ABSTRACT

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Oxidative stress and persistent DNA damage response can lead to cellular senescence and aging. The ATM kinase and p53 protein play critical roles in the DNA damage response to reactive oxygen species and other DNA-damaging agents. Although the majority of selenoproteins carry antioxidant activities, little is known about the nutritional role of selenium (Se) in aging. Previous studies indicated that selenoprotein H (SelH) is very sensitive to dietary Se deficiency. Moreover, SelH is a nuclear selenoprotein that is proposed to carry redox domains and to transactivate redox genes including one for glutathione biosynthesis. To determine the role of SelH in genome maintenance, SelH and scrambled shRNA knockdown were stably established in MRC-5 human diploid fibroblast or immortalized cancer cells. SelH shRNA MRC-5 cells showed more pronounced induction of β-galactosidase expression, autofluorescence, growth inhibition, and ATM pathway activation (γH2AX and phospho-ATM Ser-1981) as compared to scrambled shRNA cells. Interestingly, the slow proliferation in SelH shRNA MRC-5 cells was alleviated in the presence of ATM kinase inhibitors KU 55933 and KU 60019, by p53 shRNA knockdown, or by maintaining the cells in 3% O_2 incubator (vs. ambient O_2). Phospho-ATM Ser-1981 and γH2AX induction by H_2O_2 treatment (20 μM) was temporally exacerbated in SelH shRNA but reversed in the scrambled shRNA MRC-5 cells 1-5 days after recovery. GFP-SelH did not relocalize to sites of oxidative DNA damage. Results from cologenic assays indicated that SelH shRNA HeLa cells were hypersensitive to paraquat and H_2O_2 but not to other

clastogens including hydroxyurea, neocarzinostatin or camptothecin. The H_2O_2-induced cell death was attenuated in the presence of *N*-acetyl cysteine (NAC), a glutathione analogue, in SelH but not in scrambled shRNA HeLa cells. In conclusion, SelH protects against cellular senescence specifically to oxidative stress through a genome maintenance pathway involving ATM and p53.

While recent research has demonstrated that mice unable to express selenoproteins in epidermal cells or in osteo-chondroprogenitor cells showed an apparently aging phenotype characterized by alopecia or bone abnormality, respectively. Thus, a role of selenium, particularly at nutritional levels of intake, in aging is largely unknown. What is lacking is an appropriate aging model of dietary Se deprivation displaying many features of normal aging. Telomere attrition provokes DNA damage response and, subsequently, replicative senescence. Because the chromosomes of mice carry longer telomeres than those of humans, the proposed hypothesis is that lengthy telomeres preclude mice deprived of Se to display aging phenotypes and age-related disorders. To test this hypothesis, weanling late generation *Terc*$^{-/-}$ mice were fed a Se-deficient diet or the diet supplemented with selenate (0.15 ppm) throughout their life. The objectives are to elucidate the role of Se in reducing age-related loss of function and begin to identify the key molecular mediators and selenoproteins during the aging process. As evidenced by changes in metabolic markers (body weight, glucose intolerance, insulin resistance and bone structure) and aging phenotypes (gray hair, alopecia, wound healing and telomere attrition), these data strongly indicate health span deterioration by dietary Se deficiency in the short telomere mice. MicroRNAs (miRNAs) are regulators of messenger RNA stability and translation and have been proposed as biomarkers for a variety of diseases and physiological conditions, including aging. A high-throughput platform, TaqMan low density array, was used to profile more than 800

miRNAs in plasma whose expression were validated by using individual quantitative PCR. The expression of a couple of miRNAs were induced both by dietary Se deprivation and aging. Altogether, a very interesting model of aging is established in this project by deprivation of Se that displays many hallmarks of human aging and can reveal the roles of Se at nutritional levels, in contrast with previous approaches, in which these essential roles in delaying health span deterioration may have been masked by lengthy telomeres.

Nutritional roles of selenium in cellular and mouse aging

By

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Dedication

I dedicate this dissertation to my great parents. Learning is a life long journey. I am so lucky to always have your support to enjoy this trip.

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1.1 Introduction

Healthy aging with minimum age-related disorders is the aspiration of our aging society. The average life expectancy in modern society is getting longer and longer because of the improvements on the knowledge of medicine and technology; however, this trend may be compromised when elderlies suffer from age-related disorders and live with poor quality of life (Westendorp, 2006). Thus, it is critical and required to understand how and why we age.

Aging is a complicated process with sophisticated changes at different levels, resulting in age-related disorders and shortened lifespan. Currently, there are various theories of aging to explain this phenomenon at the level of molecule, cell, system, and evolution (Weinert and Timiras, 2003). Our genome constantly accumulates DNA damage and mutations, thus impacting on cell survival. Proliferating cells with persistent DNA damage may be arrested at certain stages of cell cycle permanently, which can interfere with the systems in the body and jeopardize the quality of life. On the other hand, evolution selects those phenotypes for specific habitat and behavior, which may either accelerate or attenuate the aging process. These changes as a whole decide the life expectancy of the species. Thus, securing genome stability is of fundamental importance for healthy aging by protecting against aging and age-related disorders.

Available lines of scientific evidence on the protection against aging and age-related disorders by bioactive compounds and nutrients shed lights on possible interventional

strategies for healthy aging. Arising from French Paradox, the red wine phytochemical, resveratrol, has been shown to extend the lifespan of *Saccharomyces cerevisiae* through the activation of Sirtuins (Howitz et al., 2003; Wood et al., 2004). Additionally, resveratrol improves the health span and lifespan of mice on a high fat diet (Baur et al., 2006). Moreover, vitamin C, the natural antioxidant that prevents scurvy, is known to counteract many aging phenotypes found in a mouse model for Werner syndrome in humans, suggesting a potential intervention approach for Werner syndrome patients to age with quality of life (Massip et al., 2010). Because the potential of dietary bioactive components on healthy aging is largely unknown, understanding their mechanisms of action during the aging process is necessary and significant.

Selenium, a trace mineral, is of great potential to prevent aging and age-related disorders. Selenium exists in the cells as chemical derivatives or selenium-containing proteins at nutritional level. According to free radical theory of aging, reactive oxygen species accumulate with age (Harman, 1956, 1960). Interestingly, the majority of selenoproteins in humans exhibits antioxidant functions, and there are eleven selenoproteins thought to be associated with aging and age-related disorders (McCann and Ames, 2011). On the other hand, the supranutritional selenium consumption may counteract a major age-related disorder, carcinogenesis (Ip et al., 1991; Wu et al., 2010). To protect against aging and age-related disorders by a nutritional intervention approach, the cell- and animal-based studies are useful and critical to understand the functions of selenium and selenoproteins in health span and lifespan.

1.2 Genome Stability

Genome stability is secured by DNA damage response pathways, resulting in checkpoint activation, senescence and apoptosis at the cellular level. In the nuclei and mitochondria, DNA damage is addressed by direct DNA repair, epigenetic modifications, telomere protection and perhaps lesion bypass. Thus, genome maintenance is important to prevent aging and age-related disorders. DNA damage and epigenetic alteration may generate gene mutations or alter the transcriptome. Telomere attrition occurs naturally after each round of DNA replication. Chromosome ends are looped back to from the "T-loop" structures that mask the otherwise signals for DNA damage response. When reaching a critically short level, telomeres are presented in a form reminiscent of DNA breaks, thus triggering a DNA damage response. Although we have well developed DNA repair mechanisms and epigenetic regulation to maintain genome stability, DNA damage accumulation, declined DNA repair, or both occur on the genome as we age, resulting in aging and age-related disorders.

1.2.1 DNA Damages and DNA Repair Mechanisms

DNA repair mechanisms are required to secure genome stability for maintenance of normal cellular functions, but they are challenged by various endogenous and exogenous DNA damaging agents, including oxidative stress, UV light, carcinogens and mutagens, and ionized irradiation. By estimation, there are about 10^5 events of DNA damage generated per cell per day (Ciccia and Elledge, 2010), most of which, however, are corrected by the various DNA repair pathways. Oxidized bases, single strand breaks and pyrimidine dimers are repaired by base excision repair (BER) or nucleotide excision repair (NER), mismatched bases are repaired by mismatch repair (MMR), and interstrand crosslinks and doubled strand breaks are repaired by homologous recombination (HR) or

non-homologous end-join (NHEJ). When there are DNA damage accumulation or declined DNA repair activities, unrepaired DNA damage sets the stage for genome instability and cellular aging.

Genome instability through continuous accumulation of unrepaired DNA damage is linked to aging and age-related disorders; however, the underlying mechanism is largely unknown. In particular, the linkage between genome maintenance and antioxidant nutrients such as selenium is not well studied. According to the free radical theory of aging, reactive oxygen species (ROS) mainly accumulated and produced through respiratory chain reaction in mitochondria are thought to be causative with age. The increased oxidative stress may generate base oxidation, DNA single and double strand breaks (Halliwell and Aruoma, 1991). Indeed, oxidative DNA damage has been found to be accumulated with age (Sohal et al., 1994). Noteworthy, animals with higher level of oxidative stress produced from their mitochondria have shorter maximum lifespan (Sohal and Brunk, 1992). Moreover, as one of the major age-related disorders, cancer at precancerous stage is thought to be susceptible to oxidative stress and accumulate unrepaired DNA damage to a point when significant mutations are formed that drive the transition from precancerous to malignant cells (Stratton et al., 2009). Eventually, malignant cells carry severely mutated and unstable genome and are resistant to oxidative stress, DNA damage and cell death (Cairns et al., 2011).

In order to maintain genome stability, mammalian cells are evolved to repair various types of damage through BER, NER, MMR, HR and NHEJ. Defective DNA repair is the etiology of some progeroid syndromes, which are characterized by early onset of aging and age-related disorders with shortened lifespan. The hallmark progeroid syndromes is

Werner syndrome; others include Bloom syndrome, Cockayne syndrome, Rothmund-Thomson syndrome, ataxia-telangiectasia, Nijmegen breakage syndrome, xeroderma pigmentosum and trichothiodystrophy. As aging is the accumulative phenotypes of various genomic, physiological and pathophysiological declines, none of the progeroid syndromes fully mimic normal aging, although they share various degree of similarities. For example, both ataxia-telangiestasia and Nijmegen breakage syndrome show immunodeficiency, genome instability, clinical radiosensitivity and cancer predisposition (Shiloh, 1997), and Werner syndrome and Nijmegen breakage syndrome both show graying of hair, short stature, a "bird-like" face, increased cancer susceptibility and reduced lifespan (reviewed in (Cheng et al., 2004)). In particular, the five members of the RecQ family of DNA helicases in humans seem to play a key role in the maintenance of general DNA metabolism including damage response, repair and replication. Werner syndrome, Bloom syndrome and Rothmund-Thomson syndrome are caused by mutations in the specific RecQ helicases (Bohr, 2008). Xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy are caused by defective NER (de Boer and Hoeijmakers, 2000). On the other hand, lack of general DNA repair capacity was found in older individuals and age-related disorders. Primary hepatocytes isolated from old rats are more sensitive to oxidative stress induced DNA damage than those from younger rats, owing to declined DNA repair activities in the old hepatocytes (Kennah et al., 1985). In human samples, the capacity of DNA DSB repair progressively decreases during the aging process (Garm et al., 2013). Additionally, the patients with Alzheimer's disease, Parkinson's disease and stroke are found to be defective in the repair of acute oxidative

damages in the neurons (Canugovi et al., 2013). Altogether, declined DNA repair contributes to aging and age-related disorders through increased genome instability.

1.2.2 Epigenetic regulation

Epigenetic modifications regulate the conformation and function of chromosomes. In the genome, DNA double helix is wrapped with histones to form nucleosome, which in turn is assembled to the more compact chromatin. The 23 pairs of chromosomes are observed only in the mitotic phase of cell cycle when chromatin fibers are further packed. The degree of compactness determines whether a gene can be expressed or not on the chromatin. The relaxed form of chromatin is known as euchromatin, which is accessible for transcriptional machinery to act on RNA transcription. On the other hand, DNA in heterochromatin is buried inside and inactive for RNA transcription. Therefore, chromatin conformation can regulate gene expression in an epigenetic manner. Histone modifications, known as histone codes, represent another key event of epigenetic regulation of gene expression. Known histone codes that can relax chromatin structure include lysine 4 methylation, lysine 9 acetylation and lysine 14 acetylation on histone 3 and lysine 9 acetylation and arginine 3 methylation on histone 4. On the contrary, histone codes that promote chromatin condensation include histone 4 lysine 12 acetylation and histone 3 lysine 9 methylation (Jenuwein and Allis, 2001). The histone codes are modified by antagonistic enzymes for chromatin relaxation and condensation. Histone acetylation status is antagonistically regulated by histone acetyltransferase and histone deacetylase (Lee and Workman, 2007; Thiagalingam et al., 2003), and methylation by histone methyltransferase and histone demethylase (Lam et al., 2005). These epigenetic

events determine the compactness of the local chromatin that physically regulates the accessibility of transcription factors.

Chromosome structural changes play a pivotal role in the pathogenesis of aging and age-related disorders. In certain types of human diploid cells, pRb pathway activates cellular senescence and promotes the formation of heterochromatin foci (Kosar et al., 2011; Narita et al., 2003). Interestingly, chromatin relaxation in the nucleus helps DNA DSB repair to maintain genome stability (Krishnan et al., 2011). However, a recent publication indicates that maintaining heterochromatin in aging genes can attenuate the aging process (Rando and Chang, 2012). At ribosomal DNA (rDNA) loci, the heterochromatin formation promotes the extension of longevity in *Drosophila* (Larson et al., 2012). Additionally, histone 3 lysine 4 trimethylation modifiers, ASH-2, WDR-5, or SET-2, control euchromatin formation. When these modifiers are knocked down in *C. elegans*, the worms live longer due largely to the formation of heterochromatin (Greer et al., 2011). Nonetheless, the optimal chromatin conformation in favor of longevity remains unknown. The next big question in this area is to answer whether the chromatin conformation change in the aging genome is locus specific or stage specific. With that being elucidated, we will begin to develop knowledge-based intervention approaches to delay the onset of aging and age-related disorders.

DNA methylation is another key event of epigenetic modifications. DNA methylation event modifies on the cytosine of the CpG dinucleotide, and is involved in the regulation of gene expression and genome maintenance (Ehrlich, 2002). About 57~71 % of human genomes contain methylation at CpG dinucleotide (Cooper, 1983). Among all CpG dinucleotides in the genome, the methylation at CpG islands is critical to regulate gene

expression. CpG island is a cluster of CpG dinucleotide in or near the promoter regions or the exon 1 of the gene (Robertson and Wolffe, 2000), and there are about 45000 CpG islands known to exist in humans (Antequera and Bird, 1993). Over half of the CpG islands are hypomethylated at active promoter region, but hypermethylated CpG dinucleotide around the promoter region inhibits gene transcription (Toth et al., 1989). In some cases of cancer, the promoter regions of the tumor suppressor genes are hypermethylated, supporting the development of tumorigenesis (Jones and Laird, 1999). For genome defense, defective DNA methylation promotes DNA deletion in DNA methyltransferase knockout mice (Chen et al., 1998). The evidence that supports the hypothesis of CpG suppression on evolution is provided by Cooper et al., from which methylated CpG dinucleotide is proposed to stabilize genome stability (Cooper and Krawczak, 1989). Results from human lung cancer deep sequencing analyses have showed that DNA hypomethylation is specific on repetitive sequences (short and long interspersed nuclear elements and long terminal repeats), segmental duplications, and subtelomeric regions (Rauch et al., 2008). Thus, DNA methylation is of fundamental and physiological significance to maintain genome stability and to regulate the expression of oncogenes and tumor suppressor genes.

1.2.3 Telomere Maintenance

Telomere length is one of the determinants of lifespan. Telomere is composed of the TTAGGG repetitive sequence at the very end of chromosome (Henderson and Blackburn, 1989). In somatic cells, the length of telomere decreases after each round of cell division because of the natural defect in DNA replication (Greider, 1996). Telomeres lose about 55bp after each cell doubling, and telomere attrition occurs in mortal cells both *in vitro*

and *in vivo* (Aviv et al., 2001; Harley et al., 1990). When the length of telomere was shortened to the critical threshold, such dysfunctional telomere invokes DNA damage response, dampens chromosome stability and limits cell viability (Hemann et al., 2001). DNA damage response and repair at chromosome ends with short telomeres are abnormal, and can result in chromosome end-to-end joining through the NHEJ pathway of DNA DSB repair. Despite of increasing genome instability, expressing telomerase in mortal cells can rescue the cells and bypass the restriction on senescence (Greider, 1998).

Shelterin is required to structurally and functionally protect telomeres. Shelterin is composed of six proteins: TRF1, TRF2, TPP1, POT1, TIN2 and Rap1. TRF1, TRF2 and POT1 recognize and bind to TTAGGG repeats. TIN2, TPP1 and Rap1 do not directly bind to TTAGGG repeats, but appear at telomeres by association with TRF1, TRF2 and/or POT1 (Palm and de Lange, 2008). There are two specialized structures at telomeres, telomeric-loop (T-loop) and displacement-loop (D-loop), which are stabilized by shelterin. At the very end of chromosome, the duplex-repeat of telomeres bends and forms the T-loop, where shelterin binds to the duplex-repeat for stabilizing the structure. Then, POT1 unwinds a part of the duplex repeat by binding to single strand TTAGGG repeat, enabling the 3' overhang of telomeres to invade the duplex repeat of telomere. This 3' overhang containing structure is called D-loop, which is required to protect telomere ends from being recognized as DNA damage (de Lange, 2004; Griffith et al., 1999). Without the protection of shelterin, the uncapped telomere can be recognized as a form of DNA damage and provokes chromosome end-to-end fusion (Celli and de Lange, 2005). Loss of functional TRF2 or POT1 exposes telomere ends and induces DNA damage response through the ataxia telangiectasia mutated (ATM) or ataxia

telangiectasia mutated rad3 related (ATR) pathway activation (Denchi and de Lange, 2007; van Steensel et al., 1998). On the other hand, TRF1 is a negative regulator of telomere integrity as it inhibits the elongation of telomere (van Steensel and de Lange, 1997).

1.3 Cellular Senescence

Cellular senescence is a permanent withdraw from cell cycle that completely stop cellular proliferation. Cells complete a cell division cycle through G0/G1, S, and G2/M phases. Single cell organism maintains normal cell cycle in order to keep propagate. Furthermore, cellular proliferation helps to renew or replenish cells in organs and tissues for optimal functions in multicellular organisms. When cells encounter specific stress inducers, transient cell cycle arrest is activated to allow time for recover, followed by re-entry into normal cell cycle if the damage is fixed. However, if the damage is too severe to repair, the cell cycle arrests permanently, resulting in cellular senescence.

Cellular senescence appears to be a double-edged sword concerning optimal health. Because cellular senescence arrests cell cycle permanently, this is a potential strategy for anti-cancer or old tissue regeneration. Nevertheless, cellular senescence contributes to organismal aging. Hence, understanding how cellular senescence is regulated might shed lights on cancer or aging interventions. Here, the focus is on the causes and features of cellular senescence, and to discuss the molecular regulation of cellular senescence driven by DNA damage response and the key effecter protein, p53.

1.3.1 The activation of cellular senescence

Cellular senescence can be induced by unrepaired DNA damage, dysfunctional telomere, acute chromosome conformation change, and mitogenic inducers. Broadly speaking, there are two types of senescence: telomere-dependent replicative senescence and stress-induced senescence. The molecular mechanisms of cellular senescence are not fully understood, but several inducers have been reported. First, persistent DNA damage response (DDR) including unrepaired DNA damage or dysfunctional telomere can activate downstream mediators, resulting in cellular senescence. Second, the acute changes in chromosome structures, such as a switch from heterochromatin to euchromatin, can lead to cellular senescence through DDR activation in the process of chromosome structure relaxation (Ziv et al., 2006). Finally, mitogenic stimuli can promote proliferation at the expense of collapsed replication forks or misfired replications that activate DDR.

1.3.1.1 Dysfunctional telomere and persistent DNA damage responses induced cellular senescence

DNA damage compromises genome integrity. Our genome takes charge all the functions from cells to the whole body level. Unfortunately, there are various endogenous sources of DNA damage. Formation of endogenous DNA damage is inevitable as the errors can be generated during normal metabolism. By estimation, there are about 20,000 events of endogenous DNA damage per cell per day (Ciccia and Elledge, 2010). ROS, the most common inducer of endogenous DNA damage, are generated through respiratory chain reaction in mitochondria and during many immune responses. Oxidative stress can induce DNA damage, such as oxidized guanine, DNA single strand breaks (SSB) and DNA DSB (Breen and Murphy, 1995).

Another source of endogenous damage contributes to genome instability is the so-called "end-of-replication" problem. Because the very end of lagging strand DNA cannot be replicated by DNA polymerase during each round of DNA duplication, telomere length becomes shorter and shorter. Although telomeric repeats (TTAGGG) are lengthy (Greider, 1996), cell regeneration and propagation continuously fire DNA replication, resulting in telomere shortening progressively. When telomere attrition reaches a very short stage, telomeric T-loop cannot be formed and the otherwise being protected 3' tail exposed, a universal signal that triggers DNA damage response (Hemann et al., 2001; Morrish and Greider, 2009; Verdun and Karlseder, 2006).

