_3 (1 μM), MSeA (1 μM),

and MSeC (50 μM) in PC-3 cells showed an increase in cells containing pATM S1981 foci (Figure 3.8K), the extent of induction is much smaller (43% vs. 14.6-fold) as compared to the non-cancerous MRC-5 cells (Figures 3.8A-C, 3.8K). Noticeably, the PC-3 cells exhibit high levels of intrinsic γH2AX foci, and treatment of the cells with selenium did not further increase γH2AX expression (Figure 3.8L). Thus, the PC-3 prostate cancer cells are predisposed to genomic instability, which may prevent the cells from responding to the selenium treatment for the activation of senescence and the ATM tumor-suppressing pathways.

A previous report indicates that senescent MRC-5 cells arrest in the G1 phase of the cell cycle 7-10 days after cellular exposure to H_2O_2 at a concentration of 500 mM (von Kobbe *et al.* 2004). Thus, we measured MRC-5 cell cycle profiles 1, 3 and 7 days post-treatment of the selenium compounds. We found that 1 and 3 days post-treatment of MRC-5 cells with MSeA and MSeC resulted in significant increases in cells in the S and G2/M population, followed by cell cycle arrest in the G1 phase at Day 7 in cells treated with any of the three Se compounds (Tables 3.2-3.4). The G1 cell cycle arrest in MRC-5 cells after selenium treatment is consistent with the observed senescence phenotype.

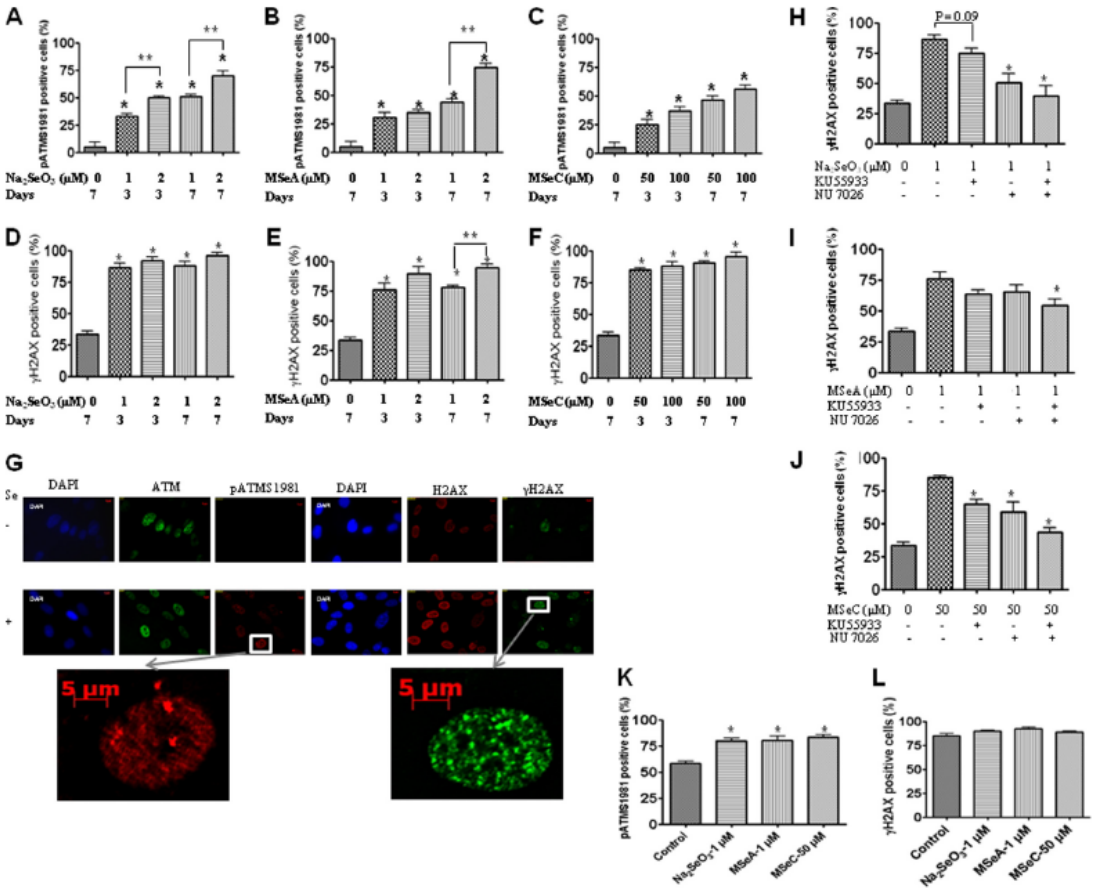


Figure 3.8 The phosphorylation of ATM at Ser-1981 (*pATMS1981*) and H2AX at Ser-139 (γH2AX) is induced in selenium-treated cells. MRC-5 cells were cultured on coverslips and treated with 1–2 μM Na_2SeO_3, 1–2 μM MSeA, and 50–100 μM MSeC, followed by a 3- or 7-day recovery. Ratios of cells expressing pATM Ser-1981 (*A–C*) or γH2AX foci (*D–F*) to cells expression total ATM or total H2AX are presented with their respective S.D. values. *, $p<0.01$ compared with the no selenium controls. **, $p< 0.05$. *G*, representative pictures showing immunofluorescent signals of DAPI (*blue*), total ATM or H2AX (*red*), and pATM Ser-1981 or γH2AX (*green*) in MRC-5 cells with or without selenium treatment. *H–J*, MRC-5 cells were treated with the selenium compounds, together with the ATM kinase inhibitor KU55933 (10 μM) and/or the DNA-PK_{cs} kinase inhibitor NU 7026 (50 μM). *, $p<0.01$ compared with the cells treated with only selenium. *K* and *L*, PC-3 cells were treated with the selenium compounds, followed by the immunofluorescent analysis. *, $p < 0.01$ compared with cells treated with selenium only.

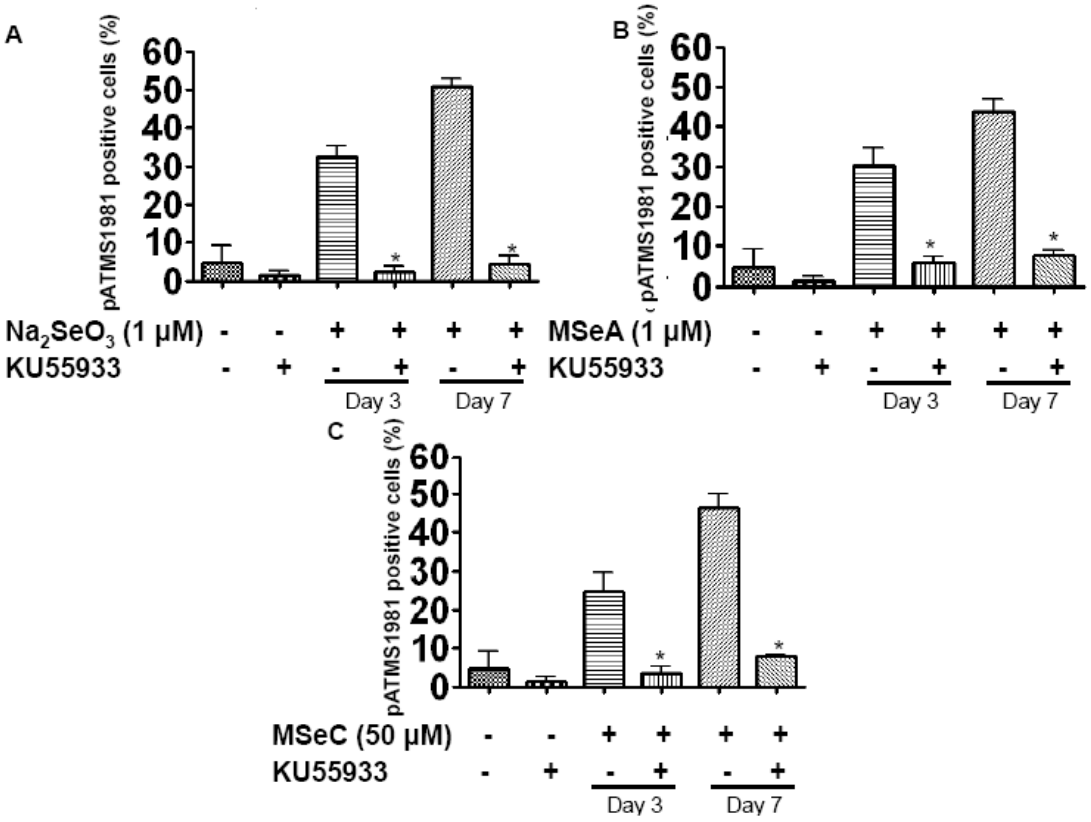


Figure 3.9 KU55933 inhibits formation of pATMS1981 in selenium-treated MRC-5 cells. The immunofluorescent analysis and quantification were described in Figure 9. *, $p < 0.05$ compared with the cells treated with selenium only at the respective day.

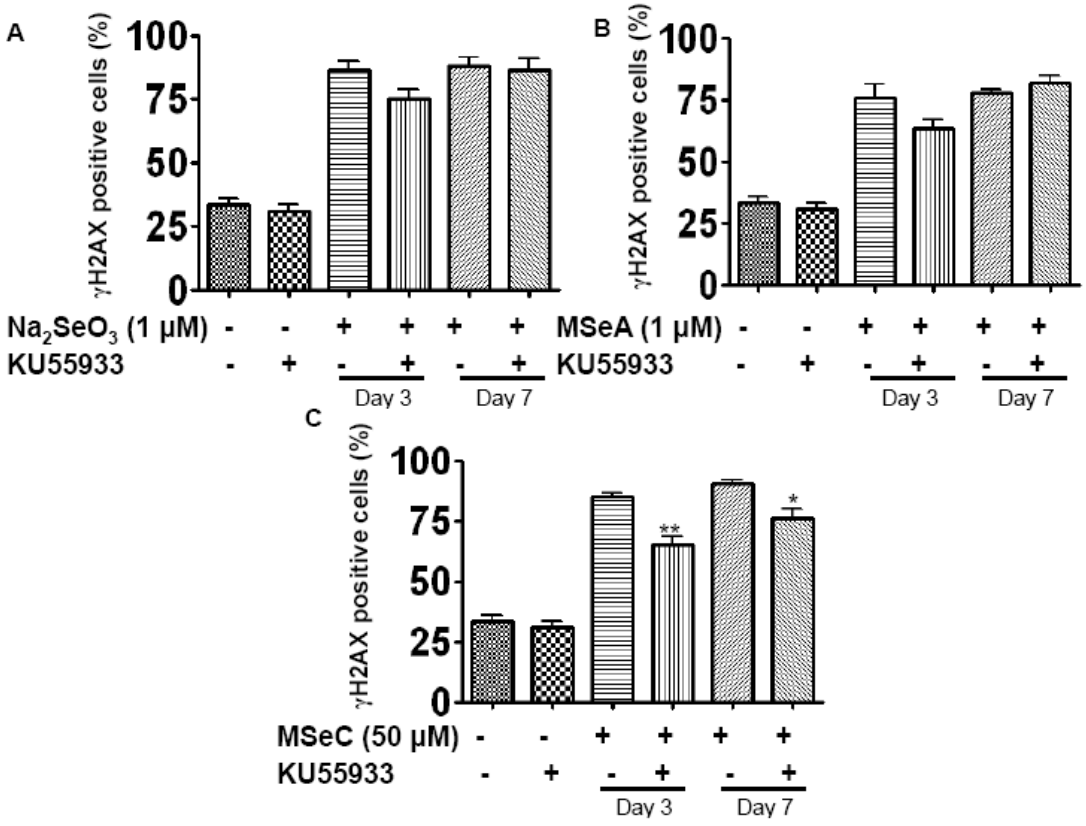
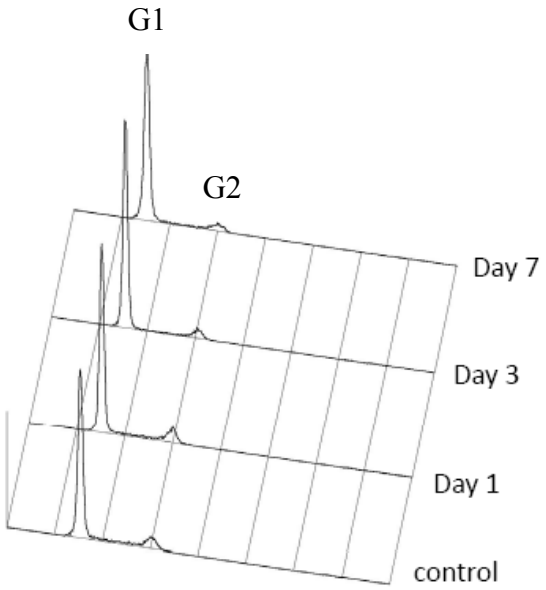
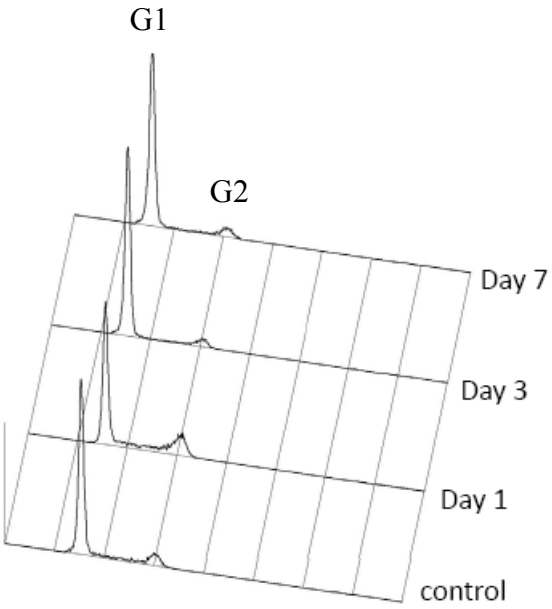


Figure 3.10 KU55933 inhibits formation of γH2AX in selenium-treated MRC-5 cells differentially. The immunofluorescent analysis and quantification were described in Figure 9. *, $p < 0.05$ or **, $p < 0.01$ compared with the cells treated with selenium only at the respective day.



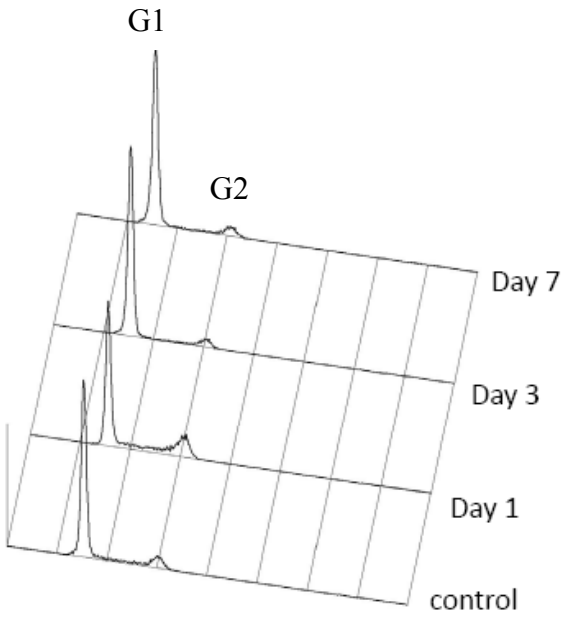
Na_2SeO_3 (1 μM)	G0/G1	S	G2/M
Control	78.4% (±0.4%)	12.5% (±0.8%)	9.1% (±0.4%)
Day 1	78.5% (±2.5%)	12.2% (±1.4%)	9.3% (±1.4%)
Day 3	87.2% (±0.3%)*	5.6% (±0.4%)*	7.3% (±0.4%)*
Day 7	85.4% (±1.0%)	7.4% (±0.4%)	7.3% (±0.7%)

Table 3.2 Effect of Na_2SeO_3 on cell cycle profiles in MRC-5 cells. Cells were treated with 1 μM Na_2SeO_3 for 48 h and followed by a 1-7 days recovery. Cells were stained with propidium iodide for cell cycle analysis. The overlay histograms represented the portions of MRC-5 cells in the phase of
the cell cycle. The tables represented the percentage of MRC-5 cells in the phase of the cell cycle as their respective mean±S.D. values analyzed by Student's t-test. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls.



MSeA (1 μM)	G0/G1	S	G2/M
Control	78.4% (±0.4%)	12.5% (±0.8%)	9.1% (±0.4%)
Day 1	72.0% (±0.4%)**	16.2% (±0.5%)**	11.8% (±0.2%)**
Day 3	83.3% (±0.8%)	8.5% (±0.6%)	8.3% (±0.2%)**
Day 7	83.2% (±1.2%)	7.9% (±0.7%)	8.8% (±0.4%)**

Table 1.3 Effect of MSeA on cell cycle profiles in MRC-5 cells. Cells were treated with 1 μM MSeA for 48 h and followed by a 1-7 days recovery. Cells were stained with propidium iodide for cell cycle analysis. The overlay histograms represented the portions of MRC-5 cells in the phase of the cell cycle. The tables represented the percentage of MRC-5 cells in the phase of the cell cycle as their respective mean±S.D. values analyzed by Student's t-test. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls.



MSeC (50 µM)	G0/G1	S	G2/M
Control	78.4% (±0.4%)	12.5% (±0.8%)	9.1% (±0.4%)
Day 1	61.6% (±1.3%)**	22.9% (±1.5%)**	15.3% (±0.4%)**
Day 3	86.4% (±0.6%)	6.3% (±0.6%)	7.3% (±1.2%)
Day 7	84.7% (±0.4%)	6.6% (±0.7%)	8.6% (±0.3%)**

Table 3.4 Effect of MSeC on cell cycle profiles in MRC-5 cells. Cells were treated with 50 µM MSeC for 48 h and followed by a 1-7 days recovery. Cells were stained with propidium iodide for cell cycle analysis. The overlay histograms represented the portions of MRC-5 cells in the phase of the cell cycle. The tables represented the percentage of MRC-5 cells in the phase of the cell cycle as their respective mean±S.D. values analyzed by Student's t-test. *, $p < 0.05$; **, $p < 0.01$ compared with their respective controls.