Acute DNA damage is usually induced by exogenous clastogens, including ionizing irradiation, UV light, mutagens, and carcinogens. UV irradiation from sunlight exposure is the most common form of clastogen that generates about 10^5 events of DNA damage per cell per day (Ciccia and Elledge, 2010). Although most of the UV-induced DNA damage can be faithfully repaired, the same is not true for some other clastogens such as DSB or DNA crosslinks. Different from endogenous DNA damage, DNA breaks from exogenous clastogens are usually induced in a prompt manner. Nonetheless, if left unrepaired, both endogenous and exogenous DNA damage accumulate and gradually compromise genome integrity.

DNA damage response, including checkpoint activation, DNA repair, senescence and apoptosis, collectively maintains genome stability and determine the fate of the cells. In general, checkpoint activation allows time for DNA repair machinery to fix the lesion. If the DNA damage is too severe or the DNA repair capability is compromised, unrepaired DNA damage signals the cell to go to senescence or apoptosis. Depending

on the types of damage, they are addressed by different pathways of DNA repair. BER corrects mutated bases generated by depurination, deamination, or oxidation through short patch or long patch sub-pathways (Frosina et al., 1996). NER fixes bulky helix distortions of DNA by two pathways, global genome NER or transcription-coupled NER (de Laat et al., 1999). MMR is a post-replication pathway that removes mis-incorporated bases or mutated base and matches with a correct one (Modrich, 1997). DNA DSB are primarily repaired by HR and NHEJ. HR requires end-processing of the broken ends to generate a 3' single-stranded tail in search for homologous sister chromatid, resulting in error-free repair of DNA DSB. NHEJ is considered an error-prone repair of DNA DSBs because it simply ligates two broken ends together. In addition to the many proteins that execute the four major DNA repair pathways, the genome is protected by the shelterin that protects chromosome ends from being recognized as DNA breaks as detailed in Section 1.2.3. In particular, telomere elongation needs ATR and ATM protein kinases to activate HR for completion of telomere elongation, but the unprotected telomere activates persistent DNA damage instead of transient DNA damage response (Verdun and Karlseder, 2006). The genome stability is thus maintained tightly by DNA damage repair mechanisms and by telomere stability machinery to prevent detrimental DNA damage accumulation.

Persistent DNA DSBs and dysfunctional telomeres are the most pronounced inducers of cellular senescence (Fumagalli et al., 2012; Mallette and Ferbeyre, 2007). In the events of persistent DNA DSBs and dysfunctional telomeres, the ATM kinase serves as the major mediator that senses and transmits the signal of DNA damage to

downstream DNA repair and checkpoint proteins. p53 is one of the major ATM substrates that contribute to a senescence response. Activated ATM phosphorylates p53 for proliferation arrest through the activation of p21, a gate keeper protein that controls the G1-S transition. In addition to p53, $p16^{INK4}$-Rb is another pathway that contributes to G1 arrest and cellular senescence in the response to DNA damage (Robles and Adami, 1998). Furthermore, the $p16^{INK4}$-Rb pathway is proposed as the second barrier to reinforce the growth arrest by p53 when telomere is dysfunctional (Beausejour et al., 2003).

1.3.1.2 Acute chromosome conformation changes activate cellular senescence through the regulation of the effectors in senescence pathways.

Histone deacetylase inhibitor drives acute changes on chromosome structures and activates cellular senescence. Histone acetyltransferase (HAT) and histone deacetylase (HDAC) are regulators that relax and pack the structure of chromosome (Luo and Dean, 1999). Decreased flexibility to pack chromosome in the presence of HDAC inhibitors can increase heterochromatin perturbation, which is known to induce senescence in human diploid fibroblasts (Ogryzko et al., 1996).

HDAC inhibitor activates p16- or p53-dependent senescence pathway through the increase of heterochromatin perturbation and DDR. When heterochromatin perturbation happens, DDR is activated together with increased ATM phosphorylation and γH2AX formation (Di Micco et al., 2011). In human fibroblasts, HDAC inhibitors such as sodium butyrate and trichostatin can activate $p16^{INK4}$-Rb pathway and induce cellular senescence, and this pathway does not solely depends on

p53 (Munro et al., 2004; Ogryzko et al., 1996). Although p53 is known as a substrate of SIRT1, a histone deacetylase (Kim et al., 2007), the role of p53 in cellular senescence induced by HDAT is not clear. It may depend upon species or cell types (Di Bernardo et al., 2009; Munro et al., 2004). On the other hand, p16 expression can be induced by inhibition of HDAC. HDAC can suppress p16 transcription through its recruitment to the promoter region of p16 (Zhou et al., 2009). Thus, HDAC inhibitors can initiate a senescence response by the induction of chromosome perturbation, which in turn activates DNA damage response and the $p16^{INK4}$-Rb pathway.

1.3.1.3 The mitogenic stimuli activate cellular senescence

Mitogenic stimuli are key contributors of cell proliferation, but they may also lead to cellular senescence. During cell proliferation, mitogenic stimuli are required to activate 1) cell growth by inducing DNA replication, 2) cell division in confluent cultured cells, 3) several rounds of cell division in sparse cultured cells, and 4) single cell proliferation to monolayer population. In order to control the proliferation in various cell types, mitogenic stimuli function in a cell type specific manner (Gospodarowicz and Moran, 1976). Just like senescence, mitogenic stimulation is a double-edged sword, because mitogens can promote both cell proliferation and tumorigenesis. Interestingly, some of the mitogenic stimuli also activate cellular senescence. For example, tumor necrosis factor-alpha, a proinflammatory cytokine, not only promote proliferation by the activation of activator protein-1 (Aggarwal et al., 2011) but also induce cellular senescence in endothelial progenitor cells under chronic exposure through activation of the p38 mitogen-activated protein kinase (MAPK) pathway (Zhang et al., 2009). Furthermore, angiotensin II induces cell

proliferation by induction of DNA synthesis in certain cell types, but it can also induce cellular senescence in endothelial cells through the activation of MAPK pathway (Shan et al., 2008). The other critical mitogenic stimulus is oncogene. When oncogenes, such as Ras, Raf, $BRAF^{E600}$, and STAT5A, are constitutively expressed in normal cells, cellular senescence can be induced (Mallette et al., 2007; Michaloglou et al., 2005; Serrano et al., 1997; Zhu et al., 1998). Under the condition of constitutive activation of Ras, Raf, or $BRAF^{E600}$, the activated MAPK pathway leads to p53 and $p16^{INK4}$ accumulation that induces cellular senescence. Additionally, when senescence is induced by the oncogenic Ras, the MAPK downstream component, MEK, is found to be required for the activation of cellular senescence. Furthermore, constitutively activated MEK alone also activate cellular senescence in primary murine fibroblast (Lin et al., 1998). Another pathway capable of mediating oncogene-induced cellular senescence is DDR. Oncogenic stimuli can trigger DNA hyper-replication, which increases DNA damages and then elicits DNA damage checkpoint activation, DDR, and the expression of p53 and $p16^{INK4}$ (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007). Thus, the mitogenic stimuli in cells are tightly regulated by complicated signal transduction pathways in an attempt to prevent the transformation to malignant stage by induction of cellular senescence.

1.3.2 The markers of cellular senescence

Specific phenotypes of cellular senescence have been observed *in vivo* and *in vitro*. The phenomenon of cellular senescence was first reported by Hayflick and co-authors who found that the human diploid fibroblast divides at a declined rate after certain passages, followed by complete stop of cell proliferation when cultured *in vitro* (Hayflick and

Moorhead, 1961). Based on this finding, ceased proliferation is one of the phenotypes in senescent cells; however, they did not distinguish senescent from quiescent cells. Currently, six major senescent phenotypes are known to exist: expression of senescence-associated beta-galactosidase (SA-β-gal), persistent DNA damage response (DDR) or DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS), increased $p16^{INK4}$ expression, senescence-associated heterochromatin formation (SAHF), transformation into senescence associated secretary phenotype, and decreased Lamin B1 expression. These features are also observed in senescent cells in mammalian tissues.

1.3.2.1 Senescence-associated beta-galactosidase

SA-β-gal expression is a specific senescence marker *in vitro* and *in vivo*. Beta-galactosidase is an abundant lysosomal enzyme with an optimal pH at 4 in young or immortal cells. However, SA-β-gal is activated at pH 6. The expression of SA-β-gal is highly correlated with cellular age in cultured cell or in tissues, and in various animal species (Dimri et al., 1995; van der Loo et al., 1998).

1.3.2.2 Persistent DNA damage response (DDR)/DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS)

Persistent DNA damage response is present in senescent cells. DNA damage response includes DNA repair, checkpoint activation, damage tolerance/lesion bypass, apoptosis, autophagy and senescence. To maintain genome integrity, functional DDR at the minimum requires DNA repair proteins and DNA damage sensing proteins. In senescent cells, DNA damage is not appropriately or completely repaired, resulting in persistent DNA damage that remains for days to months. Markers of DDR include

those that appear rapidly at the proximity of the lesion, such as ATM activation and the phosphorylation of its downstream substrates (Giglia-Mari et al., 2010). Senescent cells continue to assemble DNA damage sensing proteins at the site of unrepaired DNA damage, including γH2AX, pATM on Ser-1981, or ATM/ATR substrates in the case of DNA breaks (Fumagalli et al., 2012; Rodier et al., 2009). In the liver of old *Terc*$^{-/-}$ mouse, persistent γH2AX foci overlap with senescence-associated beta-galactosidase expression (Wang et al., 2009).

DNA-SCARS is a form of permanent DNA damage that is associated with deficiency in DNA repair protein and can induce cellular senescence (Rodier et al., 2011). Interestingly, dysfunctional telomeres induce replicative senescence by permanently activating the response to DNA-SCARS. Telomere attrition to a critically short level results in loss of protection by shelterin and the resultant dysfunction telomeres. Without the protection of TRF2, dysfunction telomeres are recognized and marked by DNA DSB repair machinery, resulting in persistent DNA damage foci (Takai et al., 2003). Specifically, ATM transduces DNA damage signals to downstream checkpoint kinase CHK2, which in turn activates p53 and p21 for senescence induction. Noticeably, this is distinct from another senescence pathway activated by p16^{INK4}-pRb (d'Adda di Fagagna et al., 2003; Herbig et al., 2004). Therefore, dysfunctional telomeres can provoke genome instability and the subsequent cellular senescence by formation of DNA-SCARS.

1.3.2.3 Increasing p16^{INK4} expression

$p16^{INK4}$ promotes cellular senescence by maintaining retinoblastoma (Rb) at hypophosphorylation status through inhibition of cyclin-dependent kinases. In human genome, there are two proteins encoded from the INK4/ARF locus on chromosome 9 by alternative splicing, resulting in $p16^{INK4}$ and $p19^{ARF}$ expression. These two products play important roles in cell cycle regulation and senescence induction. $p19^{ARF}$ mediates the p53 pathway, whereas $p16^{INK4}$ regulates the pRb pathway (Alcorta et al., 1996; Duan et al., 2001; Sharpless and DePinho, 1999). $p16^{INK4}$ was first identified as an inhibitor of CDK4 and CDK6 interactions with cyclin D, resulting in the inhibition of the kinase activities and cell proliferation (Guan et al., 1994; Serrano et al., 1993). To induce cellular senescence, $p16^{INK4}$ requires pRb to arrest cell cycle at G1 phase (Lukas et al., 1995) and helps to maintain the hypophosphorylated form of pRb, the active and stabilized pRB (Ezhevsky et al., 1997). The cooperation between $p16^{INK4}$ and pRb promotes cellular senescence when cells encounter persistent DNA damage or mitogenic signals (Shapiro et al., 1998; Takahashi et al., 2006).

$p16^{INK4}$ is one of the markers of cellular senescence *in vitro* or *in vivo*, and a key gate keeper controlling the transition from proliferating to senescent cells (Reznikoff et al., 1996). Because the turnover rate of $p16^{INK4}$ mRNA is greater in senescent than proliferating cells *in vitro*, $p16^{INK4}$ mRNA can also serve as an indicator of cellular senescence (Kim et al., 2002a; Wang et al., 2005). Nonetheless, both $p16^{INK4}$ mRNA and protein levels are ideal senescence markers *in vivo* (Liu et al., 2009; Ohtani et al., 2010; Ressler et al., 2006).

1.3.2.4 Senescence-associated heterochromatin formation

Senescence-associated heterochromatin foci (SAHF) are structurally different from regular heterochromatin and can be induced during the process of cellular senescence. The first observation of SAHF is observed in senescent human diploid fibroblast cells (Narita et al., 2003). Like other forms of heterochromatin, SAHF formation is accompanied with the expression of K9M-H3 (histone H3 methylated on Lysine 9) and HP1 in the pericentric regions. However, SAHF distinctly accumulate phosphorylated HP1γ and HMGA (High-mobility group A) proteins in a manner requiring HIRA, ASF1, and macroH2A (Narita et al., 2006; Zhang et al., 2007). Another distinctive feature of SAHF is the loss of histone H1, which can be explained by the observation that HMGA protein is a stronger competitor than histone H1 in binding linker DNA in senescent cells (Funayama et al., 2006). SAHF can also be formed by the induction of mitogenic stimuli. This chromatin conformation change needs the $p16^{INK4}$-pRB pathway activation to inhibit the function of E2F; interestingly, the binding of pRb to some of the E2F responsive genes is critical for heterochromatin formation and gene silencing (Narita et al., 2003). Additionally, inhibition of the Wnt signaling pathway leads to cellular senescence and SAHF formation in a manner independent of p53 or pRb (Ye et al., 2007). In summary, chromosome reorganization and the formation of SAHF are guided by pathways involved in cellular senescence but not proliferation.

The formation of SAHF in senescence cells depends upon cell types and stimuli. MRC-5 and BJ human normal fibroblasts and primary keratinocytes form SAHF under oncogenic stimulation. However, only MRC-5 cells can form SAHF under etoposide, doxorubicin, hydroxyurea, bacterial intoxication and telomere attrition

(Kosar et al., 2011). These findings raised additional questions regarding the mechanism of SAHF formation and how to appropriately use SAHF as a senescence marker. Although SAHF do not universally appear in all cell types and oncogenic stimuli, the formation of SAHF is useful to distinguish senescent from proliferative or quiescent cells (Narita et al., 2003).

1.3.2.5 Transforming into senescence-associated secretary phenotype

Senescence-associated secretary phenotype (SASP) controls the senescence microenvironment through non-autonomous regulation. SASP was first found in cells with oncogene-induced senescence (Kuilman et al., 2008), displaying persistent DDR and increased chemokine and cytokine secretion. On the contrary, knockdown of a chemokine receptor CXCR2 alleviates replicative and oncogene-induced senescence and decreases DDR (Acosta et al., 2008a; Kuilman et al., 2008). These findings support the new idea of which senescent cells dictate senescence microenvironment. The SASP secretes soluble factors that include interleukins, chemokines, growth factors/regulators, inflammatory factors, proteases, receptors, ligands, PGE2, nitric oxide and ROS. These molecules affect the innate immune response for tumor clearance, turn other cells into senescent cells for tumor suppression, or reinforce senescence through the maintenance of DDR (Acosta et al., 2008b; Coppe et al., 2010; Fumagalli and d'Adda di Fagagna, 2009).

SASP is not activated by “typical” senescent pathways such as p16-pRb and p53-p21. As described above, p16 and p21 are cyclin-dependent kinase inhibitors that lead to cell cycle arrest, a prerequisite of cellular senescence. However, the activation of

SASP is not p16-dependent or simply an inevitable consequence of p16-induced senescence (Coppe et al., 2010). In contrast, the activation of p53 inhibits SASP and promotes cell autonomous regulation (Coppe et al., 2008). With this in mind, it is helpful to extrapolate cellular senescence to organismal aging. The p53 signaling is getting insensitive with age, but the inflammatory signaling is getting sensitive. As such, SASP-mediated non-autonomous regulation dominates over the p16- or p53-dependent autonomous pathway. This supports the microenvironment theory of SASP through cell non-autonomous regulation (Salminen and Kaarniranta, 2010).

The p38-NFκB pathway also plays a pivotal role in the regulation of SASP. The p38 MAPK is a stress-induced kinase that is greatly activated upon induction of senescence. Different from DDR that may not always activate SASP, p38 induction is sufficient to turn SASP on (Freund et al., 2011). The p38 MAPK pathway activation transmits signals to NFκB, which serves as the effecter transcription factor for transactivation of secretary factor expression. Interestingly, senescence induction by the oncogenic Ras can be reversed by knocking down NFκB (Chien et al., 2011; Salminen and Kaarniranta, 2010; Salminen et al., 2011). Thus, senescence microenvironment can be modulated by inflammatory response in SASP.

1.3.2.6 Lamin B1 underexpression

Lamins are nuclear structural proteins that reinforce nuclear envelope integrity and genome stability. Nuclear envelope restricts the genome in the nucleus at the proximity of outer membrane, inner membrane, nuclear pore complex and the lamina. The A- and B-type lamins comprise lamina. In mammals, A-type lamins, lamin A and

lamin C, are encoded by the *LMNA* gene, whereas the two major types of B-type lamins, lamin B1 and lamin B2, are transcribed from *LMNB1* and *LMNB2* gene, respectively. Generally, A-type lamins mainly express in fully differentiated cells while B-type lamins express throughout the cell development (Rober et al., 1989; Schatten et al., 1985). Interestingly, mutations in A-type lamins lead to several human diseases, including Emery-Dreifuss muscular dystrophy, dilated cardiomyopathy, atypical Werner syndrome and Hutchinsom-Gilford progeria (Lammerding et al., 2006). Lamins provide critical structural barriers against genome instability through DNA repair, DNA replication, transcription control and chromatin organization (Dechat et al., 2008; Kennedy et al., 2000; Kumaran et al., 2002; Manju et al., 2006; Moir et al., 1994). However, human genetic diseases are more likely to be developed by A-type than by B-type lamin mutations because of the prominent role of lamin A in nuclear structural stability.

Lamin B1 is a newly identified marker of cellular senescence. Lamin B1 is not as ubiquitous as lamin B2 in human tissues, and its expression is highly associated with cell proliferation. Not only in senescent cells, lamin B1 expression is also maintained in proliferative and quiescent cells (Broers et al., 1997). However, the expression of lamin B1 is decreased under either p53 or pRb pathway activation. The turnover rate of lamin B1 protein and mRNA is great in senescent cells (Freund et al., 2011).

1.3.3 The many roles of cellular senescence

Cellular senescence helps tissue regeneration and protects cells against tumorigenesis. Cellular senescence arrests damaged cells from proliferation, eventually removing them

from the organs or tissues to facilitate replenishing new cells from stem cells or to prevent tumorigenesis. Cellular senescence can also counteract tumorigenesis by activation of DNA damage response (Bartkova et al., 2005; Gorgoulis et al., 2005). Additionally, based on a mouse model of aging, senescent cell removal by inducible apoptosis can protect tissues and organs against age-associated disorders in older animals (Baker et al., 2011). Similarly, cellular senescence can be activated by trace elements such as selenium compounds in the prevention against tumorigenesis. The Cheng Lab has previous shown that doses of selenium less than LD_{50} can induce senescence and DNA damage responses in non-cancerous but not cancerous cells, suggesting that tumorigenesis can be stifled at the very early stage by selenium-induced activation of early tumorigenesis barriers (Wu et al., 2010; Wu et al., 2011).

Strikingly, senescence can promote both aging and carcinogenesis. Senescent cells exhibit senescence-associated secretary phenotype that modulates the microenvironment. Such cell non-autonomous regulation may accelerate cellular senescence in surrounding cells or the proliferation of pre-malignant cells. Accumulation of senescent cells leads to tissue or organ degeneration. In lamin A-defective mice, senescence-associated secretary phenotype induces systematic inflammation that is driven by NF-κB transactivation through ATM activation. Interestingly, the aging-associated symptoms in the mice are reversed by inhibition of NF-κB transactivation. These results reinforce the theory of cell non-autonomous regulation by senescent cells that can promote aging in the whole body scale (Osorio et al., 2012). In addition, senescence-associated secretary phenotype facilitates the proliferation of pre-malignant cells through the secretary cytokines and chemokines. Through this microenvironment change, pre-malignant cells prone to

transformation into malignant cells. Thus, cellular senescence is a double-edged sword not only to prevent carcinogenesis and tissue regeneration, but also to promote aging and tumorigenesis.

Is cellular senescence beneficial or detrimental to multicellular organism? Proteins in the senescence-associated pathway are critical in determining how the cell senesce. To establish cellular senescence, activated effectors in senescence pathway are required to halt cell proliferation. However, the ATM-p53 pathway is critical for the process against tumorigenesis by the activation of cellular senescence (de Keizer et al., 2010). Compromised genome stability can activate DNA damage response. Although ATM and p53 are known to play a central role in the activation of cellular senescence in damaged cells, the detail molecular mechanism by which they modulate senescence is largely unknown. Understanding the role of ATM and p53 in the interphase between aging and longevity or between carcinogenesis and cancer suppression is critical for future biomedical research, in an attempt to redefine or redevelop the remedy of cancer and/or aging interventions (Campisi, 2011).