3.3 THE SELENIUM-INDUCED SENESCENCE REQUIRES THE ATM KINASE ACTIVITY

To determine whether ATM kinase activity is required for the Se-induced senescence, we pre-incubated the MRC-5 cells with KU55933. Analyses of SA-β-gal results demonstrated that inhibition of ATM kinase activity prevented senescence induction in MRC-5 cells exposed to Na_2SeO_3 (1 μM), MSeA (1 μM), or MSeC (50 μM) (Figure 3.11). The ATM kinase inhibitor also prevented senescence induction in MRC-5 cells exposed to lower doses of the selenium compounds (data not shown). The results indicated that ATM kinase activity is required for the induction of senescence by the selenium compounds in MRC-5 cells.

3.4 REGULATION OF THE SELENIUM-INDUCED DNA DAMAGE RESPOINSE AND SENESCENCE BY OXIDATIVE STRESS

It has been shown previously that MSeA and Na_2SeO_3 can induce ROS formation in a number of prostate cancer cells (Li *et al.* 2007). Thus, we determined the involvement of ROS in the selenium-induced DNA damage response and senescence. Treatment of MRC-5 cells with antioxidants, NAC (a H_2O_2 scavenger) and Tempo (a superoxide dismutase mimic), significantly suppressed senescence in MRC-5 cells treated with the selenium compounds (Figure 3.11). Interestingly, Tempo is more potent than NAC in the attenuation of senescence induced by Na_2SeO_3 and MSeA. ROS also involve in the selenium-induced DNA damage response, as the pATM S1981 focus formation (Figures 3.12A-C) and γH2AX formation (Figures 3.12D-F) are attenuated

by NAC treatment. Thus, selenium induces senescence and pATM S1981 focus formation in MRC-5 cells in a ROS-dependent manner.

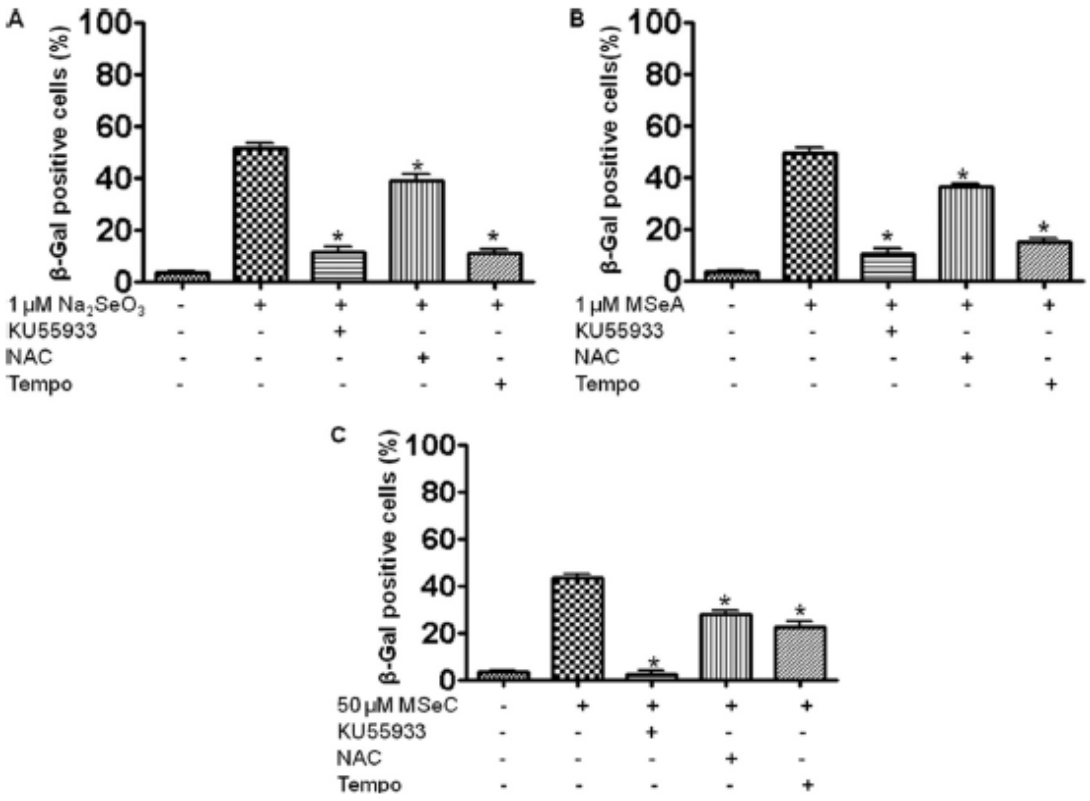


Figure 3.11 The kinase activity of ATM and ROS are involved in selenium-induced senescence in MRC-5 cells. MRC-5 cells were treated with the selenium compounds alone or together with KU55933 (10 μM), NAC (10 mM), or Tempo (1 mM). SA-β-galactosidase was measured as described in the legend to Figure 2. *, p <0.01compared with cells treated with selenium only.

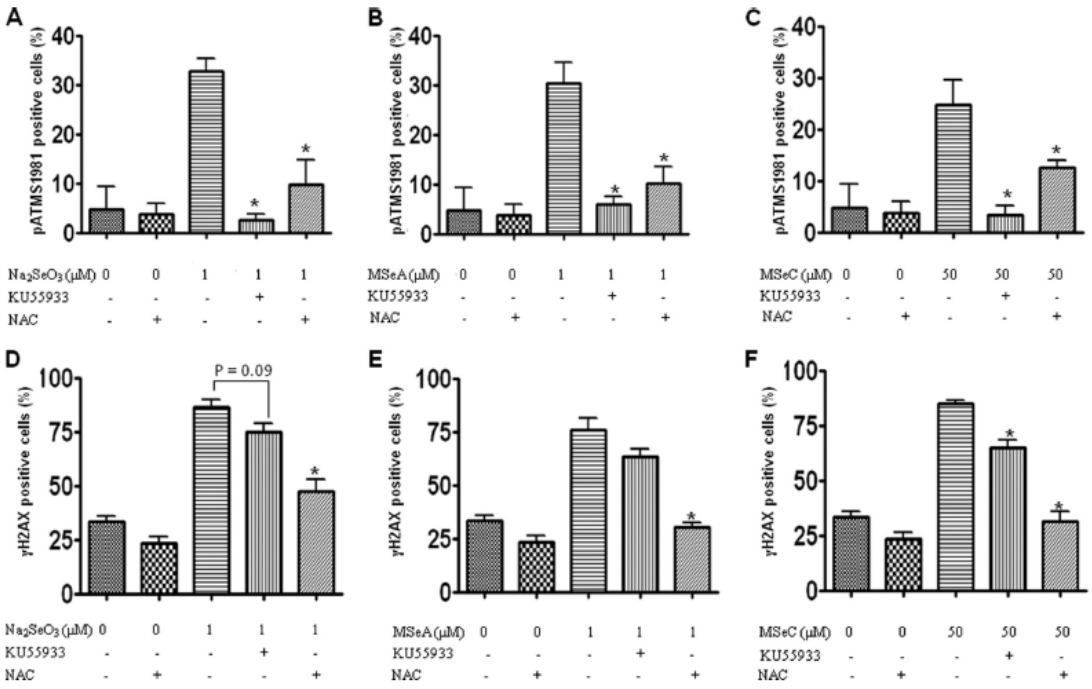


Figure 3.12 NACattenuates the formation ofpATMSer-1981 (*pATMS1981*) and γH2AXin selenium-treated MRC-5 cells. MRC-5 cells were treated with the selenium compounds alone or together with KU55933 (10 μM) or NAC (10 mM) for immunofluorescent analyses of pATM S1981 (*A–C*) and γH2AX (*D–F*) as described in the legend to Figure 9. *, $p < 0.05$ compared with cells treated with selenium only.

3.5 DISCUSSION

The evidence for selenium being a chemoprevention agent includes that from geographic, animal, and epidemiological studies (Shamberg.Rj and Frost 1969; Clark *et al.* 1996; Waters *et al.* 2005; Lippman *et al.* 2009). However, recent human clinical trials reported mixed results on the role of selenium in chemoprevention, which may potentially be explained by the differences in body selenium status of the subjects entering the trials, as well as the selenium dose and formulation between the studies (Clark *et al.* 1996; Reid *et al.* 2002; Zhuo *et al.* 2004; Hurst and Fairweather-Tait 2009; Lippman *et al.* 2009; Rayman *et al.* 2009). Whatever the reason, the molecular mechanism by which selenium mitigates tumorigenesis is largely unknown. Increasing body of recent evidence has suggested that selenium and its metabolites induce apoptotic pathways in cancer cells (Zhou *et al.* 2003; Zhou *et al.* 2003; Zhao *et al.* 2006). Nonetheless, selenium could in principle exert its chemoprevention function at the onset of tumorigenesis by other mechanisms. In line with this notion, recent reports indicate that DNA damage response and senescence are barriers of carcinogenesis that function at the interface between the precancerous and the cancerous stages (Bartkova *et al.* 2005; Gorgoulis *et al.* 2005; Bartkova *et al.* 2006). The ATM kinase plays a pivotal role in the DNA damage-induced senescence (di Fagagna *et al.* 2004; Heiss *et al.* 2007). Our findings suggest a new role of selenium in tumorigenesis: selenium induces an ATM-dependent senescence response in a manner dependent on ROS in non-cancerous, but not in cancerous cells.

How do sub-lethal ($\leq LD_{50}$) doses of selenium compounds mount the ATM-dependent senescence response? One possibility is to induce ROS generation that impacts on genome stability. Ample evidence points to that metabolites of selenium compounds at high doses can induce ROS formation and apoptosis in cancer cells (Stewart *et al.* 1999; Hu *et al.* 2006; Zhao *et al.* 2006; Li *et al.* 2007). Our findings identify an ATM-dependent senescence response induced by doses of selenium well below LD_{50} in non-cancerous cells whereby this pathway is suppressed by antioxidant administration. Early experiments showed that doxorubicin-induced ROS formation or H_2O_2 treatment can activate the ATM pathway (Shiloh 2003; Kurz *et al.* 2004), and that astrocytes and haematopoietic stem cells isolated from *Atm*$^{-/-}$ mice exhibit increased oxidative stress, early onset of senescence, and/or suppressed self-renewal capacity (Ito *et al.* 2004; Liu *et al.* 2005). Although ATM is a prominent responder to DNA double strand breaks, this kinase can be activated by various forms of chromosome alterations (Bakkenist and Kastan 2003). Importantly, our results suggest that the kinase activity of ATM mediates the senescence phenotype in the selenium-induced MRC-5 cells. Because inhibition of ATM kinase attenuates, but not prevent, selenium-induced γH2AX formation (Figures 3.8 and 3.12), other kinases are capable of phosphorylating H2AX. Altogether, we propose that selenium-induced oxidative stress activates the ATM pathway for the subsequent senescent response. Future studies are needed to elucidate the mechanism of ATM kinase activation by selenium-induced oxidative stress.

Of note, ROS is unlikely to be the only direct activator of the senescence and ATM pathways, as we do not observe a robust G1 cell cycle arrest as reported previously in

senescent MRC-5 cells treated with H_2O_2 (von Kobbe *et al.* 2004). Rather, treatment of the cells with MSeA and MSeC at a dose ~LD_{50} resulted in a minor S and G2/M arrest prior to senescence induction. It is conceivable that the ROS-induced DNA oxidation and the subsequent formation of DNA breaks in S phase may activate the ATM pathway for a checkpoint response at the early time point. ATM is known to function in DNA damage checkpoint at G1, S, and G2/M, and is activated by replication fork collapse (Shiloh 2003; Cheng *et al.* 2008). Following ATM activation (Day 1), the cells accumulate in the G1 phase (Day 3) and eventually senesce (Day 7).

There are several forms of senescence. Replicative senescence is mainly caused by telomere attrition, while premature senescence can be triggered by damaged DNA and oxidative stress. ATM is involved in both forms of senescence (Parrinello *et al.* 2003; di Fagagna *et al.* 2004; Herbig *et al.* 2004; Heiss *et al.* 2007). The p53 protein is mutated in the majority of human malignant tumors, and is required for induction of senescence by DNA replication stress (topoisomerase inhibitors) in an array of cancer cell lines (te Poele *et al.* 2002). Interestingly, we found that the selenium-induced senescence is missing in both the p53-proficient HCT 116 and the p53-deficient PC-3 cancer cells, suggesting that the lack of selenium-induced senescence in the two cancerous cell lines is not attributable to p53 status. Another candidate target of selenium action on senescence is p21, which is a gatekeeper of the G1-S transition and is implicated in a ROS-dependent senescence response in normal human fibroblasts (Brown *et al.* 1997; Macip *et al.* 2002). In HCT 116 cells exposed to doxorubicin treatment, p21 expression is induced in both a p53-dependent and –

independent manner (Ravizza *et al.* 2004). Further studies are therefore necessary to fully understand whether and how p53 and p21 regulate the ATM- and ROS-dependent senescence response after selenium exposure.

DNA replication stress and checkpoints are associated with oncogene-induced senescence (Bartkova *et al.* 2005; Gorgoulis *et al.* 2005; Bartkova *et al.* 2006). Genotoxic stress can induce persistent DNA damage, thus triggering senescence-associated secretory phenotype (SASP) and suppressing p53 in normal cells (Coppe *et al.* 2008). SASP can change the tissue microenvironment in a p53-independent fashion and cells with SASP can secrete various factors such as interleukin-6 (IL-6) (Rodier *et al.* 2009). ATM is required for IL-6 secretion that facilitates cell communications and bypasses senescence in damaged cells (Rodier *et al.* 2009). Our MRC-5 cells developed a senescence phenotype 7 days post-treatment of selenium, suggesting that persistent DNA damage and oxidative stress exist. We propose that SASP may develop and lead to senescence by changing their microenvironment. Our data indicate that the selenium-induced senescence is not associated with a robust S-phase checkpoint, as opposed to conditions such as significant DNA double strand breaks that induce S-phase checkpoint.

In conclusion, we have provided the first evidence that selenium can mitigate tumorigenesis by mechanisms other than the well studied apoptotic pathway. The observation that selenium specifically induces senescence response in non-cancerous cells suggests a cost-effective scenario by which tumorigenesis can be stifled at the very beginning in individuals who consume selenium with a cancer prevention

perspective. It is of future interests to elucidate the mechanism by which selenium activates early barriers of tumorigenesis, especially at the interphase between the precancerous and cancerous stages by using models for initiation, promotion, and progression in carcinogenesis.

CHAPTER 4: THE ROLE OF p53 IN SELENIUM-INDUCED SENESCENCE

p53 is a tumor suppressor protein encoded by gene *TP53*. In the majority of cancer cells, this protein is found mutated or the gene *TP53* is deleted. Mutation of p53 is indicative of highly aggressive cancers. Even in the cancer cells bearing wild-type p53, there are defects in the p53 pathway. The most common mutations are found in the p53 DNA-binding domain encoding amino acids from 102 to 292 (Hollstein *et al.* 1991), thus affecting its DNA binding specificity, the transactivation function and growth suppression. Interestingly, the loss of function due to the point mutation in p53 can sometimes be rescued by mutations at other sites (Wright *et al.* 2002). Regarding its vital role in preventing carcinogenesis, several studies have been constructed to explore the strategies to restore its normal conformation and functions. For example, a small molecule SCH529074 could reactivate mutant p53 by acting as a chaperone and also by inhibiting ubiquitination of p53 by HDM2 (Demma *et al.* 2010).

Selenium has shown much promise in preventing prostate cancer and other cancers, and the effect can be dependent on its forms. p53 is involved in preventing transformation by transcriptional regulation of a range of genes that are involved in vital cell functions such as DNA repair, cell cycle arrest, and induction of apoptosis. It is of great interest and urgency to understand the mechanisms underlying selenium anti-cancer action and if any, its linkage with p53 pathway. In fact, selenium can affect p53 activity through distinctive mechanisms for DNA repair, apoptosis together with DNA damage. Na_2SeO_3 may affect the apoptotic function of p53 by

phosphorylating its serines, and MSeA targets the threonine residues in p53, while selenomethionine has no phosphorylation effect of p53 serines or threonines (Smith *et al.* 2004). Blocking p53 in LNCap prostate cancer cells, the p53-dependent cascade and apoptosis could not be triggered by Na_2SeO_3, so Na_2SeO_3 may regulate the transactivation of p53 (Sarveswaran *et al.* 2010).

In my previous research, we found that selenium compounds can activate senescence in non-cancerous cells but not in cancerous cells. Selenium compounds could induce ROS generation, maybe predominantly as superoxide. The increased redox stress activates the ATM-dependent DNA damage response pathway and DNA damage. The irreversible cell cycle arrest in non-cancerous cells eventually contributes to the senescent phenotype. Here, we further investigate the mechanisms underlying selenium-induced senescence. We seek the answer to the question whether p53 pathway is essential for selenium-induced senescence. We also address the consequences for loss of p53 on manifestation of senescence, genomic stability, cell sensitivity towards selenium and DNA damage checkpoints.

4.1 SELENIUM-INDUCED SENESCENCE WAS SUPPRESSED IN p53 shRNA MRC-5 CELLS

Our previous research has shown that selenium-induced senescence requires ATM activation (Wu *et al.* 2010). Since p53 is an important phosphorylation substrate for ATM kinase (Banin *et al.* 1998), we tested whether or not p53 is involved in selenium-induced senescence. We applied lentivirus containing p53 scramble and shRNA constructs to suppress the p53 expression in MRC-5 cells, HCT 116

cancerous cells, and HCT 116 cells with hMLH1 complementation (HCT 116+ hMLH1). After infection, several derivative clones resistant to puromycin were isolated and cultured in the presence of puromycin. The p53 knockdown efficiency of the puromycin-resistant, stable clones were further examined by RT-PCR. The E3 clones showed more than 80% reduction of the p53 mRNA level in MRC-5 cells (Figure 4.1A). This p53 shRNA construct also showed similar inhibition effect in HCT 116 and HCT 116+ hMLH1 cells (Figure 4.1B and 4.1C). In particular, E3 1-3 and E3 2-5, which showed around 90% p53 knockdown efficiency were used for all subsequent experiments.

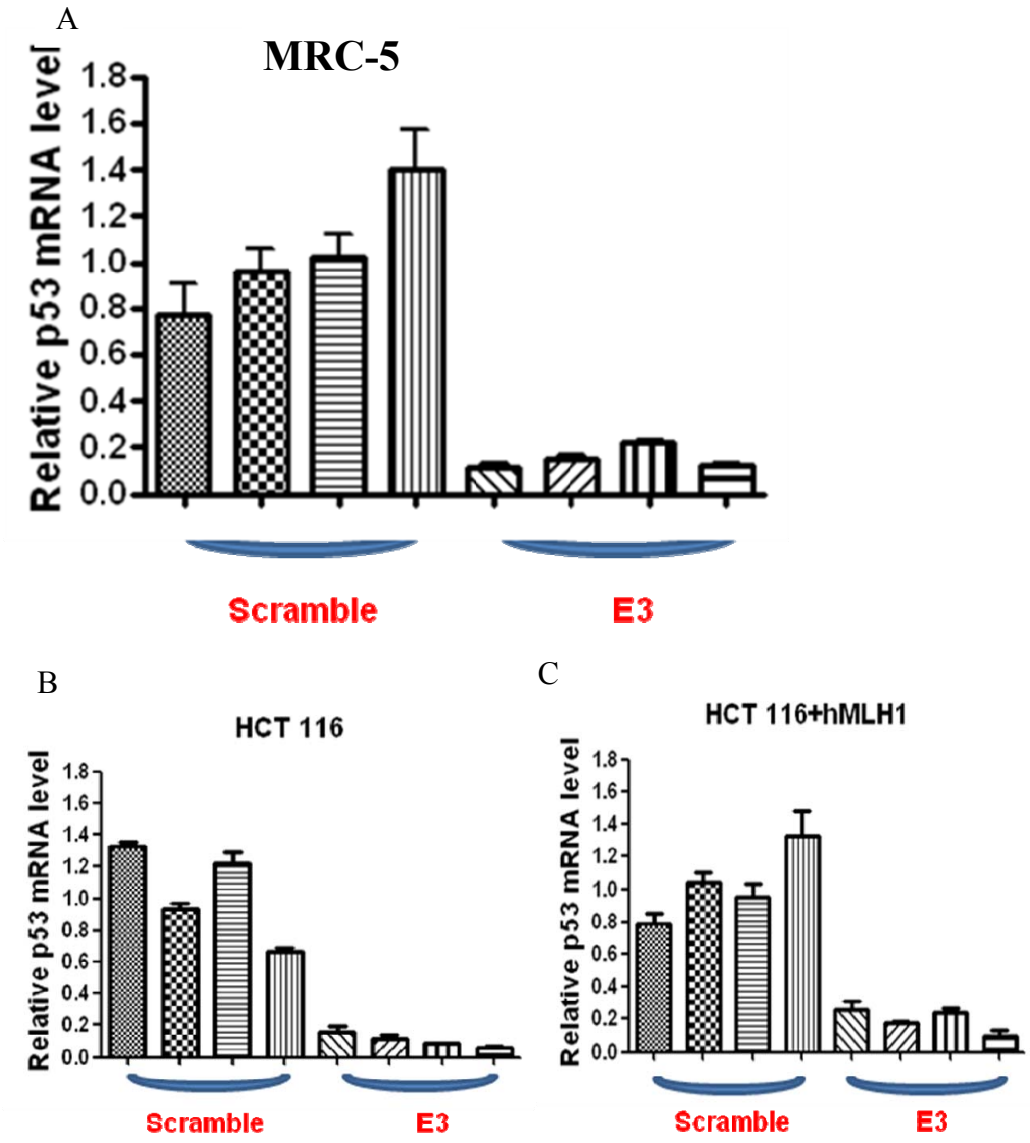
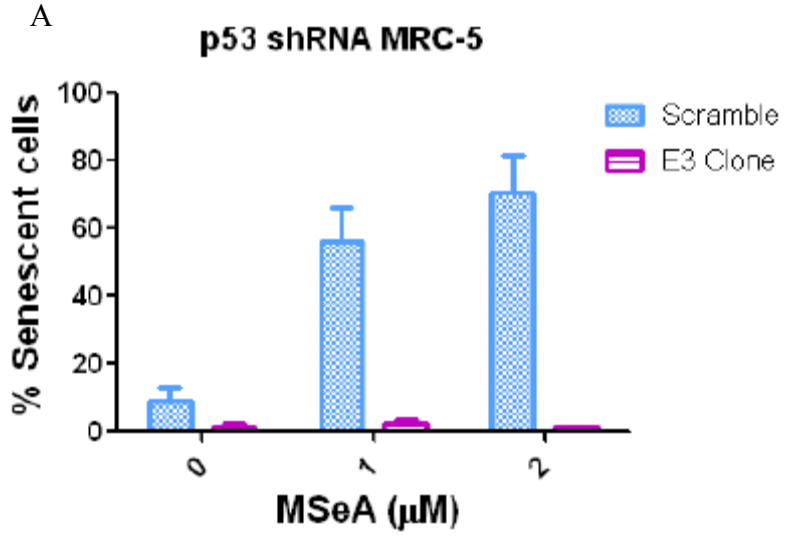
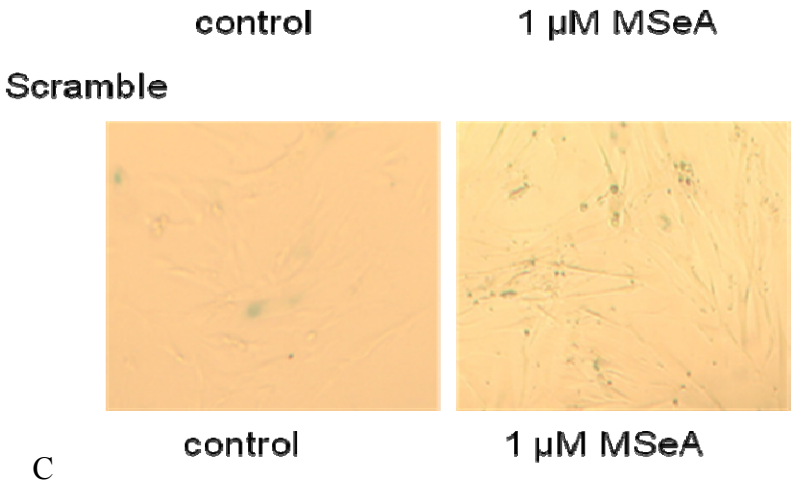


Figure 4.1 p53 knockdown efficiency. MRC-5, HCT 116 and HCT 116+hMLH1 cells were infected with p53 shRNA at a ratio of 100:1. After selecting successfully infected cells in puromycin (5 µg/mL for MRC-5 cells, and 8 µg/mL for two HCT 116 cell lines), twelve clones infected with either type of shRNA for each cell line were randomly picked for further culturing. Among them, four clones for each cell line were randomly picked for mRNA level detection using real-time PCR.

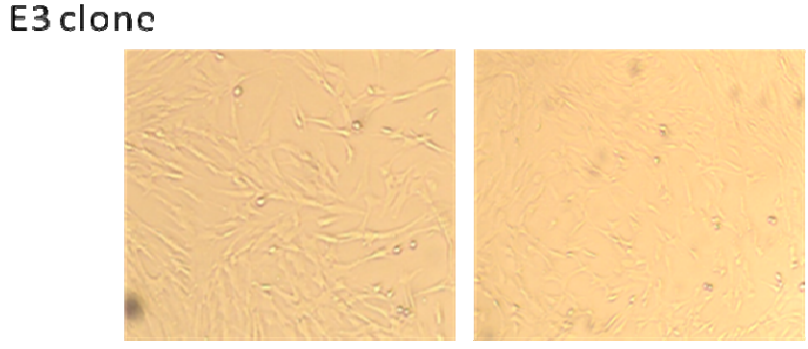
A


p53 shRNA MRC-5
% Senescent cells
100
80
60
40
20
0
Scramble
E3 Clone
0
1
2
MSeA (µM)

B


control
1 µM MSeA
Scramble
control
1 µM MSeA

C


E3 clone

We next assessed the senescence phenotype after cellular exposure to the selenium compounds. p53 shRNA and scramble MRC-5, HCT 116 and HCT 116+ hMLH1 cells were treated with MSeA (1-10 μM) for two days, followed by a 7 days recovery in normal medium in the absence of the selenium treatment. The cells were then subjected to the detection of SA-β-gal. Scramble MRC-5 cells showed a dose-dependent senescence response after MSeA exposure (Figure 4.2A and B). In contrast, the selenium treatment did not induce the expression of SA-β-gal in the p53 shRNA MRC-5 cells (Figures 4.2A and C). p53 shRNA and scramble HCT 116 and HCT 116+hMLH1 cells did not exhibit senescent phenotype. Thus, suppression of p53 might disrupt DNA replication checkpoint response in MRC-5 cells treated with the selenium compounds. Taken together, the results suggest that senescence induced by selenium compounds in the non-cancerous requires functional p53.

4.2 LOSS OF p53 INCREASED GENOMIC INSTABILITY

Since the majority of cancer incidence invovles p53 mutation or deletion of the *TP53* gene, we tested whether loss of p53 contributes to genomic stability by immunofluorescence analysis of ATM phosphorylation and γH2AX focus formation in MRC-5 cells. Treatment of the cells with 2 μM MSeA (Figure 4.3A) resulted in a significant increase of pATM S1981 focus formation, the extent of which is greater in p53 shRNA than in scramble MRC-5 cells. The ATM protein was localized in the nucleus at a comparable level in the presence or absence of MSeA (Figure 4.3B). Noticeably, p53 shRNA MRC-5 cells exhibited greater pATMS1981 foci as compared to scramble MRC-5 cells in the absence of selenium treatments.

Analysis of the immunofluorescent results showed that 2 μM MSeA treatment resulted in significant increase in the population of cells expressing γH2AX foci in scramble cells (Figure 4.4). p53 shRNA MRC-5 cells showed extremely high levels of intrinsic γH2AX foci, and treatment of the cells did not increase γH2AX expression. This feature of pATMS1981 and γH2AX expression in MRC-5 cells resembles those in PC-3 prostate cancer cells (Figure 3.8K and L), which are predisposed to genomic instability. Therefore, loss of p53 would contribute to genomic instability.

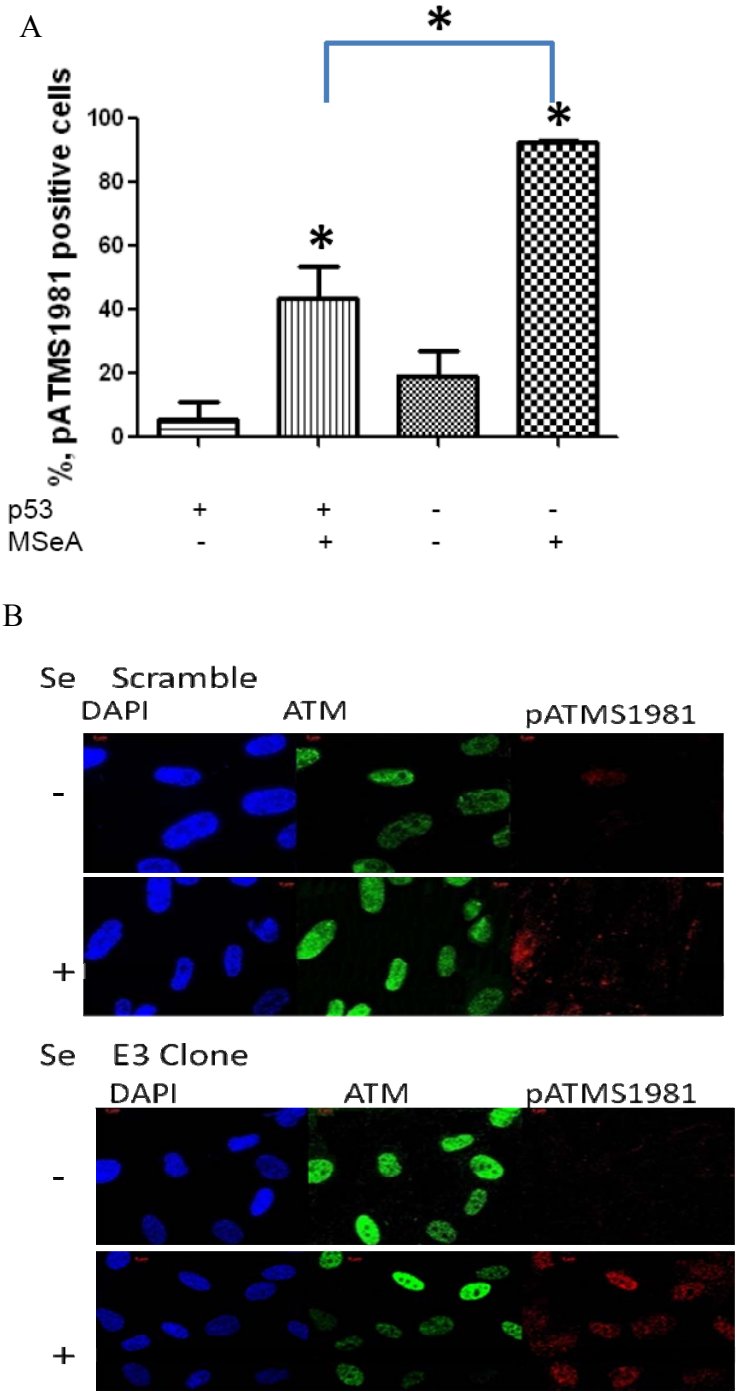


Figure 4.3 The phosphorylation of ATM at Ser-1981 (pATMS1981) is induced in MSeA-treated cells. MRC-5 cells were cultured on coverslips and treated with 2 μM MSeA followed by a 3 days or 7 days recovery. All experiments were run in duplication and performed a minimum of three times. Five pictures were randomly taken in each of the slides, and ratios of cells expressing pATMS1981 (A) to cells expression total ATM are presented with their respective S.D. values. *, $p < 0.05$ compared with the no MSeA controls.

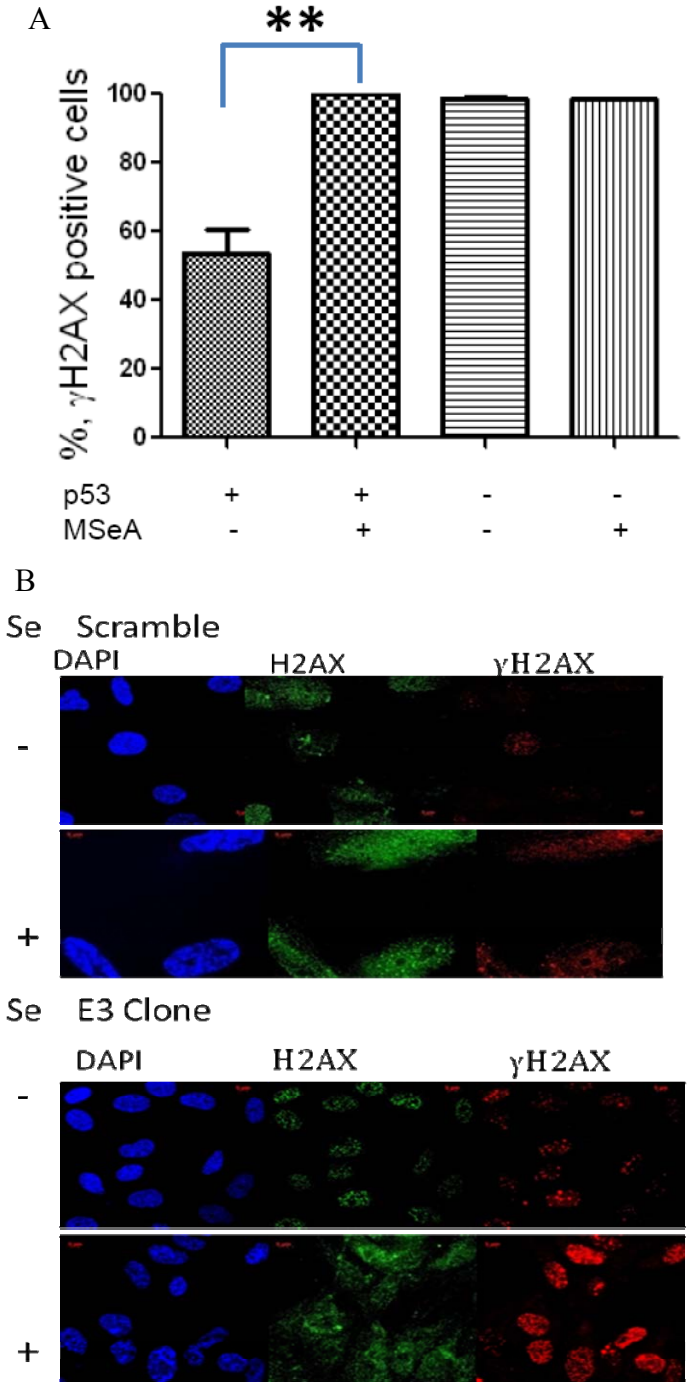


Figure 4.4 The phosphorylation of H2AX at Ser-139 (γH2AX) is induced in MSeA-treated cells. MRC-5 cells were cultured on coverslips and treated with 2 μM MSeA followed by a 3 days or 7 days recovery. All experiments were run in duplication and performed a minimum of three times. Five pictures were randomly taken in each of the slides, and ratios of cells expressing γH2AX foci (A) to cells expression total H2AX are presented with their respective S.D. values. **, $p < 0.01$ compared with the no MSeA controls.

4.3 LOSS OF p53 RENDERED MRC-5 CELLS MORE RESISTANT TO SELENIUM TREATMENT

The survival assays were applied to determine the cellular sensitivity to the MSeA treatment and to estimate the individual LD_{50}. Results from the cell proliferation analysis showed that p53 shRNA MRC-5 cells were more resistant than control shRNA MRC-5 cells to MSeA treatment (Figure 4.5A) at day 7. When p53 shRNA MRC-5 cells were treated with MSeA at 2 μM that killed 50% of the control shRNA MRC-5 cells, there were over 80% of the cells survived (Figures 4.5A). Similarly, when control shRNA MRC-5 cells were pre-treated with 5 μM KU55933, the cells became more resistant to MSeA treatment. These results showed that the ATM kinase and p53 are involved in selenium-induced senescence. In contrast, knockdown of p53 in HCT 116 or HCT 116+hMLH1 cells did not change cellular sensitivity MSeA (Figures 4.5B and C).