1.4.1 ATM activation in DDR (DNA damage response)

ATM plays a central role in DDR by transducing the DNA damage signals to downstream effectors by its catalytic activity of phosphorylation, resulting in checkpoint activation, DNA repair and cell death. Although DNA DSB has been shown to be recognized by various markers, the most significant one perhaps is poly(ADP-ribose) polymerase 1 (PARP1) that recruits Mre11-Rad50-NBS1 (MRN complex) to the DNA DSB site (Ciccia and Elledge, 2010; Haince et al., 2008). In turn, ATM is recruited by

MRN complex and subsequently phosphorylates NBS1 and other proteins for S-phase checkpoint activation (Lim et al., 2000; Wu et al., 2000; Zhao et al., 2000). By using purified proteins, it has been shown that ATM pathway activation is initiated by ATM autophosporylation at Ser-1981 in the response to DNA DSB or oxidative stress (Guo et al., 2010; Lee and Paull, 2005). Another two kinases in the same family are the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) and ataxia telangiectasia and Rad3 related (ATR). Together with the Ku70/Ku80 sensor proteins, DNA-PKcs repairs DNA DSB by NHEJ (Reynolds et al., 2012). Another kinase in this protein family is Ataxia telangiectasia and Rad3 related (ATR). These three kinases may have dependent and independent roles in DDR. It was reported that ATM and DNA-PKcs collaborate in the response to DBS (Chen et al., 2007; Martin et al., 2012; Shrivastav et al., 2008). Moreover, MRN complex cooperates with ATM to resect a DSB end for the generation of a 3'-single strand tail that recruits replication protein A (RPA), setting a stage for ATR-dependent homologous recombination repair of DNA DSB. Similarly, ATRIP is a sensor of DNA damage and recruits ATR to the site of DNA damage (Falck et al., 2005). If left unrepaired, persistent DDR follows. ATM can also phosphorylate p53 (Banin et al., 1998; Canman et al., 1998), resulting in increased levels of p53 that promotes cellular senescence or programmed cell death. Because ATM has been known to phosphorylate hundreds of substrate in the response to DDR (Kastan and Lim, 2000), ATM is critical to control various cellular functions in response to different types of DNA damage, including DSB and oxidative DNA damage.

Activated ATM regulates p53 functions directly and indirectly by different modifications on various sites. In proliferating cells, the half-life of p53 is short (30 min) because

MDM2 is constitutively associated with p53 and shuttles p53 from nucleus to cytosol for proteolytic degradation (Khosravi et al., 1999; Maya et al., 2001). However, activated ATM phosphorylates MDM2 at Ser-395, resulting in dissociation of p53 from MDM2 and the subsequent increased p53 steady state level. The dissociation of p53 from MDM2 is further reinforced by p53 phosphorylation at Ser-20 by CHK2, the residue critical for MDM2 binding (Chehab et al., 2000; Hirao et al., 2000). This modification increases steric hindrance of the MDM2-p53 interaction. Furthermore, activated ATM can phosphorylate p53 at Ser-15 and can also increase p53 stability, resulting in increased p53 transactivation activities (Canman et al., 1998; Dumaz and Meek, 1999; Khanna et al., 1998). The direct or indirect modifications by ATM determine the activity or stability of p53, promoting cellular senescence and/or apoptosis.

1.4.2 The function of p53

Although p53 serves as a tumor suppressor and prevents carcinogenesis, this protein is usually mutated during tumorigenesis. p53 was first recognized as a tumor cell specific antigen in both humans and mice, with its function being designated as cell transformation-activating protein or oncogene (Crawford et al., 1981; DeLeo et al., 1979). However, the observed transformation-activating feature of the oncogenic *Ras* or *Myc* is attributed to p53 mutations (Hinds et al., 1989). On the contrary, overexpression of wild-type p53 compromises the transformation-activating functions in tumorigenesis (Eliyahu et al., 1989; Hinds et al., 1989). In animal models, robust expression of the carboxy-terminal p53 can reduce the incidence of tumor formation (Tyner et al., 2002). On the other hand, mice introduced with additional copies of p53, known as “super p53” mice, are more resistant to tumor formation (Garcia-Cao et al., 2002). Additionally, most types

of human cancer harbor p53 mutations during tumorigenesis, and more than 80% of the mutations are missense mutations (Soussi, 2011). Based on these new strategies to analyze unbiased mutations of p53, over 80% of the breast cancer tumors harbor mutations, and in some cases there are at least three mutation sites(Edlund et al., 2012; Szymanska and Hainaut, 2003). p53 mutations can also be used as a biomarker for cancer diagnosis (Szymanska and Hainaut, 2003). In conclusion, available lines of current *in vitro* or *in vivo* evidence point to a critical role of p53 in the protection against tumorigenesis.

p53 regulates cellular functions by transactivating or repressing target genes. This is executed by the following four domains: transactivation, DNA binding, tetramerization and nuclear localization. Activated p53 forms a tetramer and serves as the transactivator targeting p53 response element, which is a consensus sequence composed of two 10-base decamers and a 0-13 base spacer in between. Although the functional response element usually has only less than 3 nucleotides at spacer, sometimes a noncanonical element exists in the active p53 response element (Menendez et al., 2009). With both transactivation domain and DNA binding domain at the N-terminus, p53 regulates the transcription of genes involved in redox regulation, metabolism regulation, DNA repair, autophagy, cell cycle arrest, cellular senescence and apoptosis (Table 1). However, in other cases, p53 works differently and suppresses the transcription of downstream genes (D'Souza et al., 2001; Li et al., 2004).

Furthermore, p53 can serve as an effecter protein in the response to DNA damage response and protects cells against tumorigenesis by the activation of cellular senescence. The majority of senescence inducers can activate DNA damage response. When DNA

damage response is activated, p53 stability is increased by ATM phosphorylation on Ser-15 and other sites. Then, p53 transactivates p21 expression that inhibits CDK2-cyclin E, resulting in G1/S arrest (Brugarolas et al., 1995). Additionally, p53 also possibly promotes G2/M arrest through 14-3-3 complex (Hermeking et al., 1997). Because the activation of cell cycle arrest is a pre-requisite of cellular senescence, p53 is the key regulator of cellular senescence that receives and transmits signals of DNA damage response.

Target	Activation/ Inhibition	Species	Reference
Anti-stress			
FLJ11259/DRAM	Activation	human	(Kerley-Hamilton et al., 2007)
Glutathione S-transferase P1 (GSTP1)	Activation	human	(Lo et al., 2008)
Interferon regulatory factor-2-binding protein-2 (IRF2BP2)	Activation	human	(Koeppel et al., 2009)
Serine/Threonine kinases 17A	Activation	human	(Mao et al., 2011)
Glutathione peroxidase 1	Activation	human	(Tan et al., 1999)
Metabolism			
Metalloproteinases-3	Activation	mouse	(Bian et al., 1996)
Brain-specific angiogenesis inhibitor 1	Activation	human	(Nishimori et al., 1997)
Smooth muscle alpha-actin	Activation	human	(Comer et al., 1998)
Human Metalloproteinase-1	Inhibition	human	(Sun et al., 1999b)
Thiamine transporter 1	Activation	mouse	(Lo et al., 2001)
Staf50 (TRIM22)	Activation	human	(Obad et al., 2004)
Podocalyxin	Inhibition	human	(Stanhope-Baker et al., 2004)
Fatty acid synthase	Activation	worm to human	(D'Erchia et al., 2006)
Vitamin D receptor	Activation	human	(Maruyama et al., 2006)
Polycystic Kidney Disease-1 Gene	Inhibition	human	(Van Bodegom et al., 2006)
Notch1	Activation	human	(Lefort et al., 2007)

Brain-expressed RING finger protein	Activation	human	(Cheung et al., 2010)
SPATA 18	Activation	human	(Bornstein et al., 2011)
Parkin	Activation	human or mouse	(Zhang et al., 2011)
Thrombospondin-1	Inhibition	human	(Dameron et al., 1994)
TIGAR	Activation	human	(Bensaad et al., 2006)
DNA damage repair			
GADD45	Activation	human	(Zhan et al., 1993)
Phosphotyrosyl phosphatase activator	Activation	human	(Janssens et al., 2000)
Ribonucleotide reductase (p53R2)	Activation	human	(Nakano et al., 2000)
DinB	Activation	mouse	(Burns and El-Deiry, 2003)
DNA polymerase eta (PolH)	Activation	Human	(Liu and Chen, 2006)
Pierce 1	Activation	Mouse	(Sung et al., 2010)
Damage-specific DNA binding Protein 2	Activation	Human	(Tan and Chu, 2002)
Fanconi anemia, complementation group C	Activation	Human	(Liebetrau et al., 1997)
PMS2	Activation	Human	(Chen and Sadowski, 2005)
MLH1	Activation	Human	(Chen and Sadowski, 2005)
Proliferating cell nuclear antigen	Activation	Human	(Jackson et al., 1994)
Xeroderma pigmentosum, complementation group	Activation	Human	(Adimoola

C			m and Ford, 2002)
Cell cycle			
Cyclin G	Activation	Rat	(Zauberman et al., 1995)
p22/PRG1	Activation	Rat	(Schafer et al., 1998)
PTGF-β	Activation	Human	(Tan et al., 2000)
DDA3	Activation	Mouse	(Lo et al., 1999)
Wig-1	Activation	Human	(Hellborg et al., 2001)
Protein regulator of cytokinesis	Inhibition	Human	(Li et al., 2004)
SMART1	Activation	Human	(Singh et al., 2007)
DEC1	Activation	Human	(Qian et al., 2008)
Prl-3 (phosphatase of regenerating liver-3)	Activation	Human	(Basak et al., 2008)
DUSP11 (dual specificity phosphatase 11)	Activation	Human	(Caprara et al., 2009)
Necdin	Activation	Mouse	(Lafontaine et al., 2012)
Murine double minute 2	Activation	Mouse	(Juven et al., 1993)
14-3-3 protein	Activation	Mouse	(Hermeking et al., 1997)
B99	Activation	Mouse	(Utrera et al., 1998)
Insulin growth factor-binding protein 3	Activation	Human	(Buckbinder et al., 1995)
GPI-anchored molecule-like protein	Activation	Human	(Furuhata et al., 1996)
Apoptosis			
MCG10	Activation	Human	(Zhu and

			Chen, 2000)
P202 (Interferon-inducible phosphoprotein)	Inhibition	Human	(D'Souza et al., 2001)
mRTVP-1	Activation	Mouse	(Ren et al., 2002)
Snk/Plk2	Activation	Mouse	(Burns et al., 2003)
CD200	Activation	Mouse	(Rosenblum et al., 2004)
SIVA	Activation	Mouse	(Fortin et al., 2004)
Decoy receptor 2	Activation	Human	(Liu et al., 2005)
Epithelial cell kinase	Activation	Human	(Jin et al., 2006)
TIS11D	Activation	Human	(Jackson et al., 2006)
RhoE (Inhibit ROCK I)	Activation	Human	(Ongusaha et al., 2006)
APLP1	Activation	Human	(Tang et al., 2007)
Beta 1, 4 GalTII	Activation	Human	(Zhou et al., 2008)
Apoptosis-enhancing nuclease (AEN)	Activation	Human	(Kawase et al., 2008)
TNFSF10 (TRAIL)	Activation	Human	(Kuribayashi et al., 2008)
S100 calcium-binding protein A9 (S100A9)	Activation	Human	(Li et al., 2009)
AlphaB-crystallin	Activation	Human	(Watanabe et al., 2009)
PUMA	Inhibition	Human	(Gomes and Espinosa, 2010)
Foxo3	Activation	Mouse	(Renault et al., 2011)
Bax	Activation	Human	(Miyashita

			and Reed, 1995)
Fas (APO-1/CD95)	Activation	Human or Mouse	(Munsch et al., 2000)
KILLER/DR5	Activation	Human	(Takimoto and El-Deiry, 2000)
PAG608	Activation	Mouse	(Israeli et al., 1997)
p53-regulated Apoptosis-Inducing Protein 1 (p53AIP1)	Activation	Human	(Oda et al., 2000)
p53-dependent damage-inducible nuclear protein (p53DINP)	Activation	Human	(Okamura et al., 2001)
Apoptosis protease-activating factor 1	Activation	Human	(Moroni et al., 2001)
PERP	Activation	Mouse	(Attardi et al., 2000)
PIDD	Activation	Human	(Lin et al., 2000)

Table 1 p53-regulated genes in anti-stress, metabolism, DNA repair, cell cycle and apoptosis.

1.4.3 Linking DNA damage to senescence

The biological function of cellular senescence remains an open field to be explored. Cellular senescence *in vivo* and in cultured cells is induced by various inducers and is characterized by various markers. Increasing lines of evidence indicate that cellular senescence can be induced by DNA damage, chromatin alteration, and DNA damage independent pathways (Nakamura et al., 2008; Pospelova et al., 2009; Prieur et al., 2011). The phenotypes of senescent cells are variable and are thought to modulate various physiological functions. The decrease in lamin B is independent of p38MAPK-NFκB pathway, ROS, or DDR, but activates p53 and p16 pathways toward senescence induction. On the contrary, cellular senescence induced by chromatin perturbation is p53 and p16 independent. Also, there may have cell type-specific senescence phenotypes in the response to various stimuli (Coppe et al., 2011; Freund et al., 2012; Kosar et al., 2011).

p53 participates in the major pathway against tumorigenesis, but the crosstalk between p53 and other pathways represent another autonomous or non-autonomous regulation of cell fate. As described above, "super p53" mice with additional copies or constitutive expression of p53 are resistant to tumorigenesis (Garcia-Cao et al., 2002; Tyner et al., 2002). As aging progressed, DDR and dysfunction telomere activate p53, resulting in restriction of pre-malignant cells in senescence instead of tumorigenesis. However, in some cases, p53/p21 and p16 are thought to initiate and maintain cellular senescence, respectively (Beausejour et al., 2003; Robles and Adami, 1998). Crosstalk between p53 and pRB pathways may involve the intermediate p16, which regulates p21 accumulation by transcriptional or post-translational regulation to reinforce cellular senescence (Al-

Mohanna et al., 2007; Mitra et al., 1999). Interestingly, in immortal cells, the overexpression of exogenous p21 promote demethylation at the promoter of p16. Thus, the expression of p16 is restored for induction of growth arrest or cellular senescence (Wu et al., 2012). The p16-pRb pathway is not a redundant pathway to activate cellular senescence for anti-tumorigenesis; rather, it is an important pathway meant to secure the cooperation with p53 and p21 and to keep normal cellular functions. Ultimately, if the regulation of cellular senescence is properly controlled by effectors, the individual will likely to age slowly with decreased incidence of cancer.

1.5 Selenium

Selenium is an essential trace element for optimal health. Current Dietary Reference Intake (DRI) information on the Recommended Dietary Allowance (RDA) of selenium for USA is below 40 μg/day for children under 13 years of age children, but RDA of selenium for those age above 14 is 55 μg/day. The special requirements during pregnancy and lactation are 60 μg/day and 70 μg/day, respectively (http://iom.edu/Activities/Nutrition/SummaryDRIs/~/media/Files/Activity%20Files/Nutrition/DRIs/RDA%20and%20AIs_Vitamin%20and%20Elements.pdf, USDA). Although certain foods are rich in selenium, such as Brazil nuts, seafood and dairy products, body selenium status is largely correlated to the level of selenium in the soil. In the United States, the intake of selenium tends to fall between 50~200 μg/day, which is considered as safe and adequacy. The extreme cases were reported elsewhere such as China, where the intake had been reported to fall between 7~3800 μg/day, resulting in selenium deficiency or toxicity (Ehrlich, 2002). However, to maintain the optimal health, there is a U-shaped relationship between selenium intake and the risk of diseases. Selenium

deficiency may cause Keshan disease or Kashin-Beck disease (Fairweather-Tait et al., 2011), which are caused by Coxsackie B virus and fungus produced mycotoxin, respectively. The proposed etiology of these two diseases is the defective antioxidant functions, as selenium deficiency decreases selenoprotein expression, the majority of which are antioxidant. On the other hand, when selenium intake reaches a toxic level, the excessive amount of selenium may induce the formation of ROS and the resultant selenosis (Yang et al., 1983). The Nutritional Prevention of Cancer (NPC) clinical trial conducted in the United States has indicated that the daily oral administration of selenium at 200 μg, which is 3-4 folds higher than nutritional needs, significantly decreases risks of prostate, lung, and colon cancers (Clark et al., 1996). The effect of chemoprevention might be resulted from excessive amount of selenium, because selenium in excess is pro-oxidant instead of antioxidant. The generation of ROS by excessive selenium may restrict pre-malignant cells from turning into malignant cells (Wu et al., 2010). Although results of the follow-up clinical trial, the Selenium and Vitamin E Cancer Prevention Trial (SELECT), do not support a beneficial role of selenium in chemoprevention, the discrepancies are largely addressed on the National Cancer Institute's website, from which it seems like that the inconsistent results may be attributed to the form of selenium (selenized-yeast vs. selenomethionine) or the basal level of selenium in volunteers (NPC, low vs. SELECT, high) (http://www.cancer.gov/newscenter/qa/2008/selectqa#que5, NCI). Clearly, additional clinical studies are needed to elucidate or re-confirm a role of selenium in cancer prevention. Therefore, in terms of obtaining optimal health, keeping selenium intake within adequate level is necessary for optimal health through the biosynthesis of selenoproteins.

1.6 Selenoproteins

1.6.1 Selenoprotein Biosynthesis

Selenium is incorporated into selenoproteins through selenocysteine. In general, there are 20 amino acids in humans for protein translation. Nevertheless, selenocysteine has been identified to be the 21st amino acid in prokaryotic and eukaryotic cells. The reactions of selenocysteinyl-tRNA biosynthesis in eukaryotes are not understood completely. It is proposed that seryl-tRNA is formed, followed by selenocysteine synthase formation to incorporate selenium in serine and generate selenocysteryl-RNA (Stadtman, 1996).

Selenocysteine incorporation into selenoproteins needs SECIS element. Since all codons are assigned to specific amino acids, selenocysteine uses another strategy to insert this amino acid into selenoproteins. First, the codon for selenocysteine is shared with one for translation termination, UGA. To distinguish these two events, there is a special sequence for selenocysteine tRNA recognition, known as SECIS element (Berry et al., 1993; Burk and Hill, 1993; Papp et al., 2007). The SECIS element forms a stem and loop structure, which can be recognized by SECIS-binding protein 2 (SBP2) that bends mRNA structures to facilitate selenocysteine tRNA recognition and (Low et al., 2000). This unique SECIS elements intrinsic in all selenoprotein genes is used as the strategy to search against the whole human and mouse genome for identification of all possible selenoproteins, resulting in the conclusion that there is a total of 25 selenoproteins in humans (Kryukov et al., 2003; Kryukov et al., 1999).

1.6.2 Selenoproteins in human genome

The expression of selenoproteins responds to body selenium status. There are three potential ways to alter the expression of selenoproteins, including transcription, post-transcriptional modification, and the sources of selenium (Burk and Hill, 1993). The post-transcriptional modification is critical for the maturation of selenocysteine-tRNA, and the sources of selenium determine the available material for selenocysteine biosynthesis. Futhermore, regulation of selenoprotein mRNA expression by dietary selenium status has been well studied (Kipp et al., 2009; Sunde, 2010; Sunde and Raines, 2011).

Selenoproteins play numerous functions in the cell, especially redox-related functions. Based on current understanding, selenoproteins play critical roles in redox regulation and signaling, thyroid hormone metabolism, selenocysteine synthesis, transportation and storage of selenium, and protein folding. In humans, the majority of the 25 selenoproteins exhibit antioxidative activities (Papp et al., 2007). Interestingly, the localizations of redox-related selenoproteins are various in the cell, suggesting that the major function of selenoproteins is to deal with oxidative stress in different compartments of the cells (Davis et al., 2012). Because of the localization divergence, the importance of selenoproteins may act on multiple pathways of redox regulation.

1.6.2.1 The selenium-dependent glutathione peroxidase family

The selenium-dependent glutathione peroxidases (GPxs) are critical antioxidant enzymes in different compartments of the cell and various tissues to protect against damages from oxidative stress. GPxs use glutathione to reduce hydrogen peroxide and organic hydroperoxides (Cohen and Hochstein, 1963). GPx (now GPx1) was first found as an enzyme to protect hemoglobin from oxidative breakdown in erythrocytes (Mills, 1957).

In February 1973, Rotruck et al. reported for the first time that GPx1 is a selenium-containing protein (Rotruck et al., 1973). This seminal contribution was confirmed by a paper published 3 months later from the Flohe group (Flohe et al., 1973). Since 2003, the consensus is that there is a total of five selenium-containing glutathione peroxidases in humans whose expression is tissue-specific. GPx1 expresses in the body ubiquitously, but is especially high in blood, kidney, liver and placenta. GPx2 is abundant in gastrointestinal tract, liver and mammary gland. The extracellular GPx3 is highly expressed in and secreted from epididymis, kidney and plasma, and resides in plasma. Phospholipid hydroperoxide GPx (GPx4) abundantly expresses in liver and testis. GPx6 is rich in embryo and olfactory epithelium (Kryukov et al., 2003). In terms of cellular distribution, current understanding indicates that 1) GPx1 localizes to cytoplasm and mitochondria, 2) GPx2 distributes in cytoplasm and possibly in Golgi apparatus, 3) GPx3 is in extra cellular space, and 4) GPx4 exists in cytoplasm, mitochondria and nucleus (Brigelius-Flohe and Maiorino, 2013; Brigelius-Flohe et al., 2001). Little is known about the cellular localization of GPx6.

GPx1 plays a pivotal role in the mitigation of oxidative damage (Cheng et al., 1998), which accounts for the majority of the peroxide-decomposing activity of selenium. A role of the GPx proteins in tumorigenesis has been extensively studied. GPx1 in principle can prevent the formation of oxidative DNA damage and suppress tumorigenesis (Brigelius-Flohe and Kipp, 2009; Esposito et al., 1999; Sandstrom and Marklund, 1990). Consistent with this notion, GPx1 can be transactivated by the tumor suppressor p53, resulting in declined oxidative stress (Tan et al., 1999). Similarly, overexpression of GPx1 counteracts tumorigenesis by the induction of Gadd45 expression for reinforcement of

cell cycle arrest (Nasr et al., 2004). GPx1 is also implicated in the protection against cardiovascular disease, neurodegeneration and autoimmune disease (Lei et al., 2007). However, the role of GPx1 in health promotion is controversial. Although increased oxidative stress is associated with many metabolic and age-related degenerations, increasing GPx1 expression above the physiological level may not necessarily beneficial. Intriguingly, GPx1 overexpression mice developed insulin resistance and glucose intolerance (McClung et al., 2004). On the contrary, GPx1 knockout mice on a high-fat diet are more resistant than wild type animals to develop insulin resistance and glucose intolerance (Loh et al., 2009). Likely, GPx1 can regulate hydrogen peroxide status in insulin signaling pathways (Veal et al., 2007). GPx2 may promote early stage carcinogenesis, but later inhibit cancer cell growth, invasion and migration (Brigelius-Flohe et al., 2001). Overexpression of GPx3 in mice may inhibit cancer cell growth and metastasis (Brigelius-Flohe and Kipp, 2009). Lastly, GPx4 overexpression in mice is known to inhibit the growth of tumor and stop metastasis (Brigelius-Flohe and Kipp, 2009).

1.6.2.2 Thioredoxin (Trx) signaling

In mammals, there are three thioredoxin reductases (TrxRs) that play various cellular and physiological functions. They are the cytosolic and extracellular TrxR1 (Tamura and Stadtman, 1996), the mitochondrial TrxR2 (Miranda-Vizuete et al., 1999), and the thioredoxin glutathione reductase, TrxR3 (Sun et al., 1999a). TrxRs are the members of the pyridine nucleotide-disulfide oxidoreductase family in mammalian cells and play critical roles in the recycling of oxidized Trx. The Trx system regulates the activity of NF-κB, AP-1, p53 and the glucocorticoid receptor, all of which are transcription factors

that contain cysteine residue(s) in their DNA-binding domains (Lu and Holmgren, 2009). TrxR1 is required for embryonic development. Knockout of TrxR1 in mice leads to the accumulation of nonfunctional oxidized rebonuleotide reductase, resulting in impaired DNA synthesis (Jakupoglu et al., 2005). Interestingly, TrxR1 was found to be overexpressed in malignant cells (Kakolyris et al., 2001; Matsutani et al., 2001; Raffel et al., 2003). Different from TrxR1, TrxR2 mainly resides in mitochondria and has been linked to the control of apoptotic signaling pathway (Nalvarte et al., 2004). Cells with TrxR2 mutations progress in an enhanced rate from G1 to S phases of the cell cycle, consistent with the proposed role of TrxR2 in cell proliferation regulation (Kim et al., 2003). Because the activities of TrxR are highly related to carcinogenesis, they are promising targets for cancer therapeutics (Klossowski et al., 2011).

1.6.2.3 Thyroid hormone metabolism

Iodothyronine deiodinases (DIO) regulate thyroid hormone maturation and maintain optimal health span. There are three DIOs in this family of proteins, which regulate the conversions between different forms of thyroid hormones, thyroxine (T4), 3,5,3'-triodothyronine (T3) and reverse triiodothyronine (rT3). DIO1 is mainly located in the liver, kidney, and thyroid. DIO2 expresses highly in brain, pituitary, thyroid, skeletal muscle and brown adipose tissue. DIO3 is abundant in placenta and uterus during pregnancy, as well as in cerebral cortex and skin (Davis et al., 2012). T4, the major hormone secreted from thyroid gland, requires DIO1 and DIO2 to catalyze the deiodination of T4, resulting in the formation of the active thyroid hormone, T3. In DIO1 or DIO2 knockout mice, the level of T3 in serum is normal (Galton et al., 2009), but

DIO2 knockout mice retain higher level of T4 in serum and show impairment in hearing, thermogenesis and neurocognition (St Germain et al., 2009). The phenotypes of DIO3 knockout mice include increased prenatal mortality, growth retardation, impaired fertility, hyperthyroidism in prenatal period and hypothyroidism in adulthood (St Germain et al., 2009). Since thyroid hormones are involved in the signaling regulation of differentiation, proliferation and apoptosis, DIOs may also mediate carcinogenesis. Increased expression of DIO3 has been shown to promote tumor proliferation (Piekielko-Witkowska and Nauman, 2011). Furthermore, DIO1 protein expression is decreased in various cancer types, including papillary thyroid cancer, papillary thyroid carcinoma, renal cell carcinoma, lung cancer, prostate cancer and hepatic adenoma. However, DIO2 protein expression is increased in follicular thyroid carcinoma, anaplastic thyroid cancer, medullary thyroid cancer, astrocytoma, gliosarcoma, glioblastoma and pituitary tumor. Furthermore, DIO3 protein expression is increased in liver hemangioma, gliomas, gliosarcoma, glioblastoma and pituitary tumors (Casula and Bianco, 2012). Because DIO1-3 change their protein expression in cancer cells, they are candidate biomarkers for cancer diagnosis and therapy.

1.6.2.4 Selenium transportation and storage

Selenoprotein P (Sepp1) transports and stores selenium to retain optimal health. Sepp1 is a plasma selenoprotein with 4 isoforms, the full-length of which contains 10 residues of selenocysteine (Burk and Hill, 2009; Himeno et al., 1996). Sepp1 is mainly synthesized in liver, heart, lung and kidney and secreted to extracellular fluids including plasma (Burk and Hill, 1994). Sepp1 is critical for selenium retention and transportation in the body. Sepp1 knockout rats show an increased level of selenium excreted through the

urine system (Burk et al., 2006). Sepp1 is responsible for selenium delivery in testis and brain (Hill et al., 2003; Schomburg et al., 2003) through the receptor-mediated uptake by apolipoprotein E receptor-2 (apoER2), whereas Sepp1 is uptaken by megalin in kidney (Olson et al., 2008; Olson et al., 2007). In addition to the well accepted role as a Se transporter, Sepp1 is also implicated in the development of spermatogenesis and the prevention of brain degeneration (Burk and Hill, 2009).

1.6.2.5 Other notable selenoproteins

Proteins in the Sep15 family, Sep15 and selenoprotein M, control the quality of protein folding in endoplasmic reticulum (ER) (Ferguson et al., 2006). Sep15 expresses highly in liver, kidney, testes and prostate (Davis et al., 2012), whereas selenoprotein M is mainly found in brain. Sep15 cooperates with the chaperon enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT) to regulate protein folding in ER (Korotkov et al., 2001). Sep15 may also participate in the disulfide bond modification of unfolded and misfolded proteins (Labunskyy et al., 2007). Furthermore, a recent report implicates Sep15 in the promotion of tumorigenesis and metastasis of colon cancer (Irons et al., 2010).

Selenoprotein H (SelH) is potentially a dual function protein involved in redox regulation and transactivation of phase II antioxidants; however, the mechanism of actions are mainly unknown. In mice, SelH mRNA is mainly expressed in brain, thymus, lung, testes and uterus (Novoselov et al., 2007). In humans, some cancer cell lines, including the colorectal HCT116 and prostate LNCaP cells, show increased expression of SelH mRNA. SelH was initially identified as a thioredoxin reductase homologue with glutathione peroxidase-like activity (Novoselov et al., 2007). Overexpression of human SelH in mouse HT22 neuronal cells improves cell survival after UVB irradiation through the

suppression of superoxide production (Ben Jilani et al., 2007). Moreover, SelH-overexpressed cells show elevated mitochondrial biogenesis and functions (Mendelev et al., 2010). In addition to being an antioxidant, SelH has been demonstrated as a potential transcription factor (Panee et al., 2007). Based on the protein domain searching, SelH carries an AT-hook domain, such that it can potentially bind DNA minor grooves for transactivation of the targeted genes. Panee et al used chromatin immunoprecipitation assays to demonstrate that GFP-tagged SelH protein appeares on heat shock element (HSE) and stress response element (STRE) (Panee et al., 2007). Additionally, results from a recent publication have shown that metal transcription factor-1 binds to metal response element (MRE) on SelH promoter for transactivation (Stoytcheva et al., 2009). Because oxidative stress can possibly be induced by heavy metals, SelH expression in principle is upregulated upon oxidative stress. Lastly, SelH is known to be localized to nucleoli under the condition of GFP-tagged SelH overexpression in NIH 3T3 cells (Novoselov et al., 2007), suggesting a role for SelH in rDNA metabolism.

Available lines of recent evidence suggest critical physiological roles of other less-characterized selenoproteins in optimal health. Selenoprotein N (SelN) mutations lead to a human genetic disorder, rigid spine muscular dystrophy. SelN potentially suppresses muscle disorder through the protection against protein oxidation, calcium handling abnormalities and predisposition to oxidative stress (Arbogast and Ferreiro, 2010; Castets et al., 2012). Selenoprotein W (SelW) is highly expressed in muscle, heart, spleen and brain, and plays important roles in human immune responses and thioredoxin-dependent redox pathway (Whanger, 2009). Selenoprotein R (SelR), also known as methionine sulfoxide reductase B (MsrB), is one of the enzymes in the Msr system, and the

expression of SelR is closely associated with the level of dietary selenium. Maintaining functional Msr system in the cell prevents protein-carbonyl adducts and protects cells against aging and age-related neurodegereration (Moskovitz and Oien, 2010; Oien and Moskovitz, 2009). Future studies on mechanistic and physiological investigation of these and other newly identified selenoproteins will provide critical insight into the battle against genetic and chronic diseases.

Chapter 2: Materials and Methods

2.1 Cell culture and reagents

The MRC-5 human diploid lung fibroblasts (Coriell Institute, Camden, NJ) were cultured at 37□ in 20% O_2 or in 3% O_2 in minimum Eagle's medium (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 units/ml penicillin and streptomycin. HeLa cervical cancer cells (ATCC, Manassas, VA) and HCT116 human colorectal adenocarcinoma cells complemented with hMLH1-expressing vector (HCT116+hMLH1) (Koi et al., 1994; Yanamadala and Ljungman, 2003) were maintained at 37□ in 20% O_2 in Dulbecco's modified Eagle's medium (Mediatech Inc., Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin and streptomycin. *N*-acetylcysteine (NAC) (Sigma-Aldrich), a glutathione mimic agent, was dissolved in water. KU 60019 and KU 55933 (Tocris, Ellisville, MO) were dissolved in Dimethyl sulfoxide (DMSO).

2.2 Stably knockdown by shRNA

The lentivirus-based strategy was used to deliver specific short hairpin RNA (shRNA)-containing cassette for the generation of stable knockdown cells. The human non-target scrambled sequence was adapted from Addgene Organization, and SelH and SelH2 shRNA sequences that target SelH mRNA 333-353 and 503-523, respectively, were designed based on the Invitrogen Block-it^{TM} RNAi designer. SelH and scrambled targeting sequences are listed in **Figure 3.1A**. For SelH and scrambled shRNA knockdown, the viral particles that contain SelH or scrambled shRNA cassette were

produced by BLOCK-iT™ Lentiviral RNAi Expression System (Life technologies). The viral particles were used to infect MRC-5, HeLa and HCT116+hMLH1 cells, followed by clonal selection. After 24-hour infection, one day recovery and 14-day blasticidin selection, a total of 36 clones (3 knockdown sequences) were picked and sub-cultured to confirm knockdown efficiency by using real-time PCR. A viable colony is defined as the colony with more than 50 cells. p53 shRNA knockdown was performed as described previously (Wu et al., 2011).

2.3 Reactive oxygen species (ROS) detection

The level of intracellular ROS is detected by using 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen). H_2DCFDA stock (1 M/ml) was prepared and dissolved in DMSO. MRC-5 cells on cover slips were washed with 1 X PBS twice and incubated in phenol red free RPMI medium containing 5 μM/ml H_2DCFDA for 15 minutes. Then, the H_2DCFDA loaded cells were washed twice with 1 X PBS and incubated for additional 15 minutes in dark at 37°C. The fluorescence signal in the unfixed cells was detected immediately by the GFP channel under Zeiss AxioObserver 100 fluorescence microscope (Carl Zeiss) for image acquisition.

2.4 Senescence Assay

To evaluate cellular senescence, the activity of senescence-associated β-galactosidase (SA-β-Gal) was measured by using a Senescence Detection Kit (BioVision, San Francisco, CA). SA-β-Gal is a useful marker to distinguish proliferating and senescent cells both *in vitro* and *in vivo* (Dimri et al., 1995). MRC-5 cells were seeded onto 12-well plates with a density of 10,000 cells/well, and incubated with or without H_2O_2 (20 μM) in

either 3% or 20% O_2 incubators. Then, the cells were washed once by 1X PBS, fixed at room temperature for 15 min, washed again three times by 1X PBS, and stained with X-gal at a concentration of 1 mg/ml for 8 h. The staining process was terminated by washing the cells in 1X PBS and overlaying in 70% glycerol. The images were captured under a light microscope for quantification.

2.5 Clonogenic Assay

HeLa and HCT116+hMLH1 SelH and scrambled shRNA cells were seeded in 6-cm dish in a density of 750 cells per dish. After 24 hours, attached cells were treated with a gradient dose of DNA clastogens, including hydroxyurea (Sigma-Aldrich), neocarzinostatin (Sigma-Aldrich), camptothecin (Sigma-Aldrich), paraquat (Sigma-Aldrich) and hydrogen peroxide (Fisher Scientific) for 24 hours. Then, the drug-containing media were replaced by a complete medium and cultured for additional 7 days. Cells were washed in 1 X PBS, fixed in 90% methanol and stained by 0.5 % crystal violet (Alfa Aesar, MA) in 25% methanol. A colony having more than 50 cells is defined as a viable one.

2.6 Immunofluorescence and immunoblotting

Immunofluorescence and immunoblotting analyses were performed as described previously (Qi et al., 2010; Wu et al., 2010). Focus positive cells were defined as those containing at least five foci within the nucleus (Maude and Enders, 2005; Qi et al., 2010; Wu et al., 2010). Briefly, permeabilized cells were incubated overnight at 4 °C with antibodies against H2AX (1:500; Abcam, Cambridge, MA), γH2AX (phospho-H2AX on Ser-139, 1:200; Abcam), ATM (1:500; Epitomics, Burlingame, CA), and phospho-ATM

on Ser-1981 (pATM Ser-1981, 1:500; Rockland, Gilbertsville, PA). γH2AX and pATM Ser-1981 are well-defined markers for DNA breaks and ATM pathway activation, respectively (Bakkenist and Kastan, 2003; Lobrich et al., 2010). Six pictures were randomly taken from each slide for statistical analysis. For immunoblotting, nuclear fraction was isolated by nuclear and cytoplasmic extraction kit (G-Bioscisnce, MO). The nuclear extracts were separated by SDS-PAGE and transferred onto a PVDF membrane. The blot was incubated sequentially with anti-phospho-Nrf-2 polyclonal antibodies (pNrf-2, 1:1000; Epitomics), anti-Nrf-2 polyclonal antibodies (Nrf-2, 1:1000; Santa Cruz Biotechnology, Dallas, TX), or anti-Lamin B polyclonal antibodies (Lamin B (C-20), 1:1000; Santa Cruz Biotechnology, Dallas, TX). After washing, the blots were incubated with HRP conjugated secondary antibodies, followed by chemiluminescent reagents (Super Signal, Pierce) for signal acquisition. All experiments were performed in duplicate and a minimum of three times.

2.7 Laser micro-irradiation and live cell imaging

A mixture of oxidative DNA damage and DNA breaks were generated in live cell nuclei by laser-induced micro-irradiation using a pulsed nitrogen laser as previously described (Chen et al., 2007). The laser system was coupled to a Zeiss Axiovert microscope for live cell, time lapse image capture. SelH-GFP (Novoselov et al., 2007) and proliferating cell nuclear antigen (PCNA)-DsRed vectors were transiently transfected into MRC-5 cells.

2.8 Total intracellular glutathione (GSH) detection and apoptotic cells

Total intracellular GSH is measured by monochlorobimane (mBCl), which forms fluorescence adducts with GSH (Franco and Cidlowski, 2006; Sebastia et al., 2003).

Adherent cells were collected and preloaded with mBCl (40 μM) in PBS for 10 minutes. Then, propyl iodide was added for differentiating live and dead cells. Only dead cells are permeable and contain positive propyl iodide signal. The stained cells were immediately applied to FACSCanto II flow cytometric analyses (BD Bioscience, CA) to determine the level of GSH. The signal was excited with a violet 405 nm laser, and emission was acquired with a 450/50 filter.

Apoptotic cells were determined by mitocapture kit (Biovision, San Francisco, CA) staining. Mitocapture in live cells accumulated in mitochondria and showed red fluorescence. In apoptotic cells, there is no aggregation of mitocapture so green fluorescence was shown. The cells on cover slip were incubated with diluted mitocapture (1 μl mitocapture in 1 ml pre-warmed incubation buffer) at incubator for 15 min, and washed three times with the incubation buffer. The stained cells were subjected to fluorescence microscopy, and observed by GFP and DsRed channels. Eight pictures were randomly taken from each slide for statistical analysis.

2.9 Generation of short telomere mouse

The *Terc*$^{+/-}$ mice were provided by Dr. Mark Mattson at the National Institute on Aging (Baltimore, Maryland). The *Terc*$^{+/-}$ mice had been established by backcrossing to >99% pure C57BL/6 background before the progressively interbred of *Terc*$^{-/-}$ mice for the generation of late generation short telomere mice used in this study (Blasco et al., 1997). The genotypes of G1 progenies were identified by tail-biopsy genomic PCR to select G1 Terc$^{-/-}$ and Terc$^{+/+}$ control mice. Then, following the mating scheme (**Figure 2.1**), the G3 Terc$^{-/-}$ mice with shorter telomere mice were housed under aseptic conditions in

individually ventilated cages within a controlled-temperature (22°C) animal room utilizing a 12-h dark:night cycle. Mice had ad libitum access to food and water. These experiments were approved by the IACUC Committee at the University of Maryland, College Park, an AAALAC accredited campus, and were conducted in accordance with the NIH guidelines for the care and use of experimental animals.

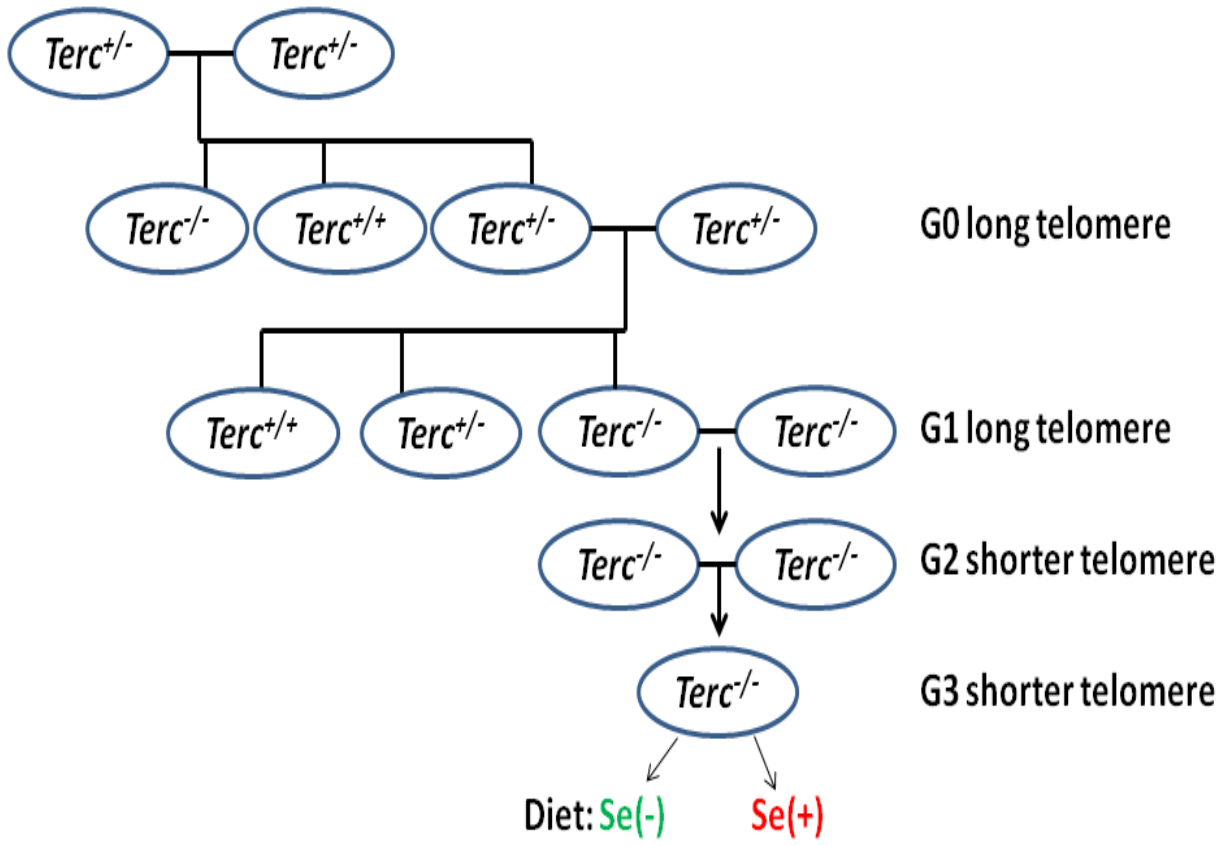


Figure 2.1 Breeding scheme *Terc*$^{+/-}$, *Terc* knockout heterozygous; *Terc*$^{-/-}$, *Terc* knockout homozygous; Se (-), Se-deficient diet; Se (+), Se-adequate diet.