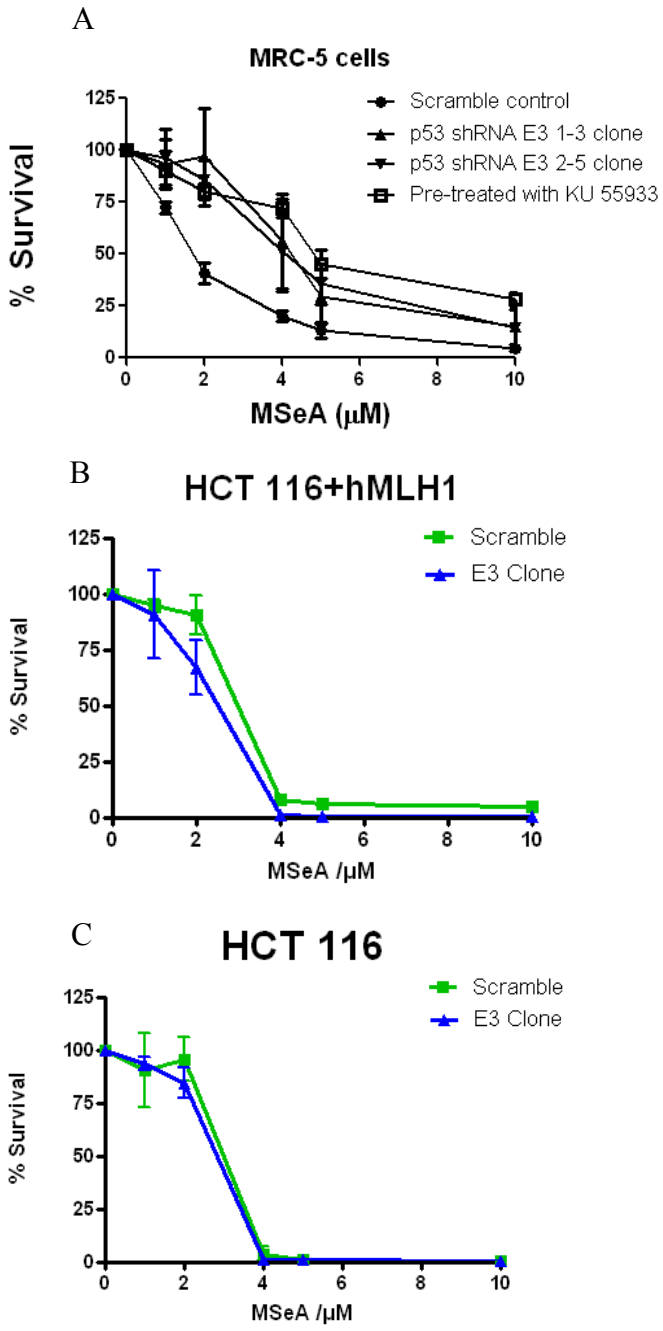


Figure 4.5 Sensitivity of MRC-5, HCT116 and HCT 116 complemented with hMLH1with and without p53 to MSeA exposure. A. For the pre-treatment, cells were incubated with 5 µM KU55933 for one day. Cells were cultured in 6-well plates, treated with 1-10 µM MSeA for 48 h, followed by a for 7 days recovery (n = 3). The number of cells in the untreated conditions was set as 100%. B-C. Cells were seeded in 24-well plates, treated with 1-10 µM MSeA for 48 h, followed by a for 7 days recovery (n = 3). The survival value was determined from the MTT assay. The number of cells in the untreated conditions was set as 100%.

4.4 SELENIUM TREATMENT RESULTED IN CELL CYCLE ARREST IN CONTROL AND p53 shRNA MRC-5 CELLS AT DIFFERENT PHASES

p53 has been well understood as a key tumor suppresser arresting cells at G1 phase (Shieh *et al.* 1997; Canman *et al.* 1998; Zeng *et al.* 2009) and G2/M phase (Agarwal *et al.* 1995; Taylor and Stark 2001). Here, we determined the role of p53 in cell cycle status 1 day after MSeA treatment (2 μM) or 1-7 days after recovery in scramble MRC-5 cells and p53 shRNA MRC-5 cells. Flow cytometric analyses showed increased G2/M population in scramble MRC-5 cells (Figures 4.6A) and G0/G1 population in p53 shRNA MRC-5 cells at 24h (Figure 4.6B). One day after recovery, both control and p53 shRNA MRC-5 cells showed an increase S phase population. The increase in S phase population after MSeA treatment was consistent with the results in normal MRC-5 cells (Table 3.3) and scramble MRC-5 cells (Figure 4.6A). Seven days after recovery, p53 shRNA MRC-5 cells but not scramble shRNA MRC-5 cells, almost resumed the cell cycle population as of the untreated cells. The above studies implicate p53 in G2/M cell cycle arrest in selenium-treated MRC-5 cells.

Because G2/M cell cycle arrest in HCT 116 cells is independent of p53 (Jo *et al.* 2008), we analyzed the cell cycle profile in scrambled and p53 shRNA HCT 116 and HCT 116+hMLH1 cells after MSeA treatment. Knockdown of p53 did not significantly affect cell cycle profiles 6-24h after MSeA treatment or one day after recovery in HCT 116 and HCT 116+hMLH1 cells (Figure 4.7). therefore, selenium-induced cell cycle arrest is p53-independent in HCT 116 colorectal cancer cells.

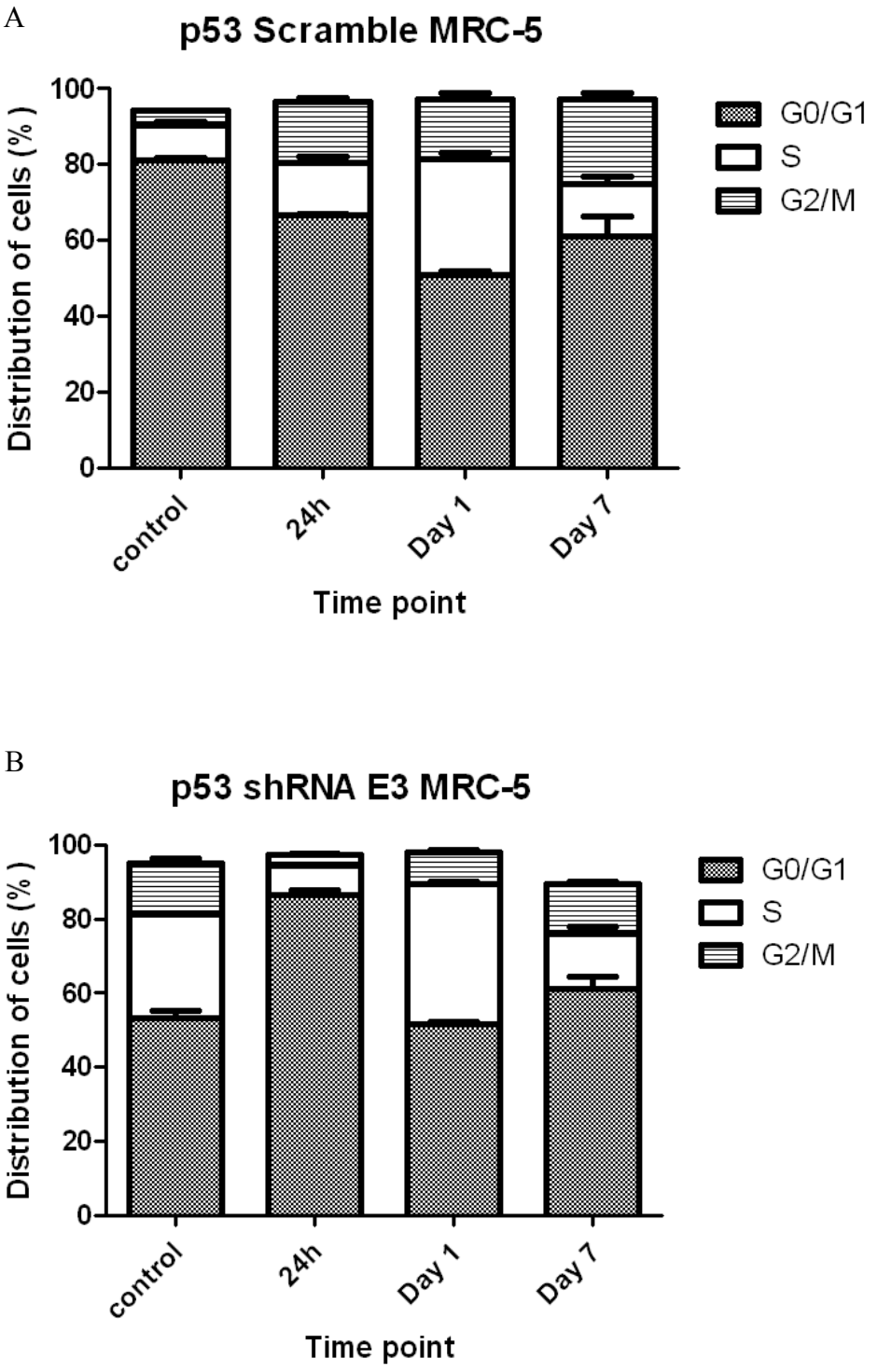


Figure 4.6 The role of p53 in cell cycle arrest in MRC-5 cells treated with MSeA (2 µM). MRC-5 scramble cells and MRC-5 p53 shRNA cells were treated with 2 µM MSeA and harvested at the indicated time points. Cell cycle profiles were analyzed by flow cytometry (n=3). Cells in G0/G1, S, and G2/M phases were quantified and presented.

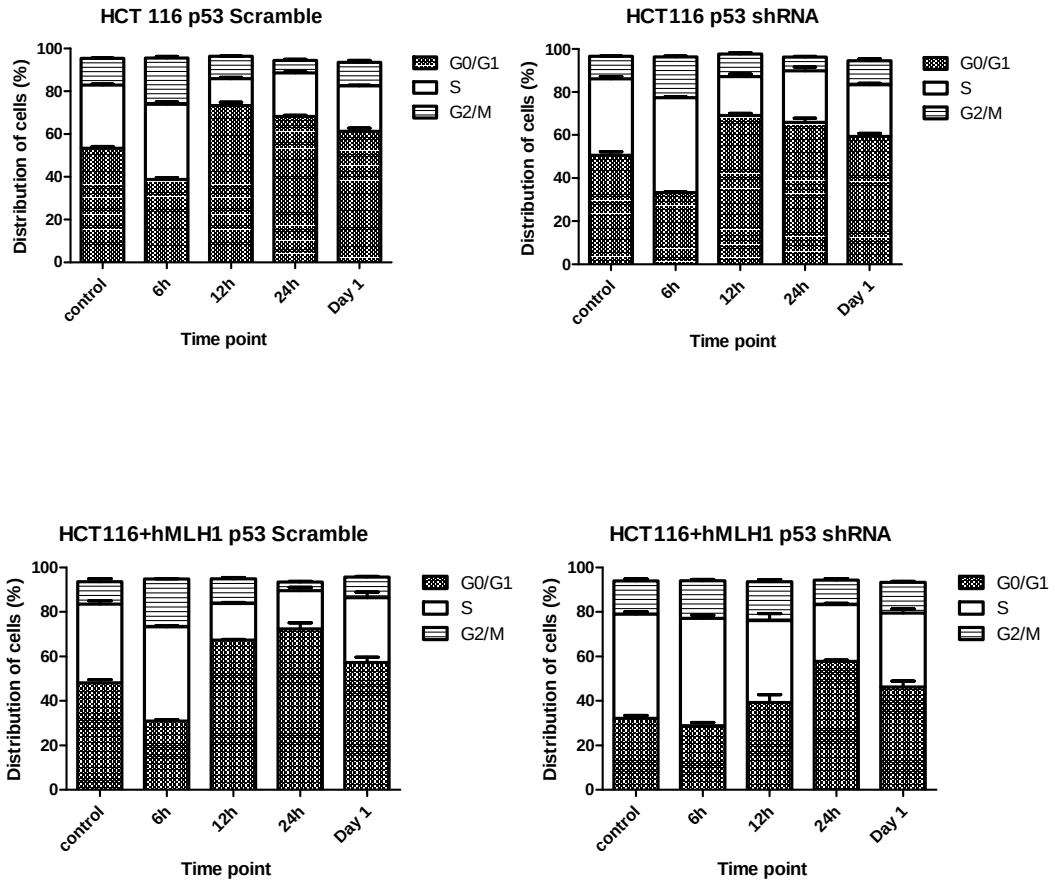


Figure 4.7 The role of p53 in cell cycle arrest in the HCT 116 and HCT 116 complemented with hMLH1 treated with MSeA (2 µM). Cells were treated with 2 µM MSeA harvested at the indicated time points. Cell cycle profiles were analyzed by flow cytometry (n=3). Cells in G0/G1, S, and G2/M phases were quantified and presented.

4.5 DISCUSSION

Previous studies have reported that selenium could induce apoptosis in cancerous cells (Zhou *et al.* 2003; Zhou *et al.* 2003; Zhao *et al.* 2006). Our lab is the first to demonstrate a role of selenium in the activation of early tumorigenesis barriers, namely senescence and DNA damage response (Wu *et al.* 2010). The ATM kinase plays a pivotal role in the selenium-induced senescence in a manner dependent on ROS in non-cancerous, but not in cancerous cells. The p53 tumor suppressor is the first ATM phosphorylation target being identified (Banin *et al.* 1998). Greater than 50% of adult human tumors bear p53 inactivating mutations or the mutations in the *TP53* gene (Gemignani *et al.* 2004; Seemann *et al.* 2004; Mechanic *et al.* 2007; Mouchawar *et al.* 2010; Ruijs *et al.* 2010). In many cancers where p53 is wild-type (for example, HCT 116 colorectal cancerous cells), the p53 pathway may be altered by oncogene activation, suggesting that p53 response is probably defective in most cancers. Also, loss of p53 promotes tumor development (Symonds *et al.* 1994; Squatrito *et al.* 2010). The p53 protein is required for induction of senescence by DNA replication stress (topoisomerase inhibitors) in an array of cancer cell lines (te Poele *et al.* 2002). Interestingly, we found that the selenium-induced senescence is missing in p53 shRNA MRC-5 cells. Our previous data showed that both p53-deficient PC-3 prostate cancerous cells and p53-proficient HCT 116 colorectal cancerous cells did not show senescence after selenium exposure (Wu *et al.* 2010). p21 is a gatekeeper of the G1-S transition and is implicated in a ROS-dependent senescence response in normal human fibroblasts (Brown *et al.* 1997; Macip *et al.* 2002). In HCT 116 cells exposed to doxorubicin treatment, p21 expression is induced

in both a p53-dependent and –independent manner (Ravizza *et al.* 2004). Therefore, it is possible that p21 can function independently of p53 as a backup pathway in selenium-induced senescence.

p53-dependent apoptotic pathway is well understood (Lowe *et al.* 1993; Polyak *et al.* 1997; Zhao *et al.* 2006; Zhao *et al.* 2006). Interestingly, MRC-5 cells with p53 knockdown are more resistant to sub-lethal ($\leq LD_{50}$) doses of MSeA, and this situation is not found in HCT 116 or HCT 116+hMLH1 cells (Figure 4.5). Why this only happened in p53 shRNA MRC-5 cells? Did the attenuation of the p53-dependent apoptotic pathway decrease the sensitivity to MSeA treatment? In fact, p53 mutations or defective p53 pathway renders cancerous cells to escape from p53-dependent apoptosis. p53 shRNA MRC-5 cells also bypass the p53-dependent apoptosis, resulting in inappropriate proliferation with unrepaired DNA damage. Because the metabolites of selenium compounds can generate ROS and apoptosis in cancer cells (Stewart *et al.* 1999; Hu *et al.* 2006; Zhao *et al.* 2006; Li *et al.* 2007), it is possible that loss of p53 in normal cells results in resistant response to evaluated ROS level and cell survival. Further research by antioxidant administration could clarify the role of ROS in the sensitivity of MRC-5 cells with and without p53 to selenium compounds.

DNA damage response and senescence serve as the early barriers of tumorigenesis that block the precancerous and the cancerous cells from inappropriate proliferation (Bartkova *et al.* 2005; Gorgoulis *et al.* 2005; Bartkova *et al.* 2006). There are two major forms of senescence. Telomere attrition contributes to replicative senescence,

while damaged DNA and oxidative stress can cause premature senescence. ATM is involved in both forms of senescence (Parrinello *et al.* 2003; di Fagagna *et al.* 2004; Herbig *et al.* 2004; Heiss *et al.* 2007). Thereafter, the ATM kinase is of great interest in cancer research (di Fagagna *et al.* 2004; Heiss *et al.* 2007). In selenium-induced senescence, ATM activation and increased γH2AX formation were observed (Figure 3.7A-F). In p53 shRNA MRC-5 cells, γH2AX level was already high in the absence of MSeA treatment (Figure 4.3 and 4.4). Reminiscent of the high γH2AX level in PC-3 cells, p53 shRNA MRC-5 cells showed increased genomic instability. Although ATM is a prominent responder to DNA double strand breaks, this kinase can be activated by various forms of chromosome alterations (Bakkenist and Kastan 2003). Importantly, our previous results suggest that inhibition of ATM kinase attenuates, but not prevent, selenium-induced γH2AX. As high γH2AX level was observed in p53 shRNA MRC-5 cells with selenium treatment, it is possible that other PI3K kinases also phosphorylate H2AX. Nevertheless, whether this high genomic instability can be rescued or attenuated by antioxidants or PI3K kinase inhibitors needs future studies. Future studies are also needed to elucidate the mechanism of increased genomic instability by loss of p53.