2.10 Diets

The Torula yeast-based purified diets (**Figure 2.2**) were formulated based on the AIN-93G diet (Reeves et al., 1993) and made by Dyets Inc. (Bethlehem, PA, USA). The basal (Se-) diet contains 30% Torula yeast and <0.03 mg/kg Se by analysis (AOAC Method 986.15; Covance Laboratory, Madison, WI, USA). The basal diet was supplemented with 0.15 mg Se/kg (Se+) as sodium selenate (Holmstrom et al., 2012). Weanling mice were fed with the Se- or Se+ diet until they were sacrificed at 12-24 months of age or die naturally. The diets for adult mice are based on AIN-93G instead of AIN-93M because the former has a higher fat and protein content that better reflects the dietary components of Western foods. The diets were stored at -20 °C until feeding and kept for less than 6 months.

Ingredient	Amount
	g/1 kg
Torula yeast	300.00
Sucrose	100.00
Cornstarch	318.90
Dyetrose	107.00
L-Cysteine	3.52
Cellulose, microcrystalline	50.00
Soybean oil	70.00
t-Butylhydroquinone	0.01
L-Methionine	2.91
L-Tryptophan	0.16
Choline Bitartrate	2.50
Vitamin mix[2]	10.00
Mineral mix[3]	35.00

[1]Basal diet contains <0.03 mg Se/kg diet.

[2]Vitamin mix components (per kg diet): niacin, 30 mg; calcium pantothenate, 16.0 mg; pyridoxine HCl, 7.0 mg; thiamine HCl, 6.0 mg; riboflavin, 6.0 mg; folic acid, 2.0 mg; biotin, 0.20 mg; vitamin E acetate (500 IU/g), 150 mg; vitamin B12 (0.1%), 25.0 mg; vitamin A palmitate (500000 IU/g), 8.0 mg; vitamin D3 (400000 IU/g), 2.50 mg.

[3]Mineral mix components (per kg diet): $CaCO_3$, 19.43 g; NaCl, 2590 mg; Na_2O_3Si, 50.75 mg; $KCrS_2O_8$ 9.625 mg; $CuCO_3$, 5.005 mg; H_3BO_3, 2.870 mg; NaF, 2.240 mg; $NiCO_3$, 1.120 mg; LiCl, 0.595 mg; KIO_3, 0.35 mg; $(NH_4)_6Mo_7O_{24}$, 0.280 mg; NH_4VO_3, 0.245 mg.

Figure 2.2 Composition of Torula yeast basal diet[1]

2.11 Hair graying

The degree of hair graying was defined as described before (Rudolph et al., 1999). Based on the estimated percentage of gray hair on the body, degree of hair graying was scored as 0 (0~10%), 1 (10~20%), 2 (20~30%), 3 (30~40%), 4 (40~50%) or 5 (>50%).

2.12 Additional serum markers

The fasting and fed plasmas were collected from selenium-deficient and selenium-adequate mice at age of 12 months and 18 months. The biomarkers (triglyceride, cholesterol, glucose, amylase, aspartate aminotransferase, alanine aminotransferase, creatine phosphokinase, lactate dehydrogenase, alkline phosphatase, bilirubin, albumin, and creatine) were measured by AniLytics Inc. (Gaithersburg, MD).

2.13 Wound healing experiment

To perform wound healing experiment (n=5 per group), a skin surgery was operated and the recovery was monitored by measuring the size of the wound. Anaesthetic inhalation was done by isoflurane before surgery. Then, Nair (Church & Dwight, NJ) was applied to the area of skin surgery for hair removal. Then, the bald skin was cleaned by 70% alcohol and betadine. The fixed size wound was generated by a 5-mm biopsy punch to perform the round shape wound. The biopsy punch cut through the epidermis and dermis, reaching panniculus carnosus. In the following 7 days of recovery, the mice were housed individually and supplied with sulfamethoxazole/trimethoprim at 1 ml/150 ml in drinking water wrapped with aluminum foil. The size of the wound was measured everyday utill Day 7.

2.14 Bone density determination by micro microtomography (μCT) scanning

The left femurs were collected from the mice in different dietary and age groups. Adherent tissues were removed from femur. Then, the bone was placed in a holder with a 10.2 mm diameter and scanned by Scanco μCT. The structures of trabecular and cortical bone structural indices were determined to acquire bone volume, connectivity density, cortical thickness, trabecular number, separation and thickness. We collaborate with Dr. Jay Cao (Grand Forks, ND), who has direct experience on this analysis (Cao et al., 2012).

2.15 Circulating miRNA isolation, microarray and real-time PCR confirmation

To maximize confidence, a high-throughput platform, TaqMan low density array, was used, and the expression of miRNAs was validated by using individual quantitative polymerase chain reaction (qPCR) assays (Witwer et al., 2012). To this end, we have profiled over 800 miRNAs. To obtain circulating miRNA, 300 μL whole blood was collected by 3.2 % sodium citrate (1:9), and plasma was isolated by 2000 x g centrifugation for 15 minutes. The total miRNA was isolated by miRNeasy Serum/Plasma Kit (Qiagen, Germany). The purified total miRNA was transcribed into cDNA by Megaplex RT Primers (Rodent Pool Set) and TaqMan MicroRNA Reverse Transcription Kit (Life technologies, CA). Then, cDNAs from pool A and pool B were pre-amplified by TaqMan PreAmp Master Mix with Megaplex™ PreAmp Pools. Lastly, pre-amplified samples were subjected to microarray analysis by TaqMan OpenArray MicroRNA Panels (Life technologies, CA).

To confirm the differential expression of miRNAs, real-time PCR was used to determine the expression of those miRNAs that display significant changes in the microarray

analysis. The prepared cDNA from Megaplex RT Primers were pre-amplified by specific miRNA primers with TaqMan PreAmp Master Mix. Real-time PCR was performed by specific miRNA probe labeled with fluorescence and TaqMan OpenArray Real-Time PCR Master Mix (Life technology, CA).

2.16 Glucose tolerance test and insulin tolerance test

The mice subjected to glucose tolerance test were fasted overnight (n=6 per group). The glucose (1g/kg body weight) was injected into mice by intraperitoneal (ip) injection (McClung et al., 2004). For insulin tolerance test, insulin (0.25 U/kg body weight) was ip injected and blood drawn every 30 minutes. Blood glucose was measured by CONTOUR Blood Glucose Meter (Bayer, Germany).

2.17 Plasma Insulin and Plasma IGF-1 detection

Plasma insulin was determined during the time course of glucose tolerance test. Plasma insulin was measured by a Mouse Insulin ELISA Kit (ALPCO Diagnostics, NH). For each sample, 10 μL plasma was applied into a 96-well plate strip with HRP labeled monoclonal insulin antibody cross-linked. After insulin hybridization with the antibody, TMB (3,3′,5,5′-tetramethylbenzidine) was added to detect HRP, and the reaction was stopped to form yellow color. Then, the microplate reader was used to detect the density of the color by the absorbance at 450 nm.

The plasma IGF-1 was determined at fasting and fed state. Plasma IGF-1 was measured by IGF-1 (Mouse/Rat) ELISA Kit (ALPCO Diagnostics, NH). The plate was coated with anti-mouse/rat IGF-I antibody, and 50 μL plasma was applied to the plate. After IGF-1 hybridization with the antibody, a TMB-based method was performed as described before.

The results were detected by a microplate reader (Bio Tek, Winooski, VT) at the absorbance at 450 nm.

2.18 Telomere length evaluation

The length of telomere was determined by Flow-Fluorescence in situ hybridization (Fish). The telomere specific FITC-PNA probe (FITC-O O C C C T A A C C C T A A C C C T A A O O) was synthesized by Bio-Synthesis Inc. (Lewisville, TX). The colonocytes were isolated from animals as described before (Abolhassani et al., 2008). 10^6 cells were washed by 1 X PBS with 0.1% BSA and centrifuged at 1000 rpm for 7 minutes. Then, cells were resuspended in a hybridization buffer (70% Formamide, 1% BSA and 0.02 M Tris pH=7.4) with or without 0.3 µg/ml Telomere FITC-PCNA probe. For probe hybridization, cells were denatured first at 86 °C for 10 minutes. The following hybridization was performed at room temperature for 2 hours. The hybridized cells were serially washed with Wash I (70% formamide, 0.1% BSA, 0.02 M Tris pH=7.4 and 0.1% Tween-20) and Wash II (1 X PBS, 0.1% BSA and 0.1% Tween-20) buffers. The washed cells were resuspended in Staining solution (1 X PBS, 0.1% BSA, 10 U/ml RNase A and 0.6 µg/ml 7-Aminoactinomycin D) for flow cytometric analysis. The signals were obtained from both 530/30 nm FITC channel and Blue 670 nm LP channel to evaluate the telomere length in live cells.

2.19 Statistical analysis

The software, SigmaPlot 12.5 was used for the following statistical analyses. The student *t*-test was employed for analyses of SelH knockdown efficiency (**Fig. 3.1B**), the growth of shRNA MRC-5 cells (**Fig. 3.2A, B, E and F; 3.6A; 3.7A**), autofluorescence (**Fig**

3.2C), ROS (**Fig. 3.2G**), glutathione (**Fig 3.8A**) and apoptosis (**Fig. 3.8B**) in shRNA cells, senescence in shRNA cells (**Fig 3.2D; 3.3C; 3.6D; 3.7B**), γH2AX and pATM Ser-1981 positive cells (**Fig. 3.3A and B; 3.6B and C**), cologenic analysis (**Fig. 3.4A, B, C, D and E; 3.5; 3.8C**), the foci number between SelH/p53 shRNA and SelH/scrambled shRNA (**Fig. 3.6E**), pNrf2 expression (**Fig. 3.9A and B**), scores of skin abnormality (**Fig. 4.1A and B**), wound healing (**Fig. 4.1C**), the fluorescence analysis of telomere Flow-FISH (**Fig. 4.1D**), glucose tolerance test (**Fig. 4.2C, D, E and F**), food intake (**Fig. 4.2B**), insulin tolerance test (**Fig. 4.2G and H**) and the amount of plasma insulin (**Fig. 4.2I and J**). The average monthly body weight (**Fig. 4.2A**) was analyzed by paired *t*-test. To determine the effects of age and dietary selenium, two-way ANOVA was employed for analyses of plasma selenium (**Table 4.1**), chemical factors in plasma (**Table 4.2**), connectivity density in bone (**Fig. 4.3B**), and qPCR of miRNAs (**Fig. 4.4**) . In the figures presented below, "*" represents $p<0.05$ and "**" represents $p<0.01$.

Chapter 3: Selenoprotein H suppresses cellular senescence through genome maintenance and redox regulation via ATM and p53.

Cellular senescence restricts cell proliferation through permanent withdraw from cell cycle and has been implicated in tissue regeneration, aging, and tumorigenesis (Rodier and Campisi, 2011). Persistent DNA damage, dysfunctional telomere, and acute chromosome conformation change can lead to a senescent stage (Fumagalli et al., 2012; Mallette and Ferbeyre, 2007; Takahashi et al., 2006). It is thought that cellular senescence prevents oncogenesis (Halazonetis et al., 2008) or promotes tissue regeneration in early stage of life (Jun and Lau, 2010), but the accumulation of senescent cells contributes to aging and age-related disorders later in life (Dimri et al., 1995; Ressler et al., 2006; Voghel et al., 2007; Wang et al., 2009).

ROS induce the formation of oxidative DNA damage and DNA breaks. If left unrepaired, persistent activation of DNA damage response can trigger permanent cell cycle arrest, known as stress-induced senescence. This notion of oxidative stress accumulation is consistent with that of the free radical theory of aging (Harman, 1960; Hutter et al., 2002). Ataxia telangiectasia mutated protein (ATM) appears to be a key DNA damage response kinase coordinating checkpoint proteins p53 and p21 (Suzuki et al., 2012) and the senescence response. ATM is activated in the response to DNA breaks and oxidative stress (Guo et al., 2010; Lee and Paull, 2005) and stabilizes p53 by phosphorylation at Serine 15 (Canman et al., 1998). An essential role of ATM and p53 in the senescence

response has been reported (Bartkova et al., 2005; Bartkova et al., 2006; Wu et al., 2010; Wu et al., 2011).

Selenium, an essential trace element, is required for the biosynthesis of selenoproteins. In mammal, the majority of selenoproteins carry antioxidative functions (Kryukov et al., 2003; Lu and Holmgren, 2009). Selenium deficiency decreases selenoprotein expression, with selenoprotein H (SelH), glutathione peroxidase-1, selenoprotein W and Sep15 being the most sensitive selenoproteins to body selenium fluctuations (Kipp et al., 2011; Raines and Sunde, 2011; Sunde, 2010). Conditional knockout of selenocysteine tRNA that suppresses selenoprotein expression in epidermal cells or osteo-chondroprogenitor cells showed age-related disorders including alopecia and bone abnormality (Downey et al., 2009; Sengupta et al., 2010). These observations are consistent with an estimation linking eleven selenoproteins to aging or age-related disorders (McCann and Ames, 2011).

SelH is a thioredoxin-like protein in the nucleolus exhibiting both antioxidant and transactivation functions (Novoselov et al., 2007). Studies of human SelH in HT22 mouse neuronal cells demonstrated that SelH protects against UVB-induced apoptosis and being a transactivator for glutathione biosynthesis by employing forward and reverse genetic approaches (Ben Jilani et al., 2007; Mendelev et al., 2011; Mendelev et al., 2009; Panee et al., 2007). Although human SelH protects mouse HT22 cells against H_2O_2 exposure, the mechanism by which SelH protects against oxidative stress and the biological consequences are not clear. Because SelH expression is enriched in the nucleoli where has been proposed as a possible stress-sensing center in the nucleus (Lewinska et al., 2010; Mayer et al., 2005; Panee et al., 2007), the hypothesis is that SelH protects against oxidative stress through genome maintenance and limitation of

replicative senescence. To test the hypothesis, the stable SelH shRNA knockdown was generated in human normal diploid fibroblast and cancerous cells to evaluate their cellular and biochemical responses to various clastogens. The results suggested a new role of SelH specifically in the cellular response oxidative stress to suppress replicative senescence through genome maintenance via ATM and p53.

3.1 Essential role of SelH in the inhibition of replicative senescence and oxidative stress

Mouse lung cancer LCC1 cells with transient SelH siRNA knockdown are hypersensitive to H_2O_2 exposure (Novoselov et al., 2007); however, the biological significance of SelH is largely unknown. To explore whether SelH plays a role in the replicative lifespan of normal human fibroblasts, the SelH shRNA and scrambled shRNA knockdown MRC-5 cells were generated by lentiviral delivery of shRNA cassettes. The choice of the diploid lung MRC-5 fibroblast is based on a previous mouse study showing that SelH expression is relatively abundant in the lungs and cultured lung cells (Novoselov et al., 2007). The two shRNA sequences were designed for targeting the 3' end of SelH mRNA. Results from real-time quantitative RT-PCR demonstrated >80% knockdown efficiency in both SelH shRNA MRC-5 cells (**Fig. 3.1A and B**). Strikingly, SelH shRNA MRC-5 cells proliferated poorly. By passage 4 (35 days after clonal selection), SelH shRNA MRC-5 cells accumulated non-dividing large cells and completely stopped growth, whereas scrambled shRNA MRC-5 cells proliferated exponentially (**Fig. 3.2A and B**). These results indicate that SelH deficiency restricts cell proliferation in human diploid fibroblasts.

(A)

Target	Sequence
Scrambled	5'-CCTAAGGTTAAGTCGCCCTCGC-3'
SelH	5'-GCCAAACTTCAGTCATGATCC-3'
SelH 2	5'-GGAGCTCTGGACTGGGATTAA-3'

(B)

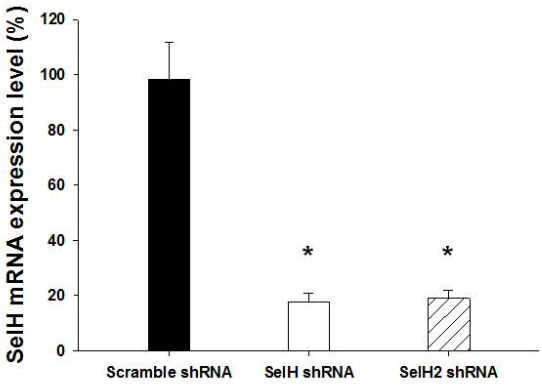
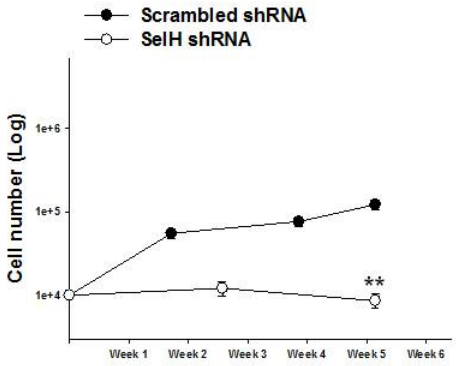
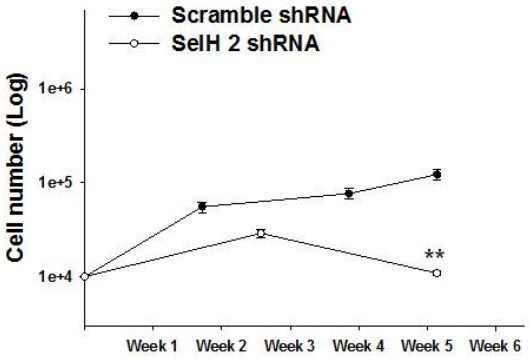


Figure 3.1 shRNA knockdown sequences and knockdown efficiency in MRC-5 cells The Sequences of shRNAs are listed in (A). SelH shRNA knockdown efficiency (B) was determined by real-time PCR. Total RNA was isolated by TRIzol (Invitrogen), and cDNA was transcribed by AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, CA). Real-time PCR was carried out using TaqMan Fast Universal PCRMaster Mix (Applied Biosystems, CA) and an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Quantification of mRNA levels was performed using the delta Ct method as previously described (Wu et al., 2011) (*, $p<0.05$, compared to scrambled shRNA)

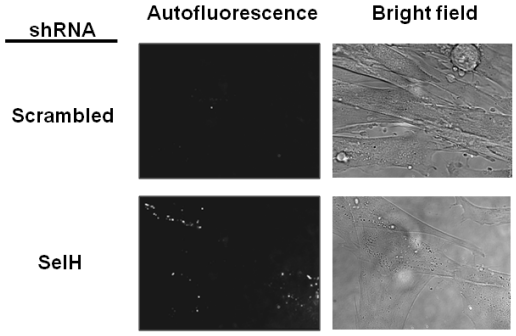
(A)

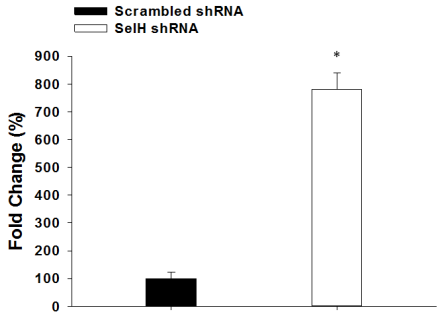

Scrambled shRNA
SelH shRNA
Cell number (Log)
1e+6
1e+5
1e+4
**
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6

(B)

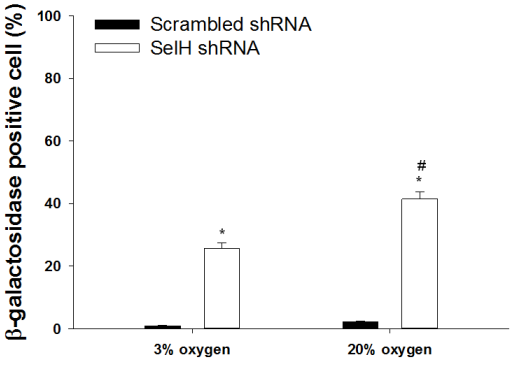

Scramble shRNA
SelH 2 shRNA
Cell number (Log)
1e+6
1e+5
1e+4
**
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6

(C)

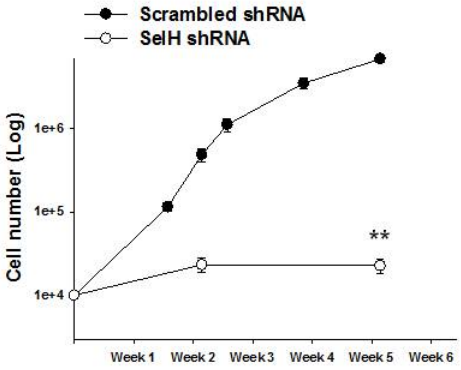

shRNA
Autofluorescence
Bright field
Scrambled
SelH


Scrambled shRNA
SelH shRNA
Fold Change (%)
900
800
700
600
500
400
300
200
100
0
*

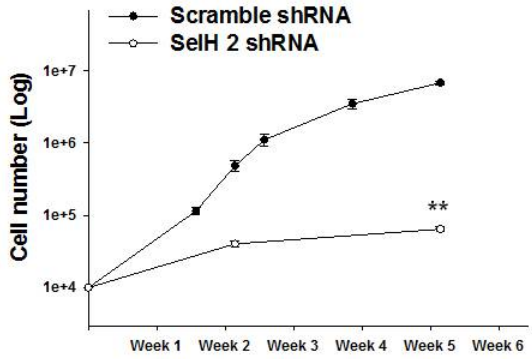
(D)


Scrambled shRNA
SelH shRNA
β-galactosidase positive cell (%)
100
80
60
40
20
0
*
#
*
3% oxygen
20% oxygen

(E)


Scrambled shRNA
SelH shRNA
Cell number (Log)
1e+6
1e+5
1e+4
**
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6

(F)



(G)

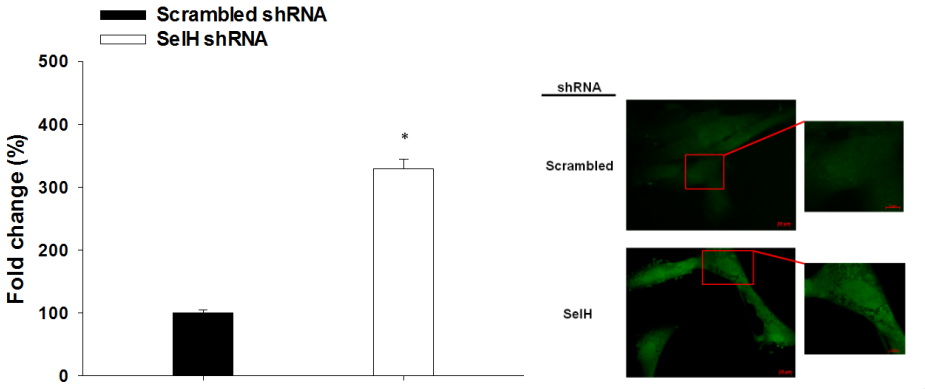


Figure 3.2 Severe inhibition of proliferation, early onset of cellular senescence and oxidative stress in SelH shRNA MRC-5 cells. SelH shRNA and scrambled shRNA MRC-5 human diploid fibroblast were seeded (10^4 cell/well) onto 12-well plates and cultured for 35 days in 20% O_2 incubator (SelH, (A); SelH2, (B)) or 3% O_2 incubator (SelH, (E); SelH2, (F)) (n=3), followed by cell counting when approaching confluence. Autofluorescence was detected in the cells under fluorescence microscope by using GFP channel and bright field (C) (*, $p<0.05$; **, $p<0.01$, compared to scrambled shRNA MRC-5 cells, n=6). SelH shRNA and scrambled shRNA MRC-5 cells were cultured in 20% O2 and 3% O2 incubators for xx days, followed by analyses of SA-β-galactosidase activity (D) (*, $p<0.05$, compared to scrambled shRNA MRC-5 cells; #, $p<0.05$, compared SelH shRNA MRC-5 cells at 20% and 3% O_2). The SA-β-galactosidase positive cells were presented with their respective mean ± S.E (n=3). The cells were at second passage after selection, and were incubated with CM-H_2DCFDA (10 µmol/L) for 15 min at 37°C. Then, the GFP signal was determined by fluorescence microscope. The fold difference was means ± S.E (n=6) and representative pictures are shown (G) (*, $p<0.05$, compared to scrambled shRNA MRC-5 cells).

Most normal mammalian cells do not divide indefinitely and undergo replicative senescence, a terminal cell cycle arrest towards the end of cellular aging (Campisi and d'Adda di Fagagna, 2007; Vijg and Campisi, 2008). It was previously demonstrated that old cells presented increased expression of autofluorescence (Wilhelm et al., 2009) and old fibroblasts were enlarged and flat in shape (Kuilman et al., 2010). To determine whether SelH shRNA MRC-5 cells display early onset of cellular aging, the autofluorescence, senescence-associated morphological changes, and the expression of SA-β-Gal were assessed in the cells, which are the second passage after clonal selection. Evaluated by fluorescence microscopy, the intensity of autofluorescence in GFP channel was 7-fold greater in SelH shRNA MRC-5 cells than in scrambled shRNA MRC-5 cells (**Fig. 3.2C**). Moreover, the flattened and enlarged phenotypes in SelH shRNA MRC-5 fibroblasts are indicative of senescence. Because oxygen tension (20% O_2, atmosphere level) contributes to replicative senescence in human fibroblasts (Parrinello et al., 2003; Poulios et al., 2007), the cells were cultured in 3% (physiological level) and 20% O_2 condition to compare the level of replicative senescence. Whereas scrambled shRNA MRC-5 cells were not apparently SA-β-Gal positive (1.0±0.2 % vs 2.3±0.1%) after 7 days in 3% or 20% O_2 incubator, there were significantly greater ($P < 0.05$) SA-β-Gal positive SelH shRNA MRC-5 cells under the same culture condition (**Fig. 3.2D**). Moreover, there were significantly greater (41.5±2.2 % vs 25.7±1.7 %) SA-β-Gal positive SelH shRNA MRC-5 cells when cultured in 20% than in 3% O_2 incubator, suggesting that SelH plays a critical role in the suppression of replicative senescence in association with oxygen tension. Furthermore, culturing cells under ambient O_2 level (20%) could activate stress-induced senescence (Moussavi-Harami et al., 2004).

Consistent with this notion, scrambled shRNA MRC-5 cells proliferated 55-fold greater in 3% than in 20% O_2 incubator after 5 weeks in culture. Interestingly, the complete growth inhibition of SelH shRNA MRC-5 cells under 20% O_2 condition can be partially rescued when grown in 3% O_2 incubator; however, SelH shRNA MRC-5 cells still grew ~300-fold slower compared to scrambled shRNA MRC-5 cells (**Fig. 3.2E and F**). To determine whether oxidative stress contributes to the observed cellular senescence and slow proliferation, the level of H_2DCFDA fluorescence was measured. It was found that intracellular ROS was 3-fold greater in SelH than in scrambled shRNA MRC-5 cells at passage 1 after clonal selection under 20% O_2 condition (**Fig. 3.2G**).

Altogether, these results suggested that SelH is required for cellular proliferation and the suppression of replicative senescence in a manner depending of ROS in human diploid fibroblast. Indeed, SelH may function as a critical antioxidant to protect against stress-induced senescence.

3.2 SelH knockdown MRC-5 cells display severe genome instability and pronounced DNA damage response

Human diploid fibroblasts maintain the senescence arrest by persistent DNA breaks and oxidative DNA damage (Campisi and d'Adda di Fagagna, 2007; Chen et al., 1995), both of which can activate the key DNA damage response kinase ATM (Guo et al., 2010; Lee and Paull, 2005). To determine a role of SelH in the DNA damage and senescence responses to oxidative stress, γH2AX and pATM Ser-1981 expression were assessed in SelH shRNA and scrambled shRNA MRC-5 cells after H_2O_2 treatment. SelH shRNA and scrambled shRNA MRC-5 cells were treated with H_2O_2 (20 μM) for 1 day, followed

by 0-5 days recovery in 20% O_2 incubator. The percentage of γH2AX or pATM Ser-1981 positive cells was greater ($P < 0.05$) in SelH shRNA than in scrambled shRNA MRC-5 cells intrinsically and 1 day after H_2O_2 treatment (**Fig. 3.3A and B**). The H_2O_2 treatment significantly induced γH2AX and pATM Ser-1981 expression in both SelH shRNA and scrambled shRNA cells. To assess a role of SelH in the repair of oxidative DNA damage, I followed γH2AX and pATM Ser-1981 expression 0-5 days after recovery from the H_2O_2 treatment. While the percentage of γH2AX and pATM Ser-1981 positive cells subsided in the scrambled shRNA MRC-5 cells, these markers were further activated during the time course of recovery in SelH shRNA MRC-5 cells. Noticeably, the reduction of pATM Ser-1981 expression was complete and faster than γH2AX in scrambled shRNA MRC-5 cells during the time course, suggesting that 20 μM is a physiological dose of H_2O_2 in normal MRC-5 cells and the ATM pathway activation mainly responds to H_2O_2 but not DNA breaks in this scenario. Therefore, SelH plays a pivotal role in the repair of oxidative DNA damage and the prevention of persistent DNA damage.

Permanent DNA damage and persistent DNA damage response are hallmarks of cellular senescence (Di Micco et al., 2008; Rodier et al., 2009). In order to further investigate whether permanent DNA damage due to acute oxidative stress contributes to senescence, the activity of SA-β-Gal was determined at 5 days after recovery from H_2O_2 treatment (20 μM, 1 day) in SelH shRNA and scrambled shRNA MRC-5 cells cultured in 3% and 20% oxygen incubators. The percent senescent cells ware increased to 17% and 70% in scrambled shRNA and SelH shRNA MRC-5 cells, respectively, under ambient oxygen level (**Fig. 3.3C**). Interestingly, in 3% O_2 condition, the percent senescent cells

significantly dropped from 70% to 34 % in SelH shRNA MRC-5 cells and from 17% to 2 % in scrambled shRNA MRC-5 cells. Therefore, SelH appears to suppress cellular senescence when persistent DNA damage response is induced by chronic and acute oxidative stress.

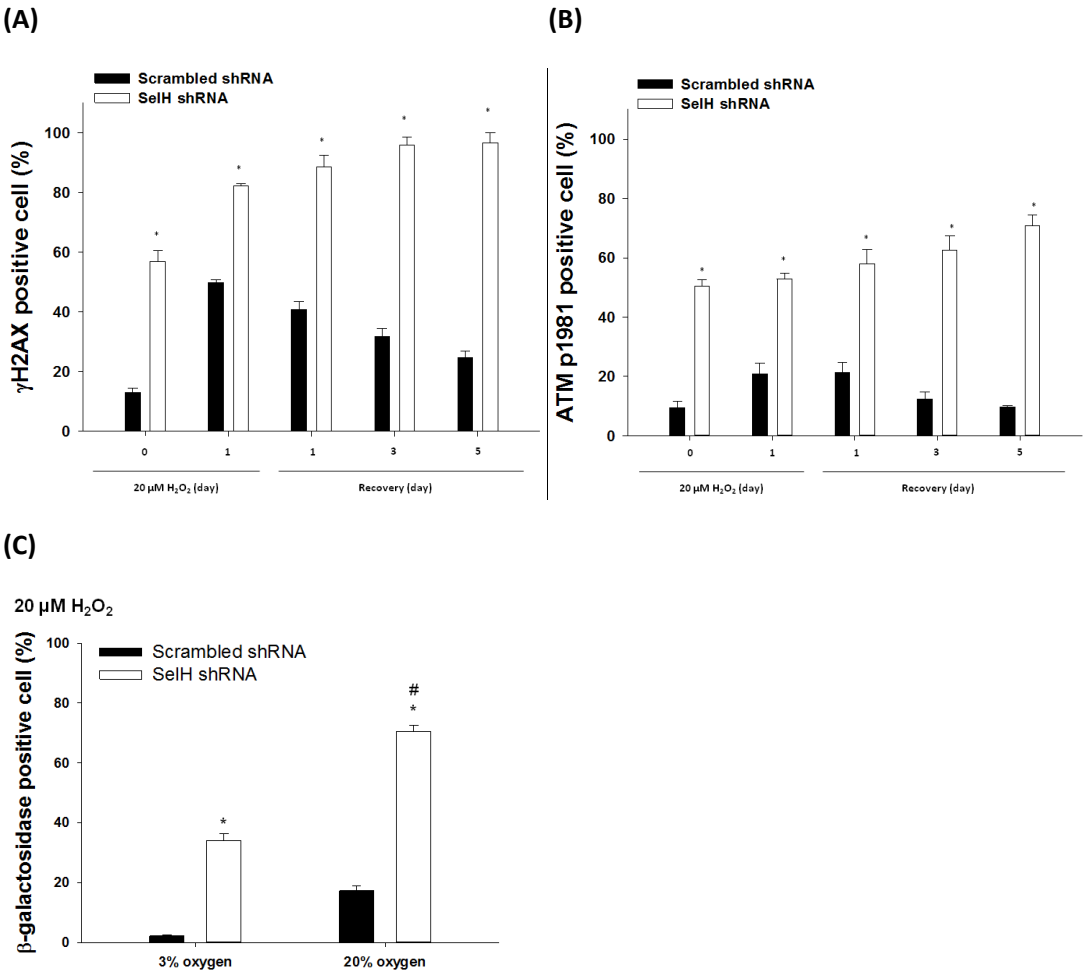


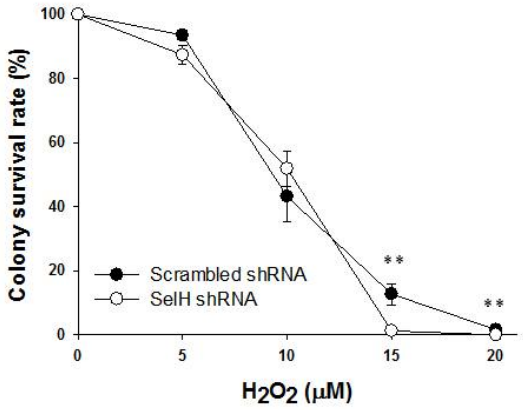
Figure 3.3 Persistent DNA damage response and increased sensitivity to chronic oxidative stress in SelH shRNA MRC-5 cells. SelH shRNA and scrambled shRNA MRC-5 cells were at second passage after selection, seeded onto coverslip, treated with 20 μM H_2O_2 for 24 hours, and followed by 5-day recovery in fresh medium for the detection of phosphorylation of H2AX on Ser-139 (γH2AX) and ATM phosphorylation on Ser-1981 (pATM Ser-1981). The percentages of γH2AX (A) or pATM Ser-1981 (B) positive cells were presented with mean ± S.E (n=6) (*, $p<0.05$, compared to scrambled shRNA MRC-5 cells at individual time point). Detection of SA-β-galactosidase positive cells was performed as described in Figure 1, and the data were presented with mean ± S.E (n=6) (C) (*, $p<0.05$, compared to scrambled shRNA MRC-5 cells; #, $p<0.05$, compared SelH shRNA MRC-5 cells at 20% and 3% O_2).

3.3 SelH deficiency specifically sensitizes cells to clastogens that induce oxidative stress

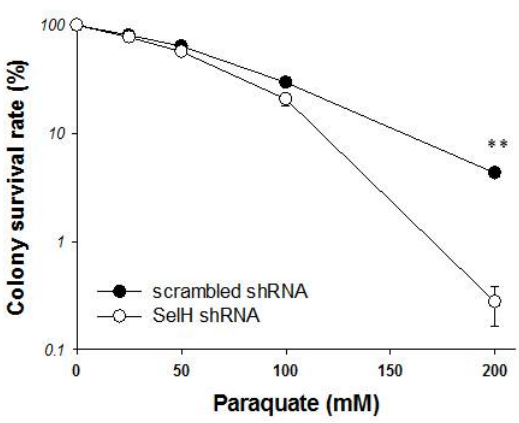
To address whether SelH protects against clastogens other than H_2O_2, cells were exposed to a variety of genotoxic agents and subjected to colonogenic assay. Although this method is considered a gold standard for assessing cell proliferation after DNA damage, not all cells, including the MRC-5 diploid fibroblasts, are capable of forming colonies from very low density seed cells. To circumvent this limitation and to determine whether SelH protection against oxidative DNA damage limits to diploid cells, the SelH shRNA and scrambled shRNA HeLa cells were generated by lentiviral delivery. Consistent with the results shown in MRC-5 cells, cologenic assay revealed that SelH shRNA HeLa cells displayed greater sensitive to oxidative stress inducers, including H_2O_2 and paraquat (**Fig. 3.4A and B**). In contrast, colony formation was comparable between SelH shRNA and scrambled shRNA HeLa cells in response to a gradient concentration of replication stress inducers hydroxyurea and camptothecin and a potent γ-irradiation mimetic, neocarzinostatin (**Fig. 3.4C, D and E**). In addition, SelH shRNA HCT116+hMLH1 colorectal cancer cells (Qi et al., 2010) displayed hypersensitivity to H_2O_2 exposure in a dose-dependent manner (**Fig. 3.5**). Therefore, SelH protects against clastogens specific to those induce ROS in various cells. To further investigate the role of SelH in the cellular response to DNA damage, the live cell imaging of SelH translocation to the site of localized DNA damage containing a mixture of DNA breaks and oxidative DNA damage was performed by monitoring fluorescent EGFP-tagged SelH and DsRed-tagged PCNA, a sensitive marker of DNA synthesis and repair (Balajee and Geard, 2001; Mortusewicz et al., 2011). Although PCNA was recruited to and enriched at the site of DNA damage, SelH did not mobilize to the site of DNA damage (**Fig. 3.4F**). Therefore, SelH

specifically protects against oxidative stress, but this is less likely to be directly involved in the early DNA damage response pathway.

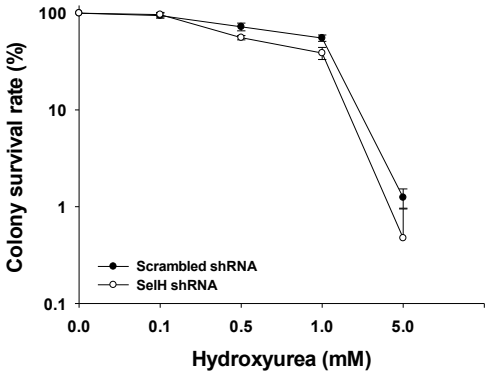
(A)


Colony survival rate (%)
100
80
60
40
20
0
0
5
10
15
20
Scrambled shRNA
SelH shRNA
**
**
H2O2 (μM)

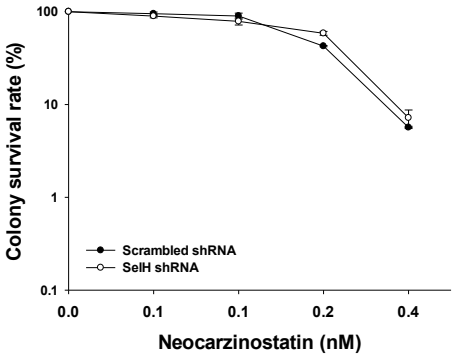
(B)


Colony survival rate (%)
100
10
1
0.1
0
50
100
150
200
scrambled shRNA
SelH shRNA
**
Paraquate (mM)

(C)


Colony survival rate (%)
100
10
1
0.1
0.0
0.1
0.5
1.0
5.0
Scrambled shRNA
SelH shRNA
Hydroxyurea (mM)

(D)


Colony survival rate (%)
100
10
1
0.1
0.0
0.1
0.1
0.2
0.4
Scrambled shRNA
SelH shRNA
Neocarzinostatin (nM)

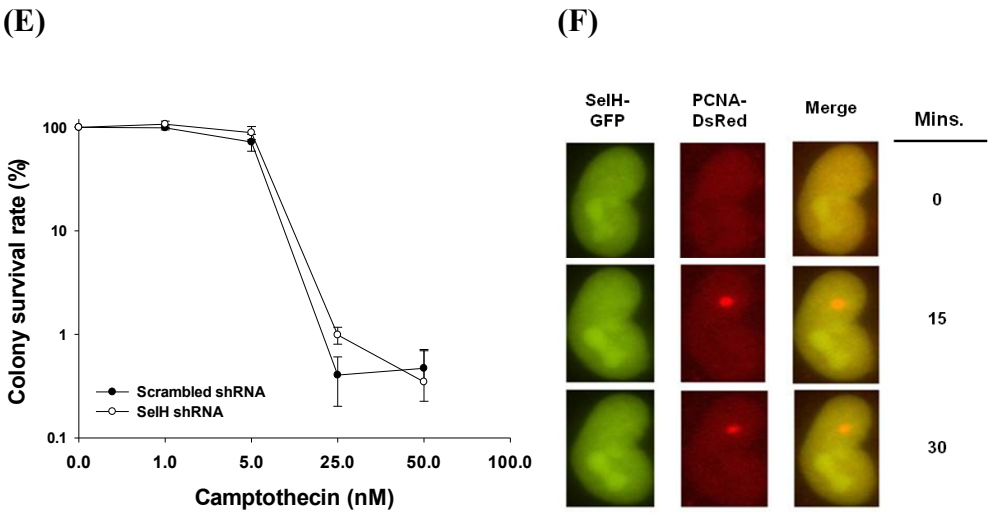


Figure 3.4 Increased sensitivity of SelH shRNA HeLa cells to H_2O_2 and paraquat but not other clastogens. Colony forming assay was performed in SelH shRNA and scrambled shRNA HeLa cells after treated with a gradient concentration of H_2O_2 (A), Paraquate (B), Hydroxyurea (C), Neocarzinostatin (D) and Camptothecin (E). The survival rates were presented with mean±S.E. (n=3) (*, $p<0.05$; **, $p<0.01$, compared to SelH shRNA HeLa cells). Localized DNA damage was generated in SelH-GFP and PCNA-DsRed overexpression MRC-5 cells. The localizations of SelH and PCNA were detected by time-lapse fluorescence microscopy by GFP and DsRed channels (F).