The p53 tumor suppressor is a potent inhibitor of cellular proliferation. In previous studies, the PA-1 ovarian carcinoma cells bearing wild-type p53 showed a high susceptibility to in G2/M arrest and an increase expression of Bax protein in the apoptotic response to γ-irradiation (Concin *et al.* 2003). In contrast, a different study using non-malignant adenomas removed from ovarian cancer patients showed that expression of wild-type p53 in chemotherapy led to cell cycle arrest in G1 phase

through reduced Bax expression and increased Bcl-2 level (Moreno *et al.* 2007). In both normal human fibroblasts and mouse embryonic fibroblasts, they passed through G1 and S phase and arrested in G2 phase after the overexpression of *MYC* in a p53- and p21-dependent manner (Felsher *et al.* 2000). In senescent MRC-5 cells after treatment with H_2O_2, a G1 arrest was observed (von Kobbe *et al.* 2004). To investigate the role of p53 in selenium-induced cell cycle profiles, we treated scramble MRC-5 cells and p53 shRNA MRC-5 cells with 2 μM MSeA, at which the two cells lines showed significant sensitivity towards MSeA. The results exhibited a G2/M phase arrest in scramble MRC-5 cells, and a G1 phase arrest in p53 shRNA MRC-5 cells. Evidence indicates that p16-Rb pathway also contributes to G1 arrest (Zhang *et al.* 1999; Wang *et al.* 2008). However, research has suggested that these two pathways are not mutually exclusive. Loss of p53 can upregulate p16 expression in mouse embryonic fibroblasts, osteoblasts, and other mouse organs, as p53 can suppress p16 through Ets1(protein C-ets-1) (Leong *et al.* 2009). p14 has been previously reported as a knot to link the two pathways (Bates *et al.* 1998). p21 is a gatekeeper of the G1-S transition and is implicated in a ROS-dependent senescence response in normal human fibroblasts (Brown *et al.* 1997; Macip *et al.* 2002). In HCT 116 cells exposed to doxorubicin treatment, p21 expression is induced in both a p53-dependent and –independent manner (Ravizza *et al.* 2004). In various mammalian cells, p16 could induce p21 level after the UV light by relocating HuR protein (ubiquitous RNA binding protein, also known as Drosophilla ELAVL1) (Al-Mohanna *et al.* 2007). Therefore, it is possible that p21 action through a p53-independent pathway or a p16-Rb pathway could compensate for the loss of p53 in p53 shRNA

MRC-5 cells, thus blocking the cells from entering S phase. In contrast, p53 pathway blocked cells with damaged DNA to entry mitosis or lead damaged cells to apoptosis in p53 expressing MRC-5 cells. The detection of p16 levels in selenium-treated cells and loss of function are needed to further confirm characterizes selenium-induced cell cycle arrest and senescence.

ATM is involved in G1, S and G2 phase checkpoint (Shiloh 2003; Cheng *et al.* 2008). Because selenium metabolism generate ROS and active ATM pathway, it is likely that selenium-induced genotoxic stress could induce DNA oxidation and the subsequent formation of DNA breaks or DNA replication fork collapse. It is conceivable that the ROS-induced DNA damage in S phase may activate the ATM pathway, leading to the increasing population in S phase in scramble and p53 shRNA MRC-5 cells for a checkpoint response one day after recovery from the MSeA treatment. Moreover, in SV-40 transformed GM847 human fibroblasts, ATR participates in p53 Ser-15 phosphorylation after γ-irradiation and UV light exposure (Tibbetts *et al.* 1999). Because DNA-PKcs null cells showed enhanced p53 phosphorylation at Ser-15 *in vivo* after ionizing radiation, DNA-PKcs does not account for p53 phosphorylation at Ser-15 in the stress response (Araki *et al.* 1999; Burma *et al.* 1999). Therefore, it is necessary to examine the functions of other PI3K kinases in addition to ATM in selenium-induced senescence and DNA damage response.

In conclusion, ROS generated by metabolizing selenium compounds impose genotoxic stress on cells and the subsequent DNA damage response (Figure 4.8).

ATM senses the DNA damage and its autophosphorylation on Ser 1981 activates the p53-p21 pathway. Also, p21 may function in a p53-independent pathway that inhibits Cdk2 and Cdc2 to block G1 and G2 cell cycle progression, respectively. However, when p53 is lost, the p16-Rb pathway can take over and keep G1 cells with damaged DNA in check. It is likely that p16 can partially compensate the loss of p53 and maintain senescence phenotype, while p53 can participate in the onset of senescence. Both pathways lead to cell cycle arrest. Clinically, suppression of senescence in p53 deficient non-cancerous cells confirms p53 as an effective chemoprevention target.

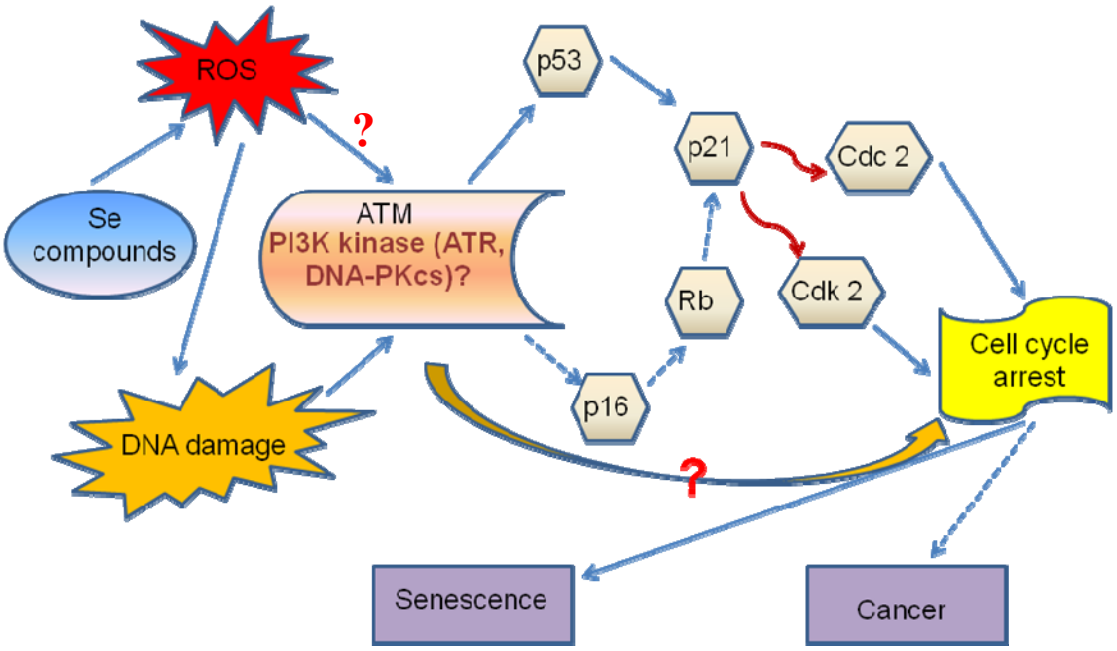


Figure 4.8 The scheme of selenium-induced senescence. Selenium compounds can generate ROS, which may induce DNA damage, leading to the activation of the ATM-dependent pathway. It is also possible that the enhanced ROS level without DNA damage may serve as a signal for ATM activation. Then, PI3K kinases activate the p53-p21 pathway for different cell cycle arrest. However, whether p16-Rb pathway or other alternative pathways are involved in cell cycle arrest is unknown. The irreversible cell cycle arrest in non-cancerous cells results in senescence. However, there is also one possibility that cells bearing damaged DNA bypass cell cycle arrest, so the unregulated cell proliferation of mutated DNA leads to cancer development.

CHAPTER 5: CONCLUSION AND PROSPECTS

On the basis of my dissertation research, the below statements that summarize key findings that conceptually advance the field of selenium chemoprevention.

A. Selenium has been proposed in chemoprevention for decades. For example, selenium can induce apoptosis in prostate cancer cells by increasing the superoxide level (Zhao *et al.* 2006; Zhao *et al.* 2006) and DNA damage regulator ATM is likely responsible for this apoptotic pathway (Zhou *et al.* 2003). However, this is still controversial due to the inconsistent between studies. Therefore, there is an urgent need to better understand the mechanism under selenium chemoprevention. My research first provides evidence that selenium compounds can serve as a barrier to tumorigenesis by activating ATM-dependent early senescence in non-cancerous cells. This result is valuable as by previous studies focus on the late stage of tumorigenesis. In most cases, in order to induce apoptosis in cancer cells, the selenium compounds doses are toxic to normal cells. While my research focuses on the early stages of tumorigenesis, showing that sub-lethal doses of selenium compounds could be effective to block the proliferation of the cells bearing damage. Selenium achieves this by functioning as a pro-oxidant.

B. There are several types of senescence, due to the oncogene activation or telomere attrition. Two parallel pathways, p53 pathway and p16-Rb pathway, are involved in the senescence (Bringold and Serrano 2000; Campisi 2001; Itahana *et al.* 2004). However, the detailed mechanism of how these two pathways function is still ambiguous. Since p53 is a target for active ATM, it will be interesting to investigate

whether p53 is required for selenium-induced senescence and what its role can be. In my research, when p53 was knockdown in MRC-5 normal cells, senescence disappeared. It then implies that p53 is required in selenium-induced senescence. Moreover, an increasing number of studies show that rather than working distinctly, p53 pathway and p16-Rb pathway interact with each other (Bates *et al.* 1998; Beausejour *et al.* 2003; Al-Mohanna *et al.* 2007; Leong *et al.* 2009). The relationship between p53 pathway and p16-Rb pathway in selenium-induced senescence is also worth examining. Interestingly, my research showed that with p53 knockdown, p16 level was upregulated in MRC-5 cells treated with sub-lethal doses of selenium. It suggests the possible compensation role of p16-Rb pathway to the p53 loss.

C. Several large-scale epidemiological studies showed an inverse relationship between cancer incidence/mortality and selenium intake level, which further boosts the interest of selenium research. It is likely that selenium can trigger genomic maintenance machinery to prevent cancer. SeMet protected cells from methyl methanesulfonate by triggering p53-depedent BER pathway in RKO cells (Kurz *et al.* 2004). Sodium selenite could induce apoptosis in cervical carcinoma cells by imposing oxidative stress to generate DNA damage followed by activation of p53 and p38 pathways (Liu *et al.* 2005). Interestingly, caspase-independent apoptosis can also be induced in the mitochondria of cervical carcinoma cells in response to selenite (Liu *et al.* 2005). Mitochondria release of cytochrome c contributes to the caspase-dependent apoptosis in DU-145 prostate cancer cells exposed to MSeA (von Kobbe *et al.* 2004). Therefore, further studies focusing on the link between genotoxic stress

imposed by selenium and pathways involved in maintaining genome stability can help understand the mechanisms underlying selenium chemopreventive effects.

Most age-related chronic diseases are associated with internal ROS imbalance, leading to genomic instability. Since most selenoproteins acquire antioxidant enzymatic functions, they should at least be able to attenuate or delay such diseases by regulating redox status. SelH is localized in the nucleus, and it can sense the redox change. Therefore, it works as a transcriptional factor, regulating genes participating in glutathione synthesis and phase II detoxification exposed to oxidative stress (Panee *et al.* 2007). In this case, selenoproteins can not only act directly in the antioxidant system to protect DNA but also function as transcription factors, indirectly maintain the genomic stability. However, current well-studied transcriptional selenoprotein is sole SelH, so more research is needed to uncover more selenoproteins and more functions of known selenoproteins. Taken together, detailed mechanisms of how selenium works are in a great urgent need in order to efficiently and precisely apply this agent for human benefits.

Glossary

1. Senescence: a biological aging process, in which cells are metabolically active but loss the capability of proliferation.
2. Selenoproteins: a group of proteins with the incorporation of selenocysteine, functioning as their active site.
3. Reactive oxygen species (ROS): free radicals generated from external sources, such as smoking, UV exposure, and internal metabolic pathways. They can cause damage to DNA, proteins and lipids.
4. Genome: the entire genetic information of an organism.
5. Carcinogenesis/tumorigenesis: a process in which normal cells are transformed to cancer cells.
6. Oncogene: a gene whose mutation can result in cancer.
7. shRNA: small-hairpin RNA, can permanently decrease the expression of some specific gene(s).
8. LD_{50}: lethal dose 50. It is the dose that 50% of cell population can be killed.

Abbreviation List

1. SA-β-gal: senescence-associated β-galactosidase
2. LD_{50}: lethal dose 50
3. ATM: ataxia telangiectasia mutated
4. MSeA: methylseleninic acid
5. MSeC: methylselenocysteine
6. BrdU: 5-bromo-2-deoxyuridine
7. TOR: target of rapamycin
8. IR: ionizing radiation
9. ROS: reactive oxygen species
10. 8-oxo-G: 8-oxo-7,8-dihydro-2'-deoxyguanosine
11. TOP1: topoisomerase 1
12. DSB: double-strand break
13. SSB: single-strand break
14. AOA1: ataxia-oculomotor apraxia 1
15. SCAN1: spinocerebellar ataxia with axonal neuropathy 1
16. O^6MeG: O^6-methylguanine
17. MGMT: O^6MeG DNA methyltransferase
18. BER: base excision repair
19. AP: apyrimidinic/apurinic
20. APE1: AP endonuclease 1
21. 5'-dRp: 5'-deoxyribose-5-phosphate

22. Pol β: DNA polymerase β

23. PCNA: proliferating cell nuclear antigen

24. FEN: flap endonuclease

25. NER: nucleotide excision repair

26. GGR: global genomic NER

27. TCR: transcription-coupled NER

28. CS: Cockayne's syndrome

29. RPA: replication protein A

30. XP: xeroderma pigmentosum

31. MMR: DNA mismatch repair

32. MutSα: MSH2/MSH6

33. MutSβ: MSH2/MSH3

34. MutLα: hMLH1/hPMS2

35. hMLH1: MutL homolog 1

36. MutLβ: hMLH1/hPMS1

37. HNPCC: hereditary non-polyposis colorectal carcinoma

38. HR: homologous recombination

39. NHEJ: non-homologous end joining

40. NBS: Nijmegen breakage syndrome

41. MRN: MRE11-RAD50-NBS1 complex

42. ATR: ATM and Rad3-related

43. CtIP: C-terminal binding protein interacting protein

44. DNA-PK: DNA-dependent protein kinase

45. Cdk2: cyclin-dependent kinase 2

46. Rb: retinoblastoma

47. SMC1: structural maintenance of chromosome protein 1

48. TERT: telomerase reverse transcriptase

49. TERC: telomerase RNA

50. DKC1: dyskerin

51. RDA: recommended daily allowance

52. Sec: selenocysteine

53. SPS2: selenophosphate synthetase 2

54. SECIS: Sec insertion sequence

55. SBP2: SECIS-binding protein 2

56. Sep15: selenoprotein 15

57. SelP: selenoprotein P

58. SelW: selenoprotein W

59. SelH: selenoprotein H

60. SelM: selenoprotein M

61. PI: propidium iodine

62. NAC: *N*-acetylcysteine

63. Tempo: 2,2,6,6-tetramethylpiperidine-1-oxyl

64. PI3K kinase: phosphatidylinositol 3-Kinase

65. GPX: glutathione peroxidase

66. TrxR: thioredoxin reductases

67. DIO: iodothyronine deiodinases

68. SELECT: selenium and vitamin E cancer prevention trial

69. MEM: minimum essential medium eagle

70. DMEM: Dulbecco's modified eagle's medium

71. PBS: phosphate-buffered saline

72. DMSO: dimethyl sulfoxide

73. X-gal: bromo-chloro-indolyl-galactopyranoside

74. MTT: 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

75. siRNA: small interfering RNA

76. shRNA: short hairpin RNA

77. pATM S1981: ATM autophosphorylation at Ser-1981

78. known as γH2AX: H2AX phosphorylation at Ser-139

Reference

Agarwal, M. L., *et al.* (1995). "p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts." Proc Natl Acad Sci U S A **92**(18): 8493-8497.

Akbaraly, T. N., *et al.* (2010). "Plasma selenium and risk of dysglycemia in an elderly French population: results from the prospective Epidemiology of Vascular Ageing Study." Nutrition & Metabolism **7**: 21-27.

Al-Mohanna, M. A., *et al.* (2007). "The p16(INK4a) tumor suppressor controls p21(WAF1) induction in response to ultraviolet light." Nucleic Acids Research **35**(1): 223-233.

Allan, C. B., *et al.* (1999). "Responsiveness of selenoproteins to dietary selenium." Annual Review of Nutrition **19**: 1-16.

Allsopp, R. C., *et al.* (1992). "Telomere length predicts replicative capacity of human fibroblasts." Proceedings of the National Academy of Sciences of the United States of America **89**(21): 10114-10118.

Ambroziak, M., *et al.* (2005). "Disturbed expression of type 1 and type 2 iodothyronine deiodinase as well as Titf1/Nkx2-1 and pax-8 transcription factor genes in papillary thyroid cancer." Thyroid **15**(10): 1137-1146.

Ames, B. N., *et al.* (1993). "Oxidants, antioxidants, and the degenerative diseases of aging." Proceedings of the National Academy of Sciences of the United States of America **90**(17): 7915-7922.

Andersen, J. K. (2004). "Oxidative stress in neurodegeneration: cause or consequence?" Nature Medicine **10**(7): S18-S25.

Ando, K., *et al.* (2010). "High expression of BUBR1 is one of the factors for inducing DNA aneuploidy and progression in gastric cancer." Cancer Science **101**(3): 639-645.

Anne-Marie, R. and A. N. Tasnime (2007). "Selenium and aging." Agro Food Industry Hi-Tech **18**(3): 59-60.

Apostolou, S., *et al.* (2004). "Growth inhibition and induction of apoptosis in mesothelioma cells by selenium and dependence on selenoprotein SEP15 genotype." Oncogene **23**(29): 5032-5040.

Araki, R., *et al.* (1999). "Enhanced phosphorylation of p53 serine 18 following DNA damage in DNA-dependent protein kinase catalytic subunit-deficient cells." Cancer Res **59**(15): 3543-3546.

Arbogast, S. and A. Ferreiro (2010). "Selenoproteins and Protection against Oxidative Stress: Selenoprotein N as a Novel Player at the Crossroads of Redox Signaling and Calcium Homeostasis." Antioxidants & Redox Signaling **12**(7): 893-904.

Arnaudeau, C., *et al.* (2001). "DNA double-strand breaks associated with replication forks are predominantly repaired by homologous recombination involving an exchange mechanism in mammalian cells." Journal of Molecular Biology **307**(5): 1235-1245.

Arthur, J. R. (2000). "The glutathione peroxidases." Cellular and Molecular Life Sciences **57**(13-14): 1825-1835.