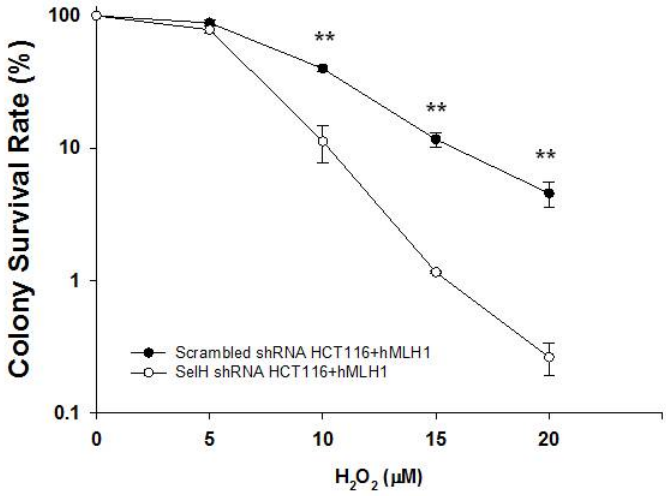


Figure 3.5 Increased sensitivity of SelH shRNA HCT116 complimented cells to H_2O_2 and paraquat but not other clastogens. Colony forming assay was performed in SelH shRNA and scrambled shRNA HCT116 complimented cells after treated with a gradient concentration of H_2O_2. The survival rates were presented with mean±S.E. (n=3) (**, $p<0.01$, compared to SelH shRNA HCT116 complimented cells).

3.4 The slow proliferation phenotype in SelH shRNA MRC-5 cells is rescued by the inhibition of ATM kinase or p53 shRNA knockdown

Both the ATM kinase and p53 are involved in the senescence response to oxidative stress in MRC-5 and endothelial cells (Wu et al., 2010; Zhan et al., 2010). Thus, the SelH and scrambled shRNA MRC-5 cells were treated with a specific ATM kinase inhibitor, Ku 60019 (5 μM, (Golding et al., 2009)), in a 20% O_2 incubator during the 28 days time course. Interestingly, Ku 60019 treatment rescued the inhibition of proliferation in SelH shRNA MRC-5 cells and resulted in a 3-fold increase in proliferation on Day 28. However, scrambled shRNA MRC-5 cells didn't proliferate faster in the presence of Ku 60019 (**Fig. 3.6A**). At day 28, the fold difference of cell number between scrambled shRNA and SelH shRNA MRC-5 cells is 14 fold in complete medium, but the fold difference of cell number was decreased to 3.6 fold with Ku 60019 treatment. After 28 days in 20% O_2 incubator, the induction of γH2AX and pATM Ser-1981 expression in SelH shRNA MRC-5 cells was suppressed in the presence of Ku 60019 (**Fig. 3.6B and C**), suggesting that the ATM kinase is responsible for the DNA break response of SelH shRNA MRC-5 cells to chronic oxidative stress. Because Ku 60019 treatment reversed γH2AX expression to the level of Day 0 but suppressed pATM Ser-1981 expression to a much lower extent compared to Day 0, these results suggest that kinases other than ATM accounts for the basal H2AX phosphorylation on Day 0 and γH2AX induction after 28 days exposure of the cells in 20% O_2 incubator is ATM-dependent. Analyses of SA-β-Gal staining confirmed that the ATM kinase is required for SelH shRNA cells to be senescent (40% vs. 5%) after being exposed to 20% O_2 for 28 days (**Fig. 3.6D**). Similar

proliferation and senescence results were observed when the cells were treated with Ku 55933, a popular but less specific ATM kinase inhibitor (**Fig. 3.7A and B**).

To further explore whether the role of SelH in the suppression of replicative senescence is mediated through p53, the SelH and p53 double knockdown was tested in MRC-5 cells. After puromycin selection, there were 6-fold greater foci in SelH and p53 double shRNA than in SelH shRNA MRC-5 cells (**Fig. 3.6E**). In particular, the cell size was much smaller and rounded in the SelH and p53 double shRNA MRC-5 cells, as opposed to the enlarged and flattened SelH shRNA cells, under a 40-fold light microscope (**Fig. 3.6F**). Altogether, the ATM kinase and p53 are necessary for the replicative senescence phenotypes in MRC-5 diploid fibroblasts under chronic oxidative stress.

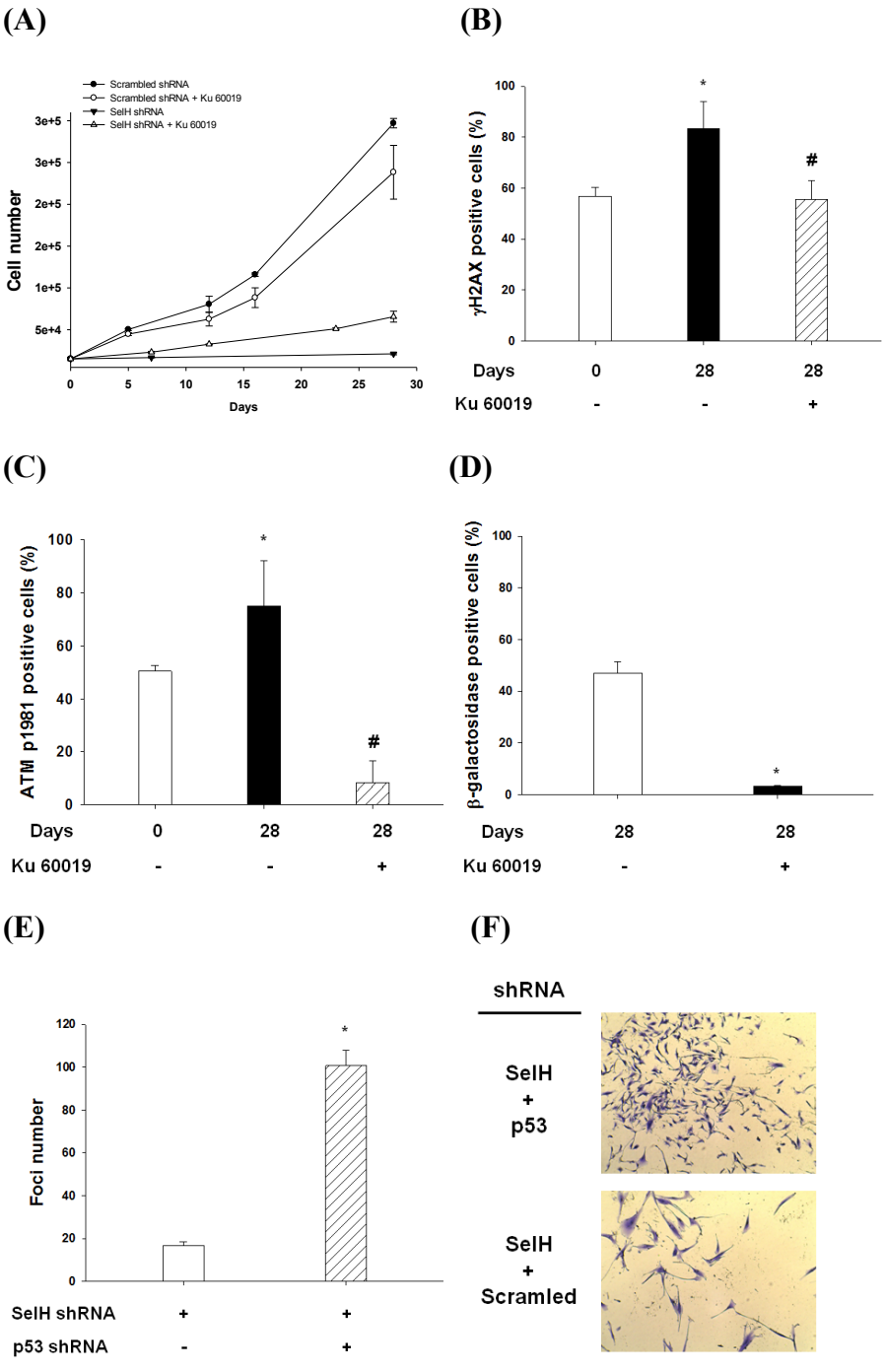

(A)
Scrambled shRNA
Scrambled shRNA + Ku 60019
SelH shRNA
SelH shRNA + Ku 60019
Cell number
Days
(B)
γH2AX positive cells (%)
Days 0 28 28
Ku 60019 - - +
(C)
ATM p1981 positive cells (%)
Days 0 28 28
Ku 60019 - - +
(D)
β-galactosidase positive cells (%)
Days 28 28
Ku 60019 - +
(E)
Foci number
SelH shRNA + +
p53 shRNA - +
(F)
shRNA
SelH + p53
SelH + Scramled

Figure 3.6 The kinase activity of ATM and p53 are involved in the protection of SelH against replicative senescence in MRC-5 cells. SelH shRNA and scrambled shRNA MRC-5 cells were seeded in 12-well plates (2 x 10^4 cells/well) and co-treated with or without 5 mM Ku60019 for 28 days, followed by cell counting (A). Immunofluorescent analyses of γH2AX (B) and pATM Ser-1981 (C) (*, $p<0.05$, compared to Day 0; #, $p<0.05$, compared to Day 28 without Ku 60019 treatment), and SA-β-galactosidase positive cells (D) (*, $p<0.05$, compared to Day 28 without Ku 60019 treatment) were detected in SelH shRNA MRC-5 cells. These assays were performed as described in Figures 1 and 2. SelH shRNA MRC-5 cells were infected with p53 shRNA or scrambled shRNA lentiviral particles, followed by puromycin selection for 14 days. The viable foci were counted after crystal violet staining. A focus is defined as one containing at least 50 cells. Values are means ± S.E. (n=3) (*, $p<0.05$, compared to SelH shRNA only MRC-5 cells) (E). Representative pictures under 40 fold bright field microscopy were shown (F).

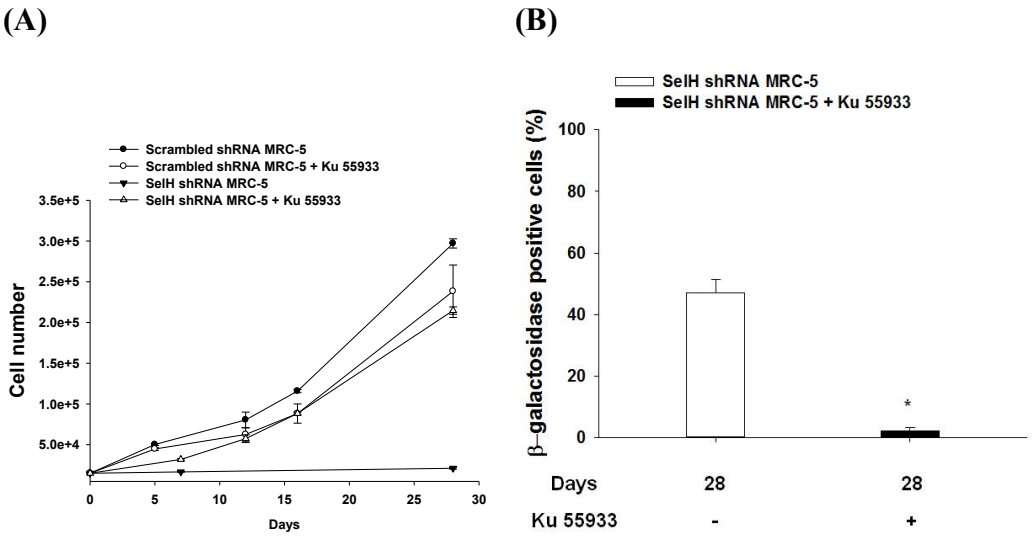


Figure 3.7 The kinase activity of ATM and p53 are involved in the protection of SelH against replicative senescence in MRC-5 cells. SelH shRNA and scrambled shRNA MRC-5 cells were seeded in 12-well plates (2 x 10^4 cells/well) and co-treated with or without 5 mM Ku55933 for 28 days, followed by cell counting (A) and SA-β-galactosidase positive cells (B). These assays were performed as described in Figures 1 and 2. Values are means ± S.E. (n=3) (*, $p<0.05$, compared to complete medium only).

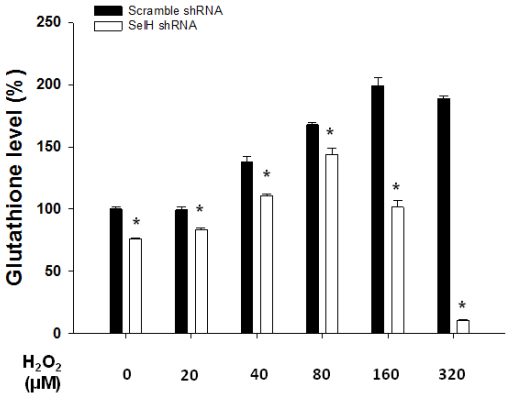
3.5 The role of transactivation on phase II antioxidant is required to protect cells against oxidative damages

It was proposed previously that SelH overexpression may transactivate the expression of a key gene for glutathione biosynthesis (Panee et al., 2007). Consistent with this notion, here the data showed that SelH shRNA HeLa cells expressed significantly less ($P < 0.05$) glutathione before and after H_2O_2 exposure for 24 hours (**Fig. 3.8A**). H_2O_2 treatment (20-80 μM) induced linearly increased glutathione levels in both SelH and scrambled shRNA HeLa cells. At the concentration of H_2O_2 at 320 μM, glutathione was almost completely depleted in SelH shRNA cells, resulting in a 17.5-fold difference as compared to scrambled shRNA HeLa cells. Therefore, SelH shRNA cells are defective in maintaining intracellular glutathione. To test the hypothesis that decreased glutathione sensitizes SelH shRNA cells to oxidative stress and limits cell proliferation, the SelH shRNA and scrambled shRNA HeLa cells were treated with H_2O_2 at 160 μM for 0-24 hours and assessed apoptotic cell death by using mitocapture. The apoptotic SelH shRNA and scrambled shRNA HeLa cells increased linearly 0-12 hours after H_2O_2 treatment (**Fig. 3.8B**). There were approximately 80% and 20-30% apoptotic SelH shRNA and scrambled shRNA HeLa cells, respectively, 12 and 24 hours after H_2O_2 treatment. Further statistical analyses showed that glutathione levels were inversely associated with apoptotic death on a certain H_2O_2 concentration. Therefore, SelH may keep glutathione level on check under oxidative stress and prevent apoptotic cell death. To test whether intracellular glutathione maintains cell survival under oxidative stress, colonogenic assay was performed by treating the cells with H_2O_2 in the presence or absence of a glutathione mimic agent, *N*-acetylcysteine (NAC). The supplement of NAC (10 mM) rescued the

proliferation of SelH shRNA HeLa cells to a level similar to that of the scrambled shRNA HeLa cells (**Fig. 3.8C**). These results suggest that SelH may counteract oxidative stress and maintain cell proliferation through maintenance of intracellular glutathione level.

It has been reported that the Nrf2-Keap1 pathway also regulates glutathione biosynthesis (Moinova and Mulcahy, 1999). Upon increased oxidative stress, phosphorylated Nrf2 disassociates from Keap1 and accumulates in the nucleus for assisting the expression of phase two antioxidants, including glutathione. To test whether the Nrf2-Keap1 pathway is differentially activated in SelH and scrambled shRNA cells after oxidative stress, Western analyses was used to determine the accumulation of phosphorylated Nrf2 in the nuclear fractions after treatment with a gradient concentration of H_2O_2 (**Fig. 3.9A**). There was significant greater phosphorylated Nrf2 in the nucleus of SelH shRNA HeLa cells than in scrambled shRNA HeLa cells. After H_2O_2 treatment, there was a dose-dependent increase in nuclear pNrf2 in both SelH shRNA and scrambled shRNA HeLa cells, but the extent of which is greater in the former than the latter cells. The same is true in MRC-5 cells (**Fig. 3.9B**).

(A)


Scramble shRNA
SelH shRNA
Glutathione level (%)
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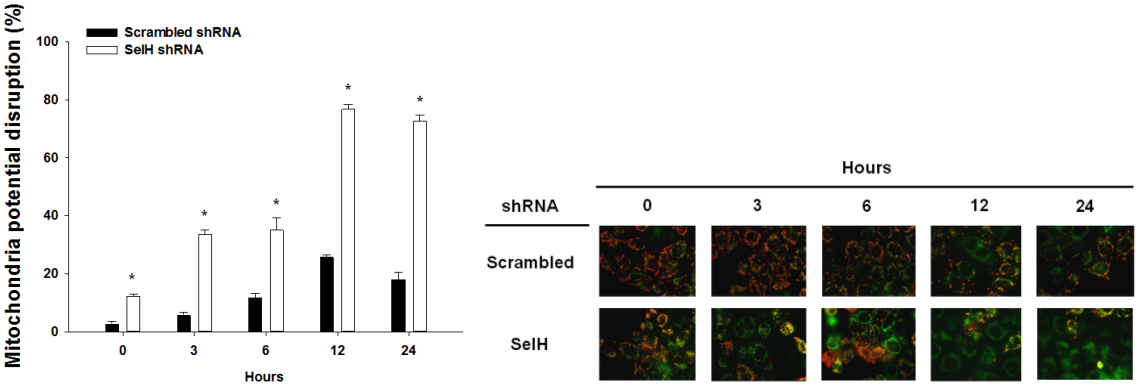
200
150
100
50
0
H_2O_2 (μM)
0
20
40
80
160
320

(B)


Scrambled shRNA
SelH shRNA
Mitochondria potential disruption (%)
100
80
60
40
20
0
0
3
6
12
24
Hours
Hours
shRNA
0
3
6
12
24
Scrambled
SelH

(C)

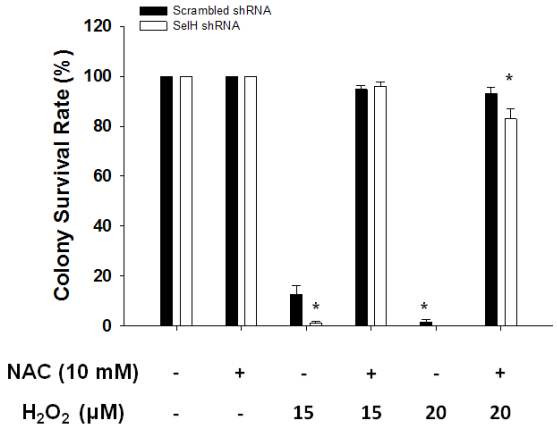
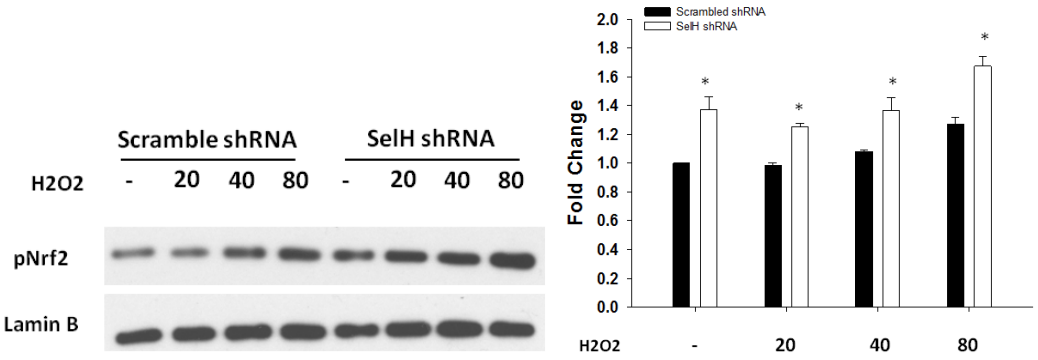

Scrambled shRNA
SelH shRNA
Colony Survival Rate (%)
120
100
80
60
40
20
0
NAC (10 mM) - + - + - +
H_2O_2 (μM) - - 15 15 20 20

Figure 3.8 Intracellular glutathione is involved in the protection of SelH against oxidative stress. SelH shRNA and scrambled shRNA HeLa cells were cultured in 10-cm dishes and treated with H_2O_2 (0-320 μM) for 24 hours, followed by staining of isolated single cells with monochlorobimane for flow cytometric analysis (A) (*, $p<0.05$, compared to Scrambled shRNA HeLa cells). Some cells were treated with H_2O_2 at 160 μM for 0-24 hours and harvested for mitocapture staining to determine apoptotic cells. The percentapoptotic cells were presented with mean ± S.E. (n=8) (*, $p<0.05$, compared to Scrambled shRNA HeLa cells), and representative pictures were shown (B). Colony forming assay was performed in SelH shRNA and scrambled shRNA HeLa cells treated with H_2O_2 (0, 15 and 20 μM) alone or together with *N*-acetylcystine (NAC, 10 mM). The number of colonies in scrambled shRNA cells without H_2O_2 or NAC treatment was set as 100%. Values are means ± S.E. (n=3) (C) (*, $p<0.05$, compared to Scrambled shRNA HeLa cells).

(A)



(B)

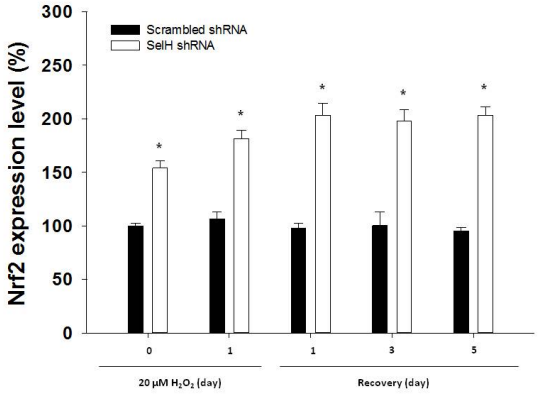


Figure 3.9 Accumulation of phosphorylated Nrf2 in the nucleus of scrambled shRNA and SelH shRNA HeLa or MRC-5 cells. SelH shRNA and scrambled shRNA HeLa cells were cultured in 15-cm dishes and treated with H_2O_2 (0-80 μM) for 24 hours. The nuclear fraction of HeLa cells were applied for immunoblotting analysis by using anti-phospho-Nrf2 (pNrf2) and anti-Lamin B antibodies. The intensity was quantified by ImageJ and presented with means ± S.E. (n=3) (A) (*, $p<0.05$, compared to Scrambled shRNA HeLa cells). SelH shRNA and scrambled shRNA MRC-5 cells were seeded onto coverslip, treated with H_2O_2 (20 μM) for 24 hours, followed by 5-day recovery in fresh medium. (B) The percentage of nucleus-specific pNrf2 was presented with means ± S.E. (n=6) (*, $p<0.05$, compared to Scrambled shRNA MRC-5 cells).