Ayaz, M. and B. Turan (2006). "Selenium prevents diabetes-induced alterations in [Zn2+](i) and metallothionein level of rat heart via restoration of cell redox

cycle." American Journal of Physiology-Heart and Circulatory Physiology **290**(3): H1071-H1080.
Azevedo, M. F., *et al.* (2010). "Selenoprotein-related disease in a young girl caused by nonsense mutations in the SBP2 gene." Journal of Clinical Endocrinology & Metabolism **95**(8): 4066-4071.
Bakkenist, C. J. and M. B. Kastan (2003). "DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation." Nature **421**(6922): 499-506.
Balass, M., *et al.* (2002). "Characterization of two peptide epitopes on Mdm2 oncoprotein that affect p53 degradation." Peptides **23**(10): 1719-1725.
Banin, S., *et al.* (1998). "Enhanced phosphorylation of p53 by ATM in response to DNA damage." Science **281**(5383): 1674-1677.
Banning, A., *et al.* (2005). "The GI-GPx gene is a target for Nrf2." Molecular and Cellular Biology **25**(12): 4914-4923.
Barker, S., *et al.* (2005). "DNA-protein crosslinks: their induction, repair, and biological consequences." Mutation Research-Reviews in Mutation Research **589**(2): 111-135.
Bartkova, J., *et al.* (2005). "DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis." Nature **434**(7035): 864-870.
Bartkova, J., *et al.* (2006). "Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints." Nature **444**(7119): 633-637.
Bates, S., *et al.* (1998). "p14ARF links the tumour suppressors RB and p53." Nature **395**(6698): 124-125.
Baur, J. A., *et al.* (2006). "Resveratrol improves health and survival of mice on a high-calorie diet." Nature **444**(7117): 337-342.
Beausejour, C. M., *et al.* (2003). "Reversal of human cellular senescence: roles of the p53 and p16 pathways." Embo Journal **22**(16): 4212-4222.
Beck, M. A., *et al.* (1994). "Increased virulence of a human enterovirus (coxsackievirus B3) in selenium-deficient mice." Journal of Infectious Diseases **170**(2): 351-357.
Beilstein, M. A., *et al.* (1996). "Selenoprotein W of rat muscle binds glutathione and an unknown small molecular weight moiety." Journal of Inorganic Biochemistry **61**(2): 117-124.
Bellinger, F. P., *et al.* (2008). "Association of selenoprotein P with Alzheimer's pathology in human cortex." Journal of Alzheimers Disease **15**(3): 465-472.
Bernstein, N. K., *et al.* (2008). "Polynucleotide kinase as a potential target for enhancing cytotoxicity by ionizing radiation and topoisomerase I inhibitors." Anti-Cancer Agents in Medicinal Chemistry **8**(4): 358-367.
Bianco, A. C. and B. W. Kim (2006). "Deiodinases: implications of the local control of thyroid hormone action." Journal of Clinical Investigation **116**(10): 2571-2579.
Bianco, A. C., *et al.* (2002). "Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases." Endocrine Reviews **23**(1): 38-89.

Biverstal, A., *et al.* (2008). "Cyclobutane pyrimidine dimers do not fully explain the mutagenicity induced by UVA in Chinese hamster cells." Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis **648**(1-2): 32-39.

Bjornstedt, M., *et al.* (1992). "Selenodiglutathione is a highly efficient oxidant of reduced thioredoxin and a substrate for mammalian thioredoxin reductase." Journal of Biological Chemistry **267**(12): 8030-8034.

Blankenberg, S., *et al.* (2003). "Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease." New England Journal of Medicine **349**(17): 1605-1613.

Blasco, M. A. (2005). "Telomeres and human disease: Ageing, cancer and beyond." Nature Reviews Genetics **6**(8): 611-622.

Bleys, J., *et al.* (2007). "Serum selenium and diabetes in US adults." Diabetes Care **30**(4): 829-834.

Bodnar, A. G., *et al.* (1998). "Extension of life-span by introduction of telomerase into normal human cells." Science **279**(5349): 349-352.

Boehme, K. A., *et al.* (2008). "p53 stabilization in response to DNA damage requires Akt/PKB and DNA-PK." Proceedings of the National Academy of Sciences of the United States of America **105**(22): 7785-7790.

Boitani, C. and R. Puglisi (2008). Selenium, a key element in spermatogenesis and male fertility. Molecular Mechanisms in Spermatogenesis. Berlin, Springer-Verlag Berlin. **636:** 65-73.

Braithwaite, E. K., *et al.* (2010). "DNA Polymerases beta and lambda Mediate Overlapping and Independent Roles in Base Excision Repair in Mouse Embryonic Fibroblasts." Plos One **5**(8): e12229.

Brigelius-Flohe, R. (1999). "Tissue-specific functions of individual glutathione peroxidases." Free Radical Biology and Medicine **27**(9-10): 951-965.

Brigelius-Flohe, R. and A. Kipp (2009). "Glutathione peroxidases in different stages of carcinogenesis." Biochimica Et Biophysica Acta-General Subjects **1790**(11): 1555-1568.

Bringold, F. and M. Serrano (2000). "Tumor suppressors and oncogenes in cellular senescence." Experimental Gerontology **35**(3): 317-329.

Brown, J. P., *et al.* (1997). "Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts." Science **277**(5327): 831-834.

Burk, R. F. and K. E. Hill (2005). "Selenoprotein P: An extracellular protein with unique physical characteristics and a role in selenium homeostasis." Annual Review of Nutrition **25**: 215-235.

Burk, R. F., *et al.* (2003). "Selenoprotein metabolism and function: Evidence for more than one function for selenoprotein P." Journal of Nutrition **133**(5): 1517S-1520S.

Burma, S., *et al.* (2001). "ATM phosphorylates histone H2AX in response to DNA double-strand breaks." Journal of Biological Chemistry **276**(45): 42462-42467.

Burma, S., *et al.* (1999). "DNA-dependent protein kinase-independent activation of p53 in response to DNA damage." J Biol Chem **274**(24): 17139-17143.

Cabelof, D. C., *et al.* (2006). "Haploinsufficiency in DNA polymerase beta increases cancer risk with age and alters mortality rate." Cancer Research **66**(15): 7460-7465.
Cadet, J., *et al.* (2004). "Radiation-induced DNA damage: Formation, measurement, and biochemical features." Journal of Environmental Pathology Toxicology and Oncology **23**(1): 33-43.
Cadet, J., *et al.* (1997). "Effects of UV and visible radiation on DNA - Final base damage." Biological Chemistry **378**(11): 1275-1286.
Caldecott, K. W. (2008). "Single-strand break repair and genetic disease." Nature Reviews Genetics **9**(8): 619-631.
Camphausen, K., *et al.* (2004). "Flavopiridol enhances human tumor cell radiosensitivity and prolongs expression of gamma H2AX foci." Molecular Cancer Therapeutics **3**(4): 409-416.
Campisi, J. (2001). "Cellular senescence as a tumor-suppressor mechanism." Trends in Cell Biology **11**(11): S27-31.
Canman, C. E., *et al.* (1998). "Activation of the ATM kinase by ionizing radiation and phosphorylation of p53." Science **281**(5383): 1677-1679.
Carlson, B. A., *et al.* (2009). "Selenoproteins regulate macrophage invasiveness and extracellular matrix-related gene expression." BMC Immunology **10**: 57-68.
Carlson, B. A., *et al.* (2010). "Role of selenium-containing proteins in T-cell and macrophage function." Proceedings of the Nutrition Society **69**(3): 300-310.
Castro, M. E., *et al.* (2004). "Cellular senescence induced by p53-ras cooperation is independent of p21(waf1) in murine embryo fibroblasts." Journal of Cellular Biochemistry **92**(3): 514-524.
Chen, C. M., *et al.* (2009). "Increased oxidative damage in peripheral blood correlates with severity of Parkinson's disease." Neurobiology of Disease **33**(3): 429-435.
Chen, J. and M. J. Berry (2003). "Selenium and selenoproteins in the brain and brain diseases." Journal of Neurochemistry **86**(1): 1-12.
Chen, P. C., *et al.* (2005). "Contributions by MutL homologues MLH3 and PMS2 to DNA mismatch repair and tumor suppression in the mouse." Cancer Research **65**(19): 8662-8670.
Cheng, W. H. (2009). "Impact of inorganic nutrients on maintenance of genomic stability." Environ Mol Mutagen **50**(5): 349-360.
Cheng, W. H., *et al.* (1998). "Cellular glutathione peroxidase is the mediator of body selenium to protect against paraquat lethality in transgenic mice." Journal of Nutrition **128**(7): 1070-1076.
Cheng, W. H., *et al.* (2008). "WRN is required for ATM activation and the S-phase checkpoint in response to interstrand cross-link-induced DNA double-strand breaks." Molecular Biology of the Cell **19**(9): 3923-3933.
Cheng, W. H., *et al.* (2003). "Low levels of glutathione peroxidase 1 activity in selenium-deficient mouse liver affect c-Jun N-terminal kinase activation and p53 phosphorylation on Ser-15 in pro-oxidant-induced aponecrosis." Biochemical Journal **370**: 927-934.

Chu, F. F., *et al.* (1993). "Expression, characterization, and tissue distribution of a new cellular selenium-depedent glutathione-peroxidase, GSHPX-GI." Journal of Biological Chemistry **268**(4): 2571-2576.
Clark, A. B., *et al.* (2000). "Functional interaction of proliferating cell nuclear antigen with MSH2-MSH6 and MSH2-MSH3 complexes." Journal of Biological Chemistry **275**(47): 36498-36501.
Clark, L. C., *et al.* (1996). "Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group." JAMA **276**(24): 1957-1963.
Cohen, S. B., *et al.* (2007). "Protein composition of catalytically active human telomerase from immortal cells." Science **315**(5820): 1850-1853.
Combs, G. F., Jr. (2004). "Status of selenium in prostate cancer prevention." Br J Cancer **91**(2): 195-199.
Concin, N., *et al.* (2003). "Role of p53 in G2/M cell cycle arrest and apoptosis in response to gamma-irradiation in ovarian carcinoma cell lines." Int J Oncol **22**(1): 51-57.
Conrad, M., *et al.* (2004). "Essential role for mitochondrial thioredoxin reductase in hematopoiesis, heart development, and heart function." Molecular and Cellular Biology **24**(21): 9414-9423.
Coon, K. D., *et al.* (2007). "A high-density whole-genome association study reveals that APOE is the major susceptibility gene for sporadic late-onset Alzheimer's disease." Journal of Clinical Psychiatry **68**(4): 613-618.
Cooper, M. L., *et al.* (2008). "Interaction between single nucleotide polymorphisms in selenoprotein P and mitochondrial superoxide dismutase determines prostate cancer risk." Cancer Research **68**(24): 10171-10177.
Coppe, J. P., *et al.* (2008). "Senescence-Associated Secretory Phenotypes Reveal Cell-Nonautonomous Functions of Oncogenic RAS and the p53 Tumor Suppressor." Plos Biology **6**(12): 2853-2868.
Cosme-Blanco, W., *et al.* (2007). "Telomere dysfunction suppresses spontaneous tumorigenesis in vivo by initiating p53-dependent cellular senescence." Embo Reports **8**(5): 497-503.
Costa, R. M. A., *et al.* (2003). "The eukaryotic nucleotide excision repair pathway." Biochimie **85**(11): 1083-1099.
Darbro, B. W., *et al.* (2006). "Methylation of the p16(INK4a) promoter region in telomerase immortalized human keratinocytes co-culturedwith feeder cells." Oncogene **25**(56): 7421-7433.
Davis, C. D. and E. O. Uthus (2003). "Dietary folate and selenium affect dimethylhydrazine-induced aberrant crypt formation, global DNA methylation and one-carbon metabolism in rats." Journal of Nutrition **133**(9): 2907-2914.
Davis, C. D., *et al.* (2002). "Selenium-enriched broccoli decreases intestinal tumorigenesis in multiple intestinal neoplasia mice." Journal of Nutrition **132**(2): 307-309.
de Lange, T. (2002). "Protection of mammalian telomeres." Oncogene **21**(4): 532-540.

Demma, M., *et al.* (2010). "SCH529074, a Small Molecule Activator of Mutant p53, Which Binds p53 DNA Binding Domain (DBD), Restores Growth-suppressive Function to Mutant p53 and Interrupts HDM2-mediated Ubiquitination of Wild Type p53." Journal of Biological Chemistry **285**(14): 10198-10212.

Demple, B., *et al.* (1991). "Cloning and expression of APE, the CDNA-encoding the major human apurinic endonuclease - definition of a family of DNA-repair enzymes." Proceedings of the National Academy of Sciences of the United States of America **88**(24): 11450-11454.

Deng, Y. B., *et al.* (2008). "Telomere dysfunction and tumour suppression: the senescence connection." Nature Reviews Cancer **8**(6): 450-458.

Dhalla, N. S., *et al.* (2000). "Role of oxidative stress in cardiovascular diseases." Journal of Hypertension **18**(6): 655-673.

di Fagagna, F. D., *et al.* (2004). "Functional links between telomeres and proteins of the DNA-damage response." Genes & Development **18**(15): 1781-1799.

Di Leonardo, A., *et al.* (1994). "DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts." Genes Dev **8**(21): 2540-2551.

Dianov, G. L., *et al.* (1999). "Role of DNA polymerase beta in the excision step of long patch mammalian base excision repair." Journal of Biological Chemistry **274**(20): 13741-13743.

Dimri, G. P., *et al.* (1995). "A BIOMARKER THAT IDENTIFIES SENESCENT HUMAN-CELLS IN CULTURE AND IN AGING SKIN IN-VIVO." Proceedings of the National Academy of Sciences of the United States of America **92**(20): 9363-9367.

Dizdaroglu, M. (1992). "Oxidative damage to DNA in mammalian chromatin." Mutation Research **275**(3-6): 331-342.

Downey, C. M., *et al.* (2009). "Osteo-chondroprogenitor-specific deletion of the selenocysteine tRNA gene, Trsp, leads to chondronecrosis and abnormal skeletal development: a putative model for Kashin-Beck disease." PLoS Genetics **5**(8): e1000616.

Drake, E. N. (2006). "Cancer chemoprevention: Selenium as a prooxidant not an antioxidant." Medical Hypotheses **67**(2): 318-322.

Duensing, S. and K. Munger (2004). "Mechanisms of genomic instability in human cancer: Insights from studies with human papillomavirus oncoproteins." International Journal of Cancer **109**(2): 157-162.

Duesberg, P. and D. Rasnick (2000). "Aneuploidy, the somatic mutation that makes cancer a species of its own." Cell Motility and the Cytoskeleton **47**(2): 81-107.

Edes, K., *et al.* (2010). "JS-K, a nitric oxide prodrug, has enhanced cytotoxicity in colon cancer cells with knockdown of thioredoxin reductase 1." Plos One **5**(1): e8786.

Esworthy, R. S., *et al.* (2005). "Epithelium-specific glutathione peroxidase, Gpx2, is involved in the prevention of intestinal inflammation in selenium-deficient mice." Journal of Nutrition **135**(4): 740-745.

Falck, J., *et al.* (2005). "Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage." Nature **434**(7033): 605-611.
Fang, L., *et al.* (1999). "p21(Waf1/Cip1/Sdi1) induces permanent growth arrest with markers of replicative senescence in human tumor cells lacking functional p53." Oncogene **18**(18): 2789-2797.
Fang, W. F., *et al.* (2010). "Tumor inhibition by sodium selenite is associated with activation of c-Jun NH2-terminal kinase 1 and suppression of beta-catenin signaling." International Journal of Cancer **127**(1): 32-42.
Felix, K., *et al.* (2004). "Selenium deficiency abrogates inflammation-dependent plasma cell tumors in mice." Cancer Research **64**(8): 2910-2917.
Felsher, D. W., *et al.* (2000). "Overexpression of MYC causes p53-dependent G2 arrest of normal fibroblasts." Proc Natl Acad Sci U S A **97**(19): 10544-10548.
Ferbeyre, G., *et al.* (2002). "Oncogenic ras and p53 cooperate to induce cellular senescence." Molecular and Cellular Biology **22**(10): 3497-3508.
Finkel, T. and N. J. Holbrook (2000). "Oxidants, oxidative stress and the biology of ageing." Nature **408**(6809): 239-247.
Flores-Mateo, G., *et al.* (2006). "Selenium and coronary heart disease: a meta-analysis." American Journal of Clinical Nutrition **84**(4): 762-773.
Fomenko, D. E. and V. N. Gladyshev (2003). "Genomics perspective on disulfide bond formation." Antioxidants & Redox Signaling **5**(4): 397-402.
Fruehauf, J. P. and F. L. Meyskens (2007). "Reactive oxygen species: A breath of life or death?" Clinical Cancer Research **13**(3): 789-794.
Galton, V. A., *et al.* (2009). "Life without Thyroxine to 3,5,3 '-Triiodothyronine Conversion: Studies in Mice Devoid of the 5 '-Deiodinases." Endocrinology **150**(6): 2957-2963.
Garcia-Solis, P. and C. Aceves (2003). "5 ' deiodinase in two breast cancer cell lines: effect of triiodothyronine, isoproterenol and retinoids." Molecular and Cellular Endocrinology **201**(1-2): 25-31.
Gasdaska, P. Y., *et al.* (1999). "Cloning, sequencing and functional expression of a novel human thioredoxin reductase." Febs Letters **442**(1): 105-111.
Gemignani, F., *et al.* (2004). "A TP53 polymorphism is associated with increased risk of colorectal cancer and with reduced levels of TP53 mRNA." Oncogene **23**(10): 1954-1956.
Gladyshev, V. N., *et al.* (1996). "Selenocysteine, identified as the penultimate C-terminal residue in human T-cell thioredoxin reductase, corresponds to TGA in the human placental gene." Proceedings of the National Academy of Sciences of the United States of America **93**(12): 6146-6151.
Gladyshev, V. N., *et al.* (1998). "A new human selenium-containing protein - Purification, characterization, and cDNA sequence." Journal of Biological Chemistry **273**(15): 8910-8915.
Gorgoulis, V. G., *et al.* (2005). "Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions." Nature **434**(7035): 907-913.
Graves, P. R., *et al.* (2000). "The Chk1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01." Journal of Biological Chemistry **275**(8): 5600-5605.

Greider, C. W. and E. H. Blackburn (1985). "Identification of a specific telomere terminal transferase-activity in the tetrahymena extracts." Cell **43**(2): 405-413.
Gresner, P., *et al.* (2009). "Expression of selenoprotein-coding genes SEPP1, SEP15 and hGPX1 in non-small cell lung cancer." Lung Cancer **65**(1): 34-40.
Guijarro, M. V., *et al.* (2007). "MAP17 enhances the malignant behavior of tumor cells through ROS increase." Carcinogenesis **28**(10): 2096-2104.
Gundimeda, U., *et al.* (2008). "Locally generated methylseleninic acid induces specific inactivation of protein kinase C isoenzymes: relevance to selenium-induced apoptosis in prostate cancer cells." Journal of Biological Chemistry **283**(50): 34519-34531.
Halazonetis, T. D., *et al.* (2008). "An oncogene-induced DNA damage model for cancer development." Science **319**(5868): 1352-1355.
Han, E. S., *et al.* (2008). "The in vivo gene expression signature of oxidative stress." Physiological Genomics **34**(1): 112-126.
Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.
Hanawalt, P. C. (2002). "Subpathways of nucleotide excision repair and their regulation." Oncogene **21**(58): 8949-8956.
Hao, L. Y., *et al.* (2004). "Phosphorylation of H2AX at short telomeres in T cells and fibroblasts." Journal of Biological Chemistry **279**(43): 45148-45154.
Harrison, D. E., *et al.* (2009). "Rapamycin fed late in life extends lifespan in genetically heterogeneous mice." Nature **460**(7253): 392-395.
Hartwell, L. (1992). "DEFECTS IN A CELL-CYCLE CHECKPOINT MAY BE RESPONSIBLE FOR THE GENOMIC INSTABILITY OF CANCER-CELLS." Cell **71**(4): 543-546.
Hawkes, W. C. and L. J. Laslett (2009). "Selenium supplementation does not improve vascular responsiveness in healthy North American men." American Journal of Physiology-Heart and Circulatory Physiology **296**(2): H256-H262.
Hayflick, L. and P. S. Moorhead (1961). "Serial cultivation of human diploid cell strains." Experimental Cell Research **25**(3): 585-621.
Hegde, M. L., *et al.* (2008). "Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells." Cell Research **18**(1): 27-47.
Heiss, E. H., *et al.* (2007). "Chronic treatment with resveratrol induces redox stress- and ataxia telangiectasia-mutated (ATM)-dependent senescence in p53-positive cancer cells." Journal of Biological Chemistry **282**(37): 26759-26766.
Herbig, U., *et al.* (2004). "Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a)." Molecular Cell **14**(4): 501-513.
Hill, R., *et al.* (2008). "p53 binding to the p21 promoter is dependent on the nature of DNA damage." Cell Cycle **7**(16): 2535-2543.
Hiyama, E. and K. Hiyama (2007). "Telomere and telomerase in stem cells." British Journal of Cancer **96**(7): 1020-1024.
Hoeijmakers, J. H. J. (2001). "Genome maintenance mechanisms for preventing cancer." Nature **411**(6835): 366-374.

Hoffmann, P. R., *et al.* (2007). "The selenoproteome exhibits widely varying, tissue-specific dependence on selenoprotein P for selenium supply." Nucleic Acids Research **35**(12): 3963-3973.
Hollstein, M., *et al.* (1991). "p53 mutations in human cancers." Science **253**(5015): 49-53.
Hu, H., *et al.* (2006). "Inorganic selenium sensitizes prostate cancer cells to TRAIL-induced apoptosis through superoxide/p53/Bax-mediated activation of mitochondrial pathway." Mol Cancer Ther **5**(7): 1873-1882.
Hurley, P. J., *et al.* (2007). "Human cancer cells require ATR for cell cycle progression following exposure to ionizing radiation." Oncogene **26**(18): 2535-2542.
Hurst, R. and S. Fairweather-Tait (2009). "Selenium and Vitamin E Supplementation for Cancer Prevention." Jama-Journal of the American Medical Association **301**(18): 1876-1877.
Hwang, D., *et al.* (2007). "Selenium acts as an insulin-like molecule for the down-regulation of diabetic symptoms via endoplasmic reticulum stress and insulin signalling proteins in diabetes-induced non-obese diabetic mice." Journal of Biosciences **32**(4): 723-735.
Iliakis, G., *et al.* (2003). "DNA damage checkpoint control in cells exposed to ionizing radiation." Oncogene **22**(37): 5834-5847.
Imai, H., *et al.* (2009). "Depletion of selenoprotein GPx4 in spermatocytes causes male infertility in mice." Journal of Biological Chemistry **284**(47): 32522-32532.
Ingram, D. K., *et al.* (2006). "Calorie restriction mimetics: an emerging research field." Aging Cell **5**(2): 97-108.
Irons, R., *et al.* (2010). "Deficiency in the 15-kDa Selenoprotein Inhibits Tumorigenicity and Metastasis of Colon Cancer Cells." Cancer Prevention Research **3**(5): 630-639.
Itahana, K., *et al.* (2004). "Mechanisms of cellular senescence in human and mouse cells." Biogerontology **5**(1): 1-10.
Ito, K., *et al.* (2004). "Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells." Nature **431**(7011): 997-1002.
Iyer, R. R., *et al.* (2008). "The MutS alpha-proliferating cell nuclear antigen interaction in human DNA mismatch repair." Journal of Biological Chemistry **283**(19): 13310-13319.
Jo, H. J., *et al.* (2008). "Diallyl disulfide induces reversible G2/M phase arrest on a p53-independent mechanism in human colon cancer HCT-116 cells." Oncology Reports **19**(1): 275-280.
Kaneko, Y., *et al.* (1999). "Cell cycle-dependent and ATM-independent expression of human Chk1 kinase." Oncogene **18**(25): 3673-3681.
Kasapoglu, M. and T. Ozben (2001). "Alterations of antioxidant enzymes and oxidative stress markers in aging." Experimental Gerontology **36**(2): 209-220.
Kastan, M. B. and J. Bartek (2004). "Cell-cycle checkpoints and cancer." Nature **432**(7015): 316-323.

Kathe, S. D., *et al.* (2004). "Single-stranded breaks in DNA but not oxidative DNA base damages block transcriptional elongation by RNA polymerase II in HeLa cell nuclear extracts." Journal of Biological Chemistry **279**(18): 18511-18520.
Katyal, S. and P. J. McKinnon (2008). "DNA strand breaks, neurodegeneration and aging in the brain." Mechanisms of Ageing and Development **129**(7-8): 483-491.
Kim, K. S., *et al.* (2009). "Interferon-gamma induces cellular senescence through p53-dependent DNA damage signaling in human endothelial cells." Mechanisms of Ageing and Development **130**(3): 179-188.
Kim, S. T., *et al.* (2002). "Involvement of the cohesin protein, Smc1, in Atm-dependent and independent responses to DNA damage." Genes & Development **16**(5): 560-570.
Kim, W. H., *et al.* (2000). "Induction of p53-independent p21 during ceramide-induced G1 arrest in human hepatocarcinoma cells." Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire **78**(2): 127-135.
Klein, E. A., *et al.* (2000). "SELECT: the selenium and vitamin E cancer prevention trial: Rationale and design." Prostate Cancer and Prostatic Diseases **3**(3): 145-151.
Knight, J. A., *et al.* (2004). "Genetic variants of GPX1 and SOD2 and breast cancer risk at the Ontario site of the breast cancer family registry." Cancer Epidemiology Biomarkers & Prevention **13**(1): 146-149.
Kohrle, J., *et al.* (2005). "Selenium, the thyroid, and the endocrine system." Endocrine Reviews **26**(7): 944-984.
Kong, B. W., *et al.* (2003). "Cloning and expression analysis of chicken phospholipid-hydroperoxide glutathione peroxidase." Animal Biotechnology **14**(1): 19-29.
Kotake, Y., *et al.* (2009). "DDB1-CUL4 and MLL1 mediate oncogene-induced p16(INK4a) activation." Cancer Research **69**(5): 1809-1814.
Kryukov, G. V., *et al.* (2003). "Characterization of mammalian selenoproteomes." Science **300**(5624): 1439-1443.
Kubota, Y., *et al.* (1996). "Reconstitution of DNA base excision-repair with purified human proteins: Interaction between DNA polymerase beta and the XRCC1 protein." Embo Journal **15**(23): 6662-6670.
Kudlow, B. A., *et al.* (2007). "Werner and Hutchinson-Gilford progeria syndromes: mechanistic basis of human progeroid diseases." Nature Reviews Molecular Cell Biology **8**(5): 394-404.
Kurz, D. J., *et al.* (2000). "Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells." J Cell Sci **113 (Pt 20)**: 3613-3622.
Kurz, E. U., *et al.* (2004). "Doxorubicin activates ATM-dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species." Journal of Biological Chemistry **279**(51): 53272-53281.
Kuzminov, A. (2001). "Single-strand interruptions in replicating chromosomes cause double-strand breaks." Proceedings of the National Academy of Sciences of the United States of America **98**(15): 8241-8246.

Lechel, A., *et al.* (2005). "The cellular level of telomere dysfunction determines induction of senescence or apoptosis in vivo." Embo Reports **6**(3): 275-281.
Lee, A. C., *et al.* (1999). "Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species." Journal of Biological Chemistry **274**(12): 7936-7940.
Lee, B. Y., *et al.* (2006). "Senescence-associated beta-galactosidase is lysosomal beta-galactosidase." Aging Cell **5**(2): 187-195.
Lee, J. H., *et al.* (2010). "The p53-inducible gene 3 (PIG3) contributes to early cellular response to DNA damage." Oncogene **29**(10): 1431-1450.
Lee, J. S., *et al.* (2006). "Breast and ovarian cancer in relatives of cancer patients, with and without BRCA mutations." Cancer Epidemiology Biomarkers & Prevention **15**(2): 359-363.
Lee, Y. J., *et al.* (2009). "Involvement of a p53-independent and post-transcriptional up-regulation for p21(WAF/CIP1) following destabilization of the actin cytoskeleton." International Journal of Oncology **34**(2): 581-589.
Leland, S., *et al.* (2009). "Heterozygosity for a Bub1 mutation causes female-specific germ cell aneuploidy in mice." Proceedings of the National Academy of Sciences of the United States of America **106**(31): 12776-12781.
Leong, W. F., *et al.* (2009). "p53 Deficiency leads to compensatory up-regulation of p16INK4a." Mol Cancer Res **7**(3): 354-360.
Li, G. X., *et al.* (2007). "Differential involvement of reactive oxygen species in apoptosis induced by two classes of selenium compounds in human prostate cancer cells." International Journal of Cancer **120**(9): 2034-2043.
Li, G. X., *et al.* (2008). "Superior in vivo inhibitory efficacy of methylseleninic acid against human prostate cancer over selenomethionine or selenite." Carcinogenesis **29**(5): 1005-1012.
Li, H., *et al.* (2010). "Transcriptional factor HBP1 targets P16(INK4A), upregulating its expression and consequently is involved in Ras-induced premature senescence." Oncogene **29**(36): 5083-5094.
Li, S., *et al.* (2007). "Selenium sensitizes MCF-7 breast cancer cells to doxorubicin-induced apoptosis through modulation of phospho-Akt and its downstream substrates." Mol Cancer Ther **6**(3): 1031-1038.
Limbo, O., *et al.* (2007). "Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination." Molecular Cell **28**(1): 134-146.
Lindahl, T. (1993). "Instability and decay of the primary structure of DNA." Nature **362**(6422): 709-715.
Lippman, S. M., *et al.* (2005). "Designing the selenium and vitamin E cancer prevention trial (SELECT)." Journal of the National Cancer Institute **97**(2): 94-102.
Lippman, S. M., *et al.* (2009). "Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT)." JAMA **301**(1): 39-51.
Lippman, S. M., *et al.* (2009). "Effect of Selenium and Vitamin E on Risk of Prostate Cancer and Other Cancers The Selenium and Vitamin E Cancer Prevention

Trial (SELECT)." Jama-Journal of the American Medical Association **301**(1): 39-51.
Lister, L. M., *et al.* (2010). "Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of cohesin and Sgo2." Current Biology **20**(17): 1511-1521.
Liu, N., *et al.* (2005). "ATM deficiency induces oxidative stress and endoplasmic reticulum stress in astrocytes." Laboratory Investigation **85**(12): 1471-1480.
Lowe, S. W., *et al.* (1993). "P53-DEPENDENT APOPTOSIS MODULATES THE CYTOTOXICITY OF ANTICANCER AGENTS." Cell **74**(6): 957-967.
Lu, J. and A. Holmgren (2009). "Selenoproteins." Journal of Biological Chemistry **284**(2): 723-727.
Lu, J., *et al.* (1995). "Dissociation of the genotoxic and growth inhibitory effects of selenium." Biochem Pharmacol **50**(2): 213-219.
Ma, A. H., *et al.* (2000). "Somatic mutation of hPMS2 as a possible cause of sporadic human colon cancer with microsatellite instability." Oncogene **19**(18): 2249-2256.
Ma, Y. M., *et al.* (2005). "Repair of double-strand DNA breaks by the human nonhomologous DNA end joining pathway - The iterative processing model." Cell Cycle **4**(9): 1193-1200.
Machwe, A., *et al.* (2004). "TRF2 recruits the Werner syndrome (WRN) exonuclease for processing of telomeric DNA." Oncogene **23**(1): 149-156.
Macip, S., *et al.* (2003). "Influence of induced reactive oxygen species in p53-mediated cell fate decisions." Mol Cell Biol **23**(23): 8576-8585.
Macip, S., *et al.* (2002). "Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence." EMBO J **21**(9): 2180-2188.
Mao, Z. Y., *et al.* (2008). "DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells." Cell Cycle **7**(18): 2902-2906.
Margolin, Y., *et al.* (2008). "DNA sequence context as a determinant of the quantity and chemistry of guanine oxidation produced by hydroxyl radicals and one-electron oxidants." Journal of Biological Chemistry **283**(51): 35569-35578.
Marshall, J. R., *et al.* (2006). "Design and progress of a trial of selenium to prevent prostate cancer among men with high-grade prostatic intraepithelial neoplasia." Cancer Epidemiology, Biomarkers and Prevention **15**(8): 1479-1484.
Marshall, J. R., *et al.* (2006). "Design and progress of a trial of selenium to prevent prostate cancer among men with high-grade prostatic intraepithelial neoplasia." Cancer Epidemiol Biomarkers Prev **15**(8): 1479-1484.
Martin, L., *et al.* (2009). "Recognition of O6MeG lesions by MGMT and mismatch repair proficiency may be a prerequisite for low-dose radiation hypersensitivity." Radiation Research **172**(4): 405-413.
Maya, R., *et al.* (2001). "ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage." Genes & Development **15**(9): 1067-1077.

McCulloch, S. D. and T. A. Kunkel (2008). "The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases." Cell Research **18**(1): 148-161.
McKinnon, P. J. and K. W. Caldecott (2007). "DNA strand break repair and human genetic disease." Annual Review of Genomics and Human Genetics **8**: 37-55.
Mechanic, L. E., *et al.* (2007). "Common genetic variation in TP53 is associated with lung cancer risk and prognosis in African Americans and somatic mutations in lung tumors." Cancer Epidemiology Biomarkers & Prevention **16**(2): 214-222.
Meydani, M. (1992). "Modulation of the platelet thromboxane A2 and aortic prostacyclin synthesis by dietary selenium and Vitamin E." Biological Trace Element Research **33**: 79-86.
Michaloglou, C., *et al.* (2005). "BRAF(E600)-associated senescence-like cell cycle arrest of human naevi." Nature **436**(7051): 720-724.
Moiseeva, O., *et al.* (2009). "Mitochondrial dysfunction contributes to oncogene-induced senescence." Molecular and Cellular Biology **29**(16): 4495-4507.
Moreno-Reyes, R., *et al.* (1998). "Kashin-Beck osteoarthropathy in rural Tibet in relation to selenium and iodine status." New England Journal of Medicine **339**(16): 1112-1120.
Moreno, C. S., *et al.* (2007). "Evidence that p53-mediated cell-cycle-arrest inhibits chemotherapeutic treatment of ovarian carcinomas." Plos One **2**(5): e441.
Mosmann, T. (1983). "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays." J Immunol Methods **65**(1-2): 55-63.
Mouchawar, J., *et al.* (2010). "Population-Based Estimate of the Contribution of TP53 Mutations to Subgroups of Early-Onset Breast Cancer: Australian Breast Cancer Family Study." Cancer Research **70**(12): 4795-4800.
Munter, L. M., *et al.* (2010). "Aberrant amyloid precursor protein (APP) processing in hereditary forms of Alzheimer Disease caused by APP familial Alzheimer Disease mutations can be rescued by mutations in the APP GxxxG motif." Journal of Biological Chemistry **285**(28): 21636-21643.
Narita, M., *et al.* (2003). "Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence." Cell **113**(6): 703-716.
Navas-Acien, A., *et al.* (2008). "Selenium intake and cardiovascular risk: what is new?" Current Opinion in Lipidology **19**(1): 43-49.
Noguchi, T., *et al.* (2008). "Requirement of reactive oxygen species-dependent activation of ASK1-p38 MAPK pathway for extracellular ATP-induced apoptosis in macrophage." Journal of Biological Chemistry **283**(12): 7657-7665.
Nowacka-Zawisza, M. and W. M. Krajewska (2009). "The role of BRCA1, BRCA2 and RAD51 proteins in the maintenance of genomic stability." Postepy Biologii Komorki **36**(4): 679-694.
Olson, E., *et al.* (2007). "The mre11-Rad50-Nbs1 complex acts both upstream and downstream of ataxia telangiectasia mutated and Rad3-related protein (ATR) to regulate the S-phase checkpoint following UV treatment." Journal of Biological Chemistry **282**(31): 22939-22952.

Olson, G. E., *et al.* (2010). "Extracellular glutathione peroxidase (Gpx3) binds specifically to basement membranes of mouse renal cortex tubule cells." American Journal of Physiology - Renal Physiology **298**(5): F1244-F1253.

Panee, J., *et al.* (2007). "Selenoprotein H is redox-sensing high mobility group family DNA-binding protein that up-regulates genes involved in glutathione synthesis and phase II detoxification." Journal of Biological Chemistry **282**(33): 23759-23765.

Papp, L. V., *et al.* (2007). "From selenium to selenoproteins: synthesis, identity, and their role in human health." Antioxid Redox Signal **9**(7): 775-806.

Pappas, A. C., *et al.* (2008). "Selenoproteins and maternal nutrition." Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology **151**(4): 361-372.

Park, J. H., *et al.* (2006). "Expression of peroxiredoxin and thioredoxin in human lung cancer and paired normal lung." Respirology **11**(3): 269-275.

Parrinello, S., *et al.* (2003). "Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts." Nature Cell Biology **5**(8): 741-747.

Pelle, E., *et al.* (2003). "Ultraviolet-B-induced oxidative DNA base damage in primary normal human epidermal keratinocytes and inhibition by a hydroxyl radical scavenger." Journal of Investigative Dermatology **121**(1): 177-183.

Penney, K. L., *et al.* (2010). "A large prospective study of SEP15 genetic variation, interaction with plasma selenium levels, and prostate cancer risk and survival." Cancer Prevention Research **3**(5): 604-610.

Perez-Campo, R., *et al.* (1998). "The rate of free radical production as a determinant of the rate of aging: evidence from the comparative approach." Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology **168**(3): 149-158.

Persson-Moschos, M. E. K., *et al.* (2000). "Selenoprotein P in plasma in relation to cancer morbidity in middle-aged Swedish men." Nutrition and Cancer-an International Journal **36**(1): 19-26.

Perssonmoschos, M., *et al.* (1995). "Selenoprotein-P in serum as a biochemical marker of selenium status." Analyst **120**(3): 833-836.

Polyak, K., *et al.* (1997). "A model for p53-induced apoptosis." Nature **389**(6648): 300-305.

Polymeropoulos, M. H., *et al.* (1997). "Mutation in the alpha-synuclein gene identified in families with Parkinson's disease." Science **276**(5321): 2045-2047.

Qi, Y., *et al.* (2010). "Selenium compounds activate ATM-dependent DNA damage response via the mismatch repair protein hMLH1 in colorectal cancer cells." Journal of Biological Chemistry **285**(43): 33010-33017.

Rajagopalan, H. and C. Lengauer (2004). "Aneuploidy and cancer." Nature **432**(7015): 338-341.

Ravizza, R., *et al.* (2004). "Role of the p53/p21 system in the response of human colon carcinoma cells to Doxorubicin." Bmc Cancer **4**: 10.

Rayman, M. P. (2002). "The argument for increasing selenium intake." Proceedings of the Nutrition Society **61**(2): 203-215.

Rayman, M. P. (2009). "Selenoproteins and human health: Insights from epidemiological data." Biochimica Et Biophysica Acta-General Subjects **1790**(11): 1533-1540.
Rayman, M. P., *et al.* (2009). "Selenium and Vitamin E Supplementation for Cancer Prevention." Jama-Journal of the American Medical Association **301**(18): 1876-1876.
Reid, M. E., *et al.* (2002). "Selenium supplementation and lung cancer incidence: An update of the nutritional prevention of cancer trial." Cancer Epidemiology Biomarkers & Prevention **11**(11): 1285-1291.
Ridley, A. J., *et al.* (2009). "Cellular and sub-cellular responses to UVA in relation to carcinogenesis." International Journal of Radiation Biology **85**(3): 177-195.
Rodier, F., *et al.* (2009). "Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion." Nature Cell Biology **11**(8): 973-U142.
Ruijs, M. W. G., *et al.* (2010). "TP53 germline mutation testing in 180 families suspected of Li-Fraumeni syndrome: mutation detection rate and relative frequency of cancers in different familial phenotypes." Journal of Medical Genetics **47**(6): 421-428.
Saito, Y., *et al.* (1999). "Selenoprotein P in human plasma as an extracellular phospholipid hydroperoxide glutathione peroxidase - Isolation and enzymatic characterization of human selenoprotein P." Journal of Biological Chemistry **274**(5): 2866-2871.
Salonen, J. T., *et al.* (1991). "Effects of antioxidant supplementation on platelet-function - a randomized pair-matched, placebo-controlled, double-blind trial in men with low antioxidant status." American Journal of Clinical Nutrition **53**(5): 1222-1229.
Sancar, A., *et al.* (2004). "Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints." Annual Review of Biochemistry **73**: 39-85.
Sarkisian, C. J., *et al.* (2007). "Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis." Nature Cell Biology **9**(5): 493-U425.
Sarveswaran, S., *et al.* (2010). "Selenite triggers rapid transcriptional activation of p53, and p53-mediated apoptosis in prostate cancer cells: Implication for the treatment of early-stage prostate cancer." International Journal of Oncology **36**(6): 1419-1428.
Schnabel, R., *et al.* (2005). "Glutathione peroxidase-1 and homocysteine for cardiovascular risk prediction: results from the AtheroGene study." Journal of the American College of Cardiology **45**(10): 1631-1637.
Schneider, M., *et al.* (2009). "Mitochondrial glutathione peroxidase 4 disruption causes male infertility." Faseb Journal **23**(9): 3233-3242.
Schomburg, L., *et al.* (2003). "Gene disruption discloses role of selenoprotein P in selenium delivery to target tissues." Biochemical Journal **370**: 397-402.
Schrauzer, G. N., *et al.* (1977). "Cancer mortality correlation studies .3. statistical associations with dietary selenium intakes." Bioinorganic Chemistry **7**(1): 23-34.

Schwaab, V., *et al.* (1998). "GPx3: The plasma-type glutathione peroxidase is expressed under androgenic control in the mouse epididymis and vas deferens." Molecular Reproduction and Development **51**(4): 362-372.
Seemann, S., *et al.* (2004). "The tumor suppressor gene TP53: Implications for cancer management and therapy." Critical Reviews in Clinical Laboratory Sciences **41**(5-6): 551-583.
Seluanov, A., *et al.* (2001). "Change of the death pathway in senescent human fibroblasts in response to DNA damage is caused by an inability to stabilize p53." Molecular and Cellular Biology **21**(5): 1552-1564.
Seyfried, J. and U. Wullner (2007). "Inhibition of thioredoxin reductase induces apoptosis in neuronal cell lines: Role of glutathione and the MKK4/JNK pathway." Biochemical and Biophysical Research Communications **359**(3): 759-764.
Shaham, J., *et al.* (2003). "DNA-protein crosslinks and p53 protein expression in relation to occupational exposure to formaldehyde." Occupational and Environmental Medicine **60**(6): 403-409.
Shamberg.Rj and D. V. Frost (1969). "POSSIBLE PROTECTIVE EFFECT OF SELENIUM AGAINST HUMAN CANCER." Canadian Medical Association Journal **100**(14): 682-&.
Shargorodsky, M., *et al.* (2010). "Effect of long-term treatment with antioxidants (vitamin C, vitamin E, coenzyme Q10 and selenium) on arterial compliance, humoral factors and inflammatory markers in patients with multiple cardiovascular risk factors." Nutrition & Metabolism **7**: 55-62.
Shen, C. L., *et al.* (2001). "Interactions of selenium compounds with other antioxidants in DNA damage and apoptosis in human normal keratinocytes." Cancer Epidemiol Biomarkers Prev **10**(4): 385-390.
Shieh, S. Y., *et al.* (1997). "DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2." Cell **91**(3): 325-334.
Shiloh, Y. (2003). "ATM and related protein kinases: Safeguarding genome integrity." Nature Reviews Cancer **3**(3): 155-168.
Shim, M. S., *et al.* (2009). "Elevation of glutamine level by selenophosphate synthetase 1 knockdown induces megamitochondrial formation in drosophila cells." Journal of Biological Chemistry **284**(47): 32881-32894.
Smith, M. L., *et al.* (2004). "Selenium compounds regulate p53 by common and distinctive mechanisms." Anticancer Research **24**(3A): 1401-1408.
Smolkova, B., *et al.* (2004). "Folate levels determine effect of antioxidant supplementation on micronuclei in subjects with cardiovascular risk." Mutagenesis **19**(6): 469-476.
Soerensen, J., *et al.* (2008). "The role of thioredoxin reductases in brain development." Plos One **3**(3): e1813.
Spallholz, J. E., *et al.* (2001). "Dimethyldiselenide and methylseleninic acid generate superoxide in an in vitro chemiluminescence assay in the presence of glutathione: implications for the anticarcinogenic activity of L-selenomethionine and L-Se-methylselenocysteine." Nutr Cancer **40**(1): 34-41.

Squatrito, M., *et al.* (2010). "Loss of ATM/Chk2/p53 pathway components accelerates tumor development and contributes to radiation resistance in gliomas." Cancer Cell **18**(6): 619-629.
Squires, J. E. and M. J. Berry (2008). "Eukaryotic selenoprotein synthesis: Mechanistic insight incorporating new factors and new functions for old factors." Iubmb Life **60**(4): 232-235.
Srivenugopal, K. S., *et al.* (1996). "Ubiquitination-dependent proteolysis of O-6-methylguanine-DNA methyltransferase in human and murine tumor cells following inactivation with O-6-benzylguanine or 1,3-bis(2-chloroethyl)-1-nitrosourea." Biochemistry **35**(4): 1328-1334.
St Germain, D. L., *et al.* (2005). "Insights into the role of deiodinases from studies of genetically modified animals." Thyroid **15**(8): 905-916.
Steinbrenner, H., *et al.* (2006). "Involvement of selenoprotein P in protection of human astrocytes from oxidative damage." Free Radical Biology and Medicine **40**(9): 1513-1523.
Stewart, M. S., *et al.* (1999). "Selenium compounds have disparate abilities to impose oxidative stress and induce apoptosis." Free Radic Biol Med **26**(1-2): 42-48.
Stiff, T., *et al.* (2004). "ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation." Cancer Res **64**(7): 2390-2396.
Stranges, S., *et al.* (2006). "Effects of selenium supplementation on cardiovascular disease incidence and mortality: Secondary analyses in a randomized clinical trial." American Journal of Epidemiology **163**(8): 694-699.
Stranges, S., *et al.* (2010). "A prospective study of dietary selenium intake and risk of type 2 diabetes." Bmc Public Health **10**: 564-571.
Sun, Q. A., *et al.* (2001). "Selenoprotein oxidoreductase with specificity for thioredoxin and glutathione systems." Proceedings of the National Academy of Sciences of the United States of America **98**(7): 3673-3678.
Sung, J. S. and B. Demple (2006). "Roles of base excision repair subpathways in correcting oxidized abasic sites in DNA." Febs Journal **273**(8): 1620-1629.
Symonds, H., *et al.* (1994). "p53-dependent apoptosis suppresses tumor growth and progression in vivo." Cell **78**(4): 703-711.
Takashima, Y., *et al.* (2009). "Dependence of DNA double strand break repair pathways on cell cycle phase in human lymphoblastoid cells." Environmental and Molecular Mutagenesis **50**(9): 815-822.
Taylor, W. R. and G. R. Stark (2001). "Regulation of the G2/M transition by p53." Oncogene **20**(15): 1803-1815.
te Poele, R. H., *et al.* (2002). "DNA damage is able to induce senescence in tumor cells in vitro and in vivo." Cancer Research **62**(6): 1876-1883.
Thompson, I. M. (2007). "Chemoprevention of prostate cancer: agents and study designs." J Urol **178**(3 Pt 2): S9-S13.
Thompson, I. M. (2007). "Chemoprevention of prostate cancer: agents and study designs." Journal of Urology **178**(3 Pt 2): S9-S13.
Thompson, K. M., *et al.* (1998). "Liver selenium and testis phospholipid hydroperoxide glutathione peroxidase are associated with growth during selenium repletion of second-generation Se-deficient male rats." Journal of Nutrition **128**(8): 1289-1295.

Tian, L., *et al.* (2009). "Distinct nucleotide binding/hydrolysis properties and molar ratio of MutS alpha and MutS beta determine their differential mismatch binding activities." Journal of Biological Chemistry **284**(17): 11557-11562.
Tibbetts, R. S., *et al.* (1999). "A role for ATR in the DNA damage-induced phosphorylation of p53." Genes Dev **13**(2): 152-157.
Turenne, G. A., *et al.* (2001). "Activation of p53 transcriptional activity requires ATM's kinase domain and multiple N-terminal serine residues of p53." Oncogene **20**(37): 5100-5110.
Unger, T., *et al.* (1999). "Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2." Embo Journal **18**(7): 1805-1814.
Ursini, F., *et al.* (1999). "Dual function of the selenoprotein PHGPx during sperm maturation." Science **285**(5432): 1393-1396.
Valko, M., *et al.* (2007). "Free radicals and antioxidants in normal physiological functions and human disease." International Journal of Biochemistry & Cell Biology **39**(1): 44-84.
Valko, M., *et al.* (2006). "Free radicals, metals and antioxidants in oxidative stress-induced cancer." Chem Biol Interact **160**(1): 1-40.
Vaziri, H. and S. Benchimol (1998). "Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span." Current Biology **8**(5): 279-282.
Vitoux, D., *et al.* (1996). "Selenium, glutathione peroxidase, peroxides and platelet functions." Annales De Biologie Clinique **54**(5): 181-187.
von Kobbe, C., *et al.* (2004). "Werner syndrome cells escape hydrogen peroxide-induced cell proliferation arrest." Faseb Journal **18**(12): 1970-+.
Vural, H., *et al.* (2010). "Alterations of plasma magnesium, copper, zinc, iron and selenium concentrations and some related erythrocyte antioxidant enzyme activities in patients with Alzheimer's disease." Journal of Trace Elements in Medicine and Biology **24**(3): 169-173.
Wang, H. L., *et al.* (2007). "Elemental selenium at nano size possesses lower toxicity without compromising the fundamental effect on selenoenzymes: Comparison with selenomethionine in mice." Free Radical Biology and Medicine **42**(10): 1524-1533.
Wang, L., *et al.* (2009). "Methyl-selenium compounds inhibit prostate carcinogenesis in the transgenic adenocarcinoma of mouse prostate model with survival benefit." Cancer Prev Res (Phila) **2**(5): 484-495.
Wang, Z., *et al.* (2008). "Methylseleninic acid inhibits microvascular endothelial G1 cell cycle progression and decreases tumor microvessel density." Int J Cancer **122**(1): 15-24.
Ward, I. M. and J. Chen (2001). "Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress." Journal of Biological Chemistry **276**(51): 47759-47762.
Waters, D. J., *et al.* (2005). "Prostate cancer risk and DNA damage: translational significance of selenium supplementation in a canine model." Carcinogenesis **26**(7): 1256-1262.

Waters, D. J., *et al.* (2005). "Prostate cancer risk and DNA damage: translational significance of selenium supplementation in a canine model." Carcinogenesis **26**(7): 1256-1262.
Wei, W. and E. W. Englander (2008). "DNA polymerase beta-catalyzed-PCNA independent long patch base excision repair synthesis: a mechanism for repair of oxidatively damaged DNA ends in post-mitotic brain." Journal of Neurochemistry **107**(3): 734-744.
Weinrich, S. L., *et al.* (1997). "Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT." Nature Genetics **17**(4): 498-502.
Westin, E. R., *et al.* (2007). "Telomere restoration and extension of proliferative lifespan in dyskeratosis congenita fibroblasts." Aging Cell **6**(3): 383-394.
Williams, L., *et al.* (2009). "Aneuploidy involving chromosome 1 may be an early predictive marker of intestinal type gastric cancer." Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis **669**(1-2): 104-111.
Wilson, D. M. (2007). "Processing of nonconventional DNA strand break ends." Environmental and Molecular Mutagenesis **48**(9): 772-782.
Wright, J. D., *et al.* (2002). "Factors governing loss and rescue of DNA binding upon single and double mutations in the p53 core domain." Nucleic Acids Research **30**(7): 1563-1574.
Wu, C. H., *et al.* (2007). "Cellular senescence is an important mechanism of tumor regression upon c-Myc inactivation." Proceedings of the National Academy of Sciences of the United States of America **104**(32): 13028-13033.
Wu, M., *et al.* (2010). "Selenium compounds activate early barriers of tumorigenesis." Journal of Biological Chemistry **285**(16): 12055-12062.
Wyllie, F. S., *et al.* (2000). "Telomerase prevents the accelerated cell ageing of Werner syndrome fibroblasts." Nature Genetics **24**(1): 16-17.
Xu, X. M., *et al.* (2007). "Selenophosphate synthetase 2 is essential for selenoprotein biosynthesis." Biochemical Journal **404**: 115-120.
Xu, X. M., *et al.* (2007). "New developments in selenium biochemistry: Selenocysteine biosynthesis in eukaryotes and archaea." Biological Trace Element Research **119**(3): 234-241.
Yan, W. and X. Chen (2006). "GPX2, a direct target of p63, inhibits oxidative stress-induced apoptosis in a p53-dependent manner." Journal of Biological Chemistry **281**(12): 7856-7862.
Yazdi, P. T., *et al.* (2002). "SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint." Genes & Development **16**(5): 571-582.
Youngman, L. D., *et al.* (1992). "Protein oxidation associated with aging is reduced by dietary restriction of protein or calories." Proceedings of the National Academy of Sciences of the United States of America **89**(19): 9112-9116.
Zachara, B. A., *et al.* (2003). "Selenium and glutathione peroxidases in blood of patients with different stages of chronic renal failure." Journal of Trace Elements in Medicine and Biology **17**(4): 291-299.

Zeng, H., *et al.* (2009). "Methylselenol, a selenium metabolite, induces cell cycle arrest in G1 phase and apoptosis via the extracellular-regulated kinase 1/2 pathway and other cancer signaling genes." J Nutr **139**(9): 1613-1618.
Zhang, H. S., *et al.* (1999). "Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFbeta, and contact inhibition." Cell **97**(1): 53-61.
Zhang, R., *et al.* (2005). "Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA." Developmental Cell **8**(1): 19-30.
Zhang, S., *et al.* (2010). "Selenoproteins and the aging brain." Mechanisms of Ageing and Development **131**(4): 253-260.
Zhao, R., *et al.* (2006). "Apoptosis induced by selenomethionine and methioninase is superoxide mediated and p53 dependent in human prostate cancer cells." Mol Cancer Ther **5**(12): 3275-3284.
Zhao, R., *et al.* (2006). "Apoptosis induced by selenomethionine and methioninase is superoxide mediated and p53 dependent in human prostate cancer cells." Molecular Cancer Therapeutics **5**(12): 3275-3284.
Zhao, R., *et al.* (2006). "Expression of p53 enhances selenite-induced superoxide production and apoptosis in human prostate cancer cells." Cancer Research **66**(4): 2296-2304.
Zhou, N., *et al.* (2003). "DNA damage-mediated apoptosis induced by selenium compounds." J Biol Chem **278**(32): 29532-29537.
Zhou, W. and P. W. Doetsch (1993). "Effects of abasic sites and DNA single-strand breaks on prokaryotic RNA-polymerases." Proceedings of the National Academy of Sciences of the United States of America **90**(14): 6601-6605.
Zhou, X. Y., *et al.* (2002). "An ATM-independent S-phase checkpoint response involves CHK1 pathway." Cancer Research **62**(6): 1598-1603.
Zhou, X. Y., *et al.* (2002). "Ku affects the ATM-dependent S phase checkpoint following ionizing radiation." Oncogene **21**(41): 6377-6381.
Zhou, X. Z., *et al.* (2003). "Role of Pin2/TRF1 in telomere maintenance and cell cycle control." Journal of Cellular Biochemistry **89**(1): 19-37.
Zhuo, H. J., *et al.* (2004). "Selenium and lung cancer: A quantitative analysis of heterogeneity in the current epidemiological literature." Cancer Epidemiology Biomarkers & Prevention **13**(5): 771-778.
Zimprich, A., *et al.* (2004). "Mutations in LRRK2 cause autosomal-dominant Parkinsonism with pleomorphic pathology." Neuron **44**(4): 601-607.