3.6 Discussion

In this study, the evidence indicated that the role of SelH in human diploid cells is protecting cells against genome instability and early onset replicative senescence through the clearance of ROS. The antioxidation activity of SelH had been demonstrated previously that the transient SelH siRNA knockdown mouse cells were more sensitive to increased oxidative stress, and human SelH overexpression in murine hippocampal HT22 cells strengthened the antioxidative function (Novoselov et al., 2007; Panee et al., 2007). However, the previous studies used immortalized cells to study the function of SelH, but the biological significance of SelH in mortal cells is still largely unknown. The results showed that SelH was required in human diploid fibroblast to prevent the early inhibition on proliferation in physiological or atmosphere level of oxygen or increased oxidative stress. The absence of SelH in MRC-5 cells promoted the accumulation of ROS robustly, and it leaded to induce higher level of DNA damage and DNA damage response, which activated replicative senescence. Additionally, the sub-lethal dose of H_2O_2 treatment further generated permanent DNA damages and persistent DNA damages response to activate severe replicative senescence in SelH knockdown MRC-5 cells, so SelH is required for the protection from chronically-increased oxidative stress induced genome instability and replicative senescence. In the mean time, ATM and p53 are necessary components in SelH deficient activated replicative senescence. According to the previous evidence, SelH was proposed to play a role as transcription factor (Panee et al., 2007), which facilitates GSH biosynthesis. Thus, the data indicated that SelH was required for maintaining the level of intracellular GSH, especially with the increased level of oxidative stress, and this function is critical for SelH to protect cells from oxidative stress

induced damages. Finally, SelH desensitized Nrf2 pathway activation, so the role of SelH is at the front line of antioxidation. Here, the data suggests that SelH has a new role on maintaining genome stability and suppressing replicative senescence as an antioxidant and transactivator in human diploid fibroblast (**Figure 3.10**).

Permanent DNA damage and persistent DNA damage response both activate replicative senescence (Campisi and d'Adda di Fagagna, 2007). ROS is an endogenous and continuous damaging source to dampen genome stability. However, the accumulation of ROS in the cell is a general trend of cellular and organismal aging (Harman, 1960; Lawless et al., 2012) because of the reduction of antioxidant capacity (Kim et al., 2002b; Kimoto-Kinoshita et al., 1999; Perez et al., 1991; Pieri et al., 2001; Sivonova et al., 2007; Williams et al., 2008) or the increase of ROS production. Hence, when the sub-lethal dose of H_2O_2 was used to mimic the chronic increase of oxidative stress, the permanent DNA damages and persistent DNA damage response presented in SelH knockdown MRC-5 cells but not in scrambled shRNA MRC-5 cells. The possible explanation is that SelH deficiency in human diploid fibroblast may already establish the prone of genome instability in the cell. Prior to the treatment with sub-lethal doses of H_2O_2, the SelH deficient cells, which are at second passage after clonal selection, already had higher level of DNA breaks and DNA damage response. SelH deficient condition may chronically result in genome instability, so the chronic oxidative stress treatment may further generate severe DNA lesions on unstable genome to activate senescence through unrepaired DNA damage and persistent DNA damage response. Noteworthy, during SelH shRNA knockdown clonal selection, the number of viable colonies of SelH shRNA MRC-5 cells in 3% O_2 was 3 fold more than in 20% O_2 (Data not shown). The evidence

supported the idea that SelH deficiency may chronically damage cells, so 20% O2, the chronic oxidative stress, lowered the number of viable clones.

SelH is a possible redox sensor to control the homeostasis of oxidative stress in the cell. Previously, nucleolar, where transcription of rRNA takes place, has been proposed to be the redox sensor in the cell. The rRNA transcription is halted when there is an acute and severe increase of oxidative stress sensed by nucleolar (Lewinska et al., 2010). The action also halts the protein translation until the cell surpasses the stress (Mayer et al., 2005). Interestingly, SelH was found to localize to nucleoli specifically with critical role on antioxidation and facilitating GSH biosynthesis (Novoselov et al., 2007; Panee et al., 2007). In the scrambled shRNA HeLa cells, the level of intracellular GSH was gradually increased with the gradient increased H_2O_2 treatment. Differently, the level of GSH in SelH shRNA HeLa cells was only induced from 0~80 μM H_2O_2, but started to drop sharply from 160 μM H_2O_2 and depleted at 320 μM H_2O_2. Although the level of GSH is still inducible by low dose of H_2O_2 in the SelH deficient condition, the induction of GSH production may be regulated solely by Nrf2, which is one of the transcription factors to promote GSH biosynthesis (Suh et al., 2004). On the other hand, the results showed that the accumulation of pNrf2 in nucleus fraction was less in the gradient H_2O_2 treatment in scrambled shRNA MRC-5 and HeLa cells, whereas pNrf2 was accumulated robustly in SelH shRNA MRC-5 and HeLa cells. The data suggested that the synergistic regulation by both SelH and pNrf2 is critical to maintain the homeostasis of oxidative stress and cell survival, and the accumulation of pNrf2 in the nucleus is less sensitive when the cells have functional SelH. Noteworthy, the evidence from Burk et al. suggested that selenium deficiency leaded to pNrf2 accumulation in nuclei in mouse liver, but the other natural

antioxidant, Vitamin E, deficiency didn't promote the same action (Burk et al., 2008). Then, pNrf2 may transactivate phase II antioxidant in the nucleus, including some selenoproteins (Burk et al., 2008; Singh et al., 2006). Since SelH is one of the most dietary selenium sensitive selenoproteins, the dietary selenium deprivation may lead to SelH deficiency. Thus, the increased pNrf2 accumulation may be resulted from SelH deficiency in dietary selenium deficiency. According to the available lines of evidence and the results here, SelH might be a possible redox sensor to control the activation of cellular defense system on oxidative stress, i.e. Keap1-Nrf2 pathway for transactivating phase II antioxidants.

The SelH deficient MRC-5 cells showed early onset cellular aging, which may sensitize the cells to DNA damage. Based on the phenotypic observation, the cellular aging phenotypes, morphological change, increased autofluorescence and increased level of ROS, were found in SelH knockdown MRC-5 cells. The capacity of DNA repair has been proposed to be decreased with age (Garm et al., 2013), especially DNA DSB repair. Thus, increased genome instability in SelH knockdown MRC-5 cells might be resulted from impaired DNA repair mechanism, because the molecular age of the cell is older than scrambled shRNA MRC-5 cells. Additionally, the older cells promotes the production of ROS, which exacerbates genome instability. Although the activation of replicative senescence plays positive role on anti-tumorigenesis in premalignant cells (Bartek et al., 2007), it is also critical to accelerate aging and age-related disorders in normal cells (Wang et al., 2009). The data suggested that SelH plays a key role to prevent advanced molecular age of the cell which may lead to sever genome instability through impaired DNA repair mechanism and increased level of ROS.

Herein, there is a proposed new role of SelH to protect human normal diploid cells against early onset replicative senescence. In the nucleus, SelH prevents the chronic oxidative stress induced DNA damages, which may activate DNA damage response and cellular senescence by ATM and p53, respectively. Moreover, SelH also serves as a transcription factor to activate glutathione, and the increased level of glutathione may further reinforce the antioxidation function to prevent oxidative DNA damages (**Figure 3.10**). To sum up, SelH serves as a oxidative stress sensor to regulate the homeostasis of oxidative stress in nucleus to prevent severe DNA damages induced senescence. Previously, 11 out of 25 selenoproteins were predicted to be related to aging (McCann and Ames, 2011). Additionally, the selenocysteine $tRNA^{[Ser]Sec}$ gene (*Trsp*) conditional knockout in epidermal cells (Sengupta et al., 2010) and osteo-chondroprogenitor cells (Downey et al., 2009) showed premature aging phenotypes. The conditional knockout generated selenoprotein deficient condition, which is also possibly created by dietary selenium deficiency. Interestingly, selenoprotein H is one of the most dietary selenium sensitive selenoprotein (Kipp et al., 2009; Sunde, 2010). Although SelH is not in the list of aging-related selenoproteins (McCann and Ames, 2011), the observed replicative senescence in SelH knockdown human diploid cells suggested SelH is a new aging-related selenoprotein needed for the protection against chronic oxidative stress induced senescence.

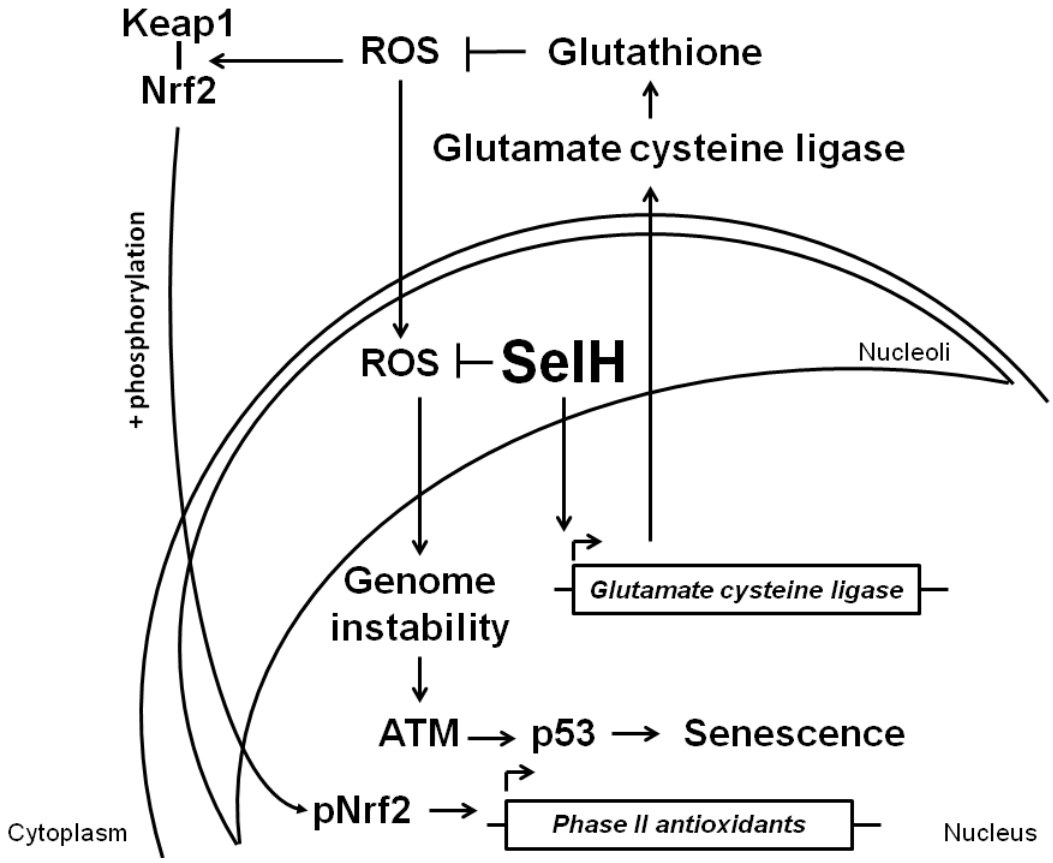


Figure 3.10 Proposed function of SelH in cellular senescence SelH is a dual function protein localized specifically in nucleolus. Firstly, SelH serves as antioxidant in nucleus to suppress ROS induced genome instability, which may activate senescence through ATM and p53. Secondly, SelH may transactivate glutamate cysteine ligase transcription to facilitate glutathione biosynthesis. This is critical for cells to protect against oxidative damage. Lastly, SelH regulates the homeostasis of oxidative stress in the nucleolus to control the accumulation of pNrf2 in the nucleus, which represents the activation of Keap1-Nrf2 pathway for phase II antioxidant transactivation.

Chapter 4: Essential role of selenium at nutritional levels of intake in mouse health span and lifespan

To better model human health span and lifespan in the mouse model, *Terc*$^{-/-}$ (telomerase RNA component) (Blasco et al., 1997) and *Tert*$^{-/-}$ mice (Yuan et al., 1999) carrying humanized telomeres are generated. Since the identification of Hayflick limit and telomere, telomere became one of the critical determinants of lifespan, which protects chromosome end against natural telomere attrition (Greider, 1998; Harley et al., 1990; Linskens et al., 1995). The length of telomere is highly correlated to the lifespan and health span of human aging (Barbieri et al., 2009; Honig et al., 2012; Kong et al., 2013). However, telomeric repeats in human cells average between 10 and 15 kb in length. In contrast, the laboratory mouse species *Mus musculus* possesses much longer telomeres, ranging in size from 40 to 80 kb (Blasco et al., 1997; Zijlmans et al., 1997). Furthermore, telomerase activity is absent in most human somatic cells, while low levels of telomerase activity are present in mouse somatic cells to attenuate telomere attrition. Since mouse telomeres are much longer than those of humans and telomerase activity is detectable in mouse somatic cells, replicative senescence is unlikely to occur during the normal aging process in mice. These telomerase-deficient mice are viable, fertile and have no significant physiological abnormalities at early generations. Progressive inbreeding of *Terc*$^{-/-}$ mice is required to gradually shorten telomere length to a range reminiscent of that in humans (Blasco et al., 1997). Rudolph et al (Rudolph et al., 1999) showed that generation 3(G3) *Terc*$^{-/-}$ mice display skin ulceration, alopecia and hair graying, but not many other disorders in association with human aging, including osteoporosis, body weight changes, shortened lifespan, and type II diabetes (Rudolph et al., 1999). The aging phenotypes worsen in G4-G6 *Terc*$^{-/-}$ mice with even shorter telomere (Blasco et al.,

1997).

Selenium is required not only for optimal health span but lifespan potentially. As an essential trace element, selenium has a U-shaped relationship with risk of diseases (Fairweather-Tait et al., 2011). The level of dietary selenium regulates the expression of selenoproteins (Burk and Hill, 1993), which play important roles in the body on antioxidation, redox signaling, thyroid hormone maturation, protein folding in ER and even more unknown functions required to be discovered (Papp et al., 2007). While recent research has demonstrated that mice have selenoprotein deficiency by selenocysteine tRNA knockout specifically in epidermal cells (Sengupta et al., 2010) or in osteo-chondroprogenitor cells (Downey et al., 2009) showed an apparently aging phenotype characterized by alopecia and bone abnormality. Of the 25 mammalian selenoproteins, 11 are believed to have functional roles related to aging (McCann and Ames, 2011); however, it is not clear whether Selenium, particularly, at nutritional levels of intake, may play a role in aging and age-related disorders.

Telomere attrition provokes DNA damage and, subsequently, replicative senescence (d'Adda di Fagagna et al., 2003). Because the chromosomes of mice carry longer telomeres than those of humans (Blasco et al., 1997), the aging phenotypes of such premature aging syndromes in humans as Werner syndrome and ataxia-telangiectasia do not appear in mice when only the responsible mutated genes (*Wrn* and *Atm*) have been knocked out. However, those aging phenotypes of $Wrn^{-/-}$ and $Atm^{-/-}$ mice can be recapitulated under a short telomere background (Chang et al., 2004; Wong et al., 2003). Similarly, mice unable to express the major selenoprotein, glutathione peroxidase-1, develop normally and are apparently healthy up to 20 months of age (Ho et al., 1997).

Clearly, what is lacking is an appropriate aging model of dietary Se deficiency displaying many features of normal aging. The hypothesis is that lengthy telomeres preclude mice deprived of Se to display aging phenotypes and age-related disorders. Closing this knowledge gap is critical to advance understanding of the health roles of dietary Se.

4.1 Dietary selenium deprivation causes early onset of aging phenotypes in short telomere mice

It had been proposed that there are 11 selenoproteins are related to aging and age-related disorders, but the expression of selenoproteins are highly correlated with dietary selenium. To dissect a specific role of dietary Se in age-related dysfunction, the short telomere G3 *Terc*$^{-/-}$ mice were employed as the animal model. The length of telomere is a key factor for human organismal aging, so the proper animal model, late generation *Terc*$^{-/-}$ mice, is a well-developed model to study human organismal aging with human aging phenotypes, such as hair graying, alopecia, weak wound healing (Rudolph et al., 1999). The short telomere mice were prepared from Se-adequate G1 *Terc*$^{-/-}$ following mating scheme (**Fig. 2.1**), and fed after weaning with a purified basal diet (<0.03 ppm of Se by analysis) or the diet supplemented with sodium selenate (0.15 ppm) throughout their life. The level of plasma Se concentrations were significantly reduced ($P < 0.001$) by dietary Se deprivation (ng/mL; Se-deficient mice: 9 mo, 156 ± 10; 18 mo, 131 ± 18; Se-adequate mice: 9 mo, 372 ± 16; 18 mo, 394 ± 14, $n = 5$/group) **(Table 4.1)**, but the reduction is not related to the effect of aging. Following the continuous observation, the age-related appearance change happened earlier and severer in animals with selenium deprivation than the animals with selenium supplementation. Deprivation of body Se accelerated the skin abnormality in male G3 *Terc*$^{-/-}$ mice starting at 7 months of age but not until 10

months in G2 Se-deficient *Terc*$^{-/-}$ mice (not shown), further supporting the hypothesis that long telomere limits Se-deficient mice to reveal aging phenotypes. The alopecia (scored 4+) was greatly accelerated by dietary Se deprivation in the male G3 *Terc*$^{-/-}$ mice (Se-deficient, 29/73 vs. Se-adequate, 8/84, $P < 0.05$, **Fig. 4.1A**). The average scores of skin abnormity were consistently higher in selenium deficient short telomere animals at different age (**Fig. 4.1B**). On the other hand, loss of the ability on wound healing is one of the age-related disorders for normal human aging, so the skin surgery was performed to test the ability of wound healing in both animals in selenium deprived or supplemented diets. During the all 7-day recovery, the animals in selenium deprived diet significantly kept continuously greater wound area (n=5, $p<0.05$) than the animals in selenium supplemented diet. At day 7, in 12 and 18 month-old mice, the average size of the wounds was found to be decreased to 7.2 % and 16.4 % of the original wound in Se-adequate mice, but it was only decreased to 21.6 % and 40.6 % in Se-adequate mice (**Fig. 4.1C**). The data suggested that the Se-deficient late generation *Terc*$^{-/-}$ mice showed skin abnormality, an aging phenotype, significantly earlier than the animals in Se-adequate diet. Furthermore, the flow FISH method was performed by a FITC-labeled $(CCCTAA)_3$ PNA probe as described in Rudolph et al (Rudolph et al., 1999). The Flow-FISH telomere analysis by using primary colonocytes isolated from G3 *Terc*$^{-/-}$ mice at 18 months of age showed that Se deprivation shortened average telomere length by 25% (**Fig. 4.1D**).

Therefore, there was a very interesting model of aging established by deprivation of Se in short telomere *Terc*$^{-/-}$ mice that displays many hallmarks of human aging and can reveal the roles of Se at nutritional levels, in contrast with previous approaches in which this

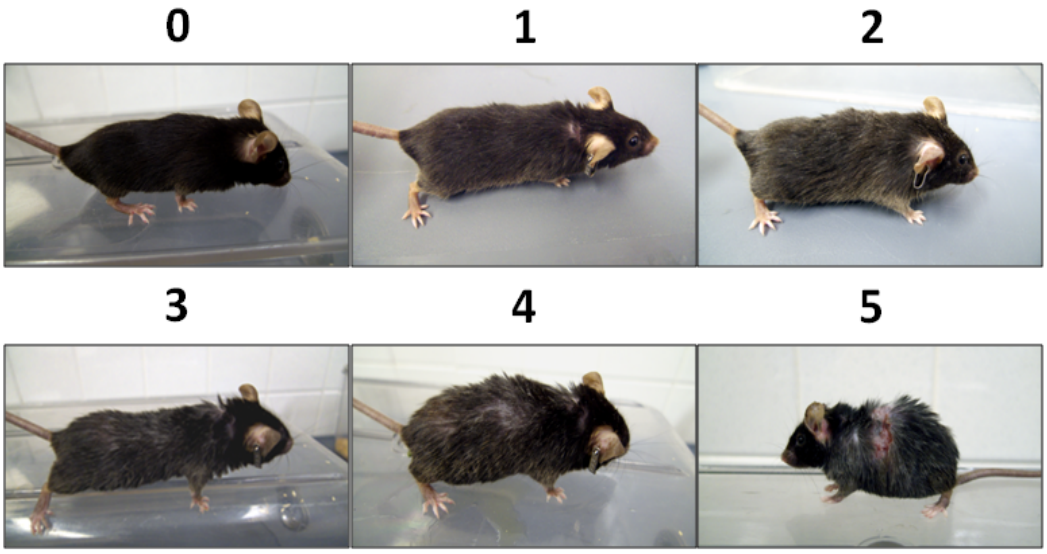
effect may have been masked by lengthy telomeres.

	Se (-)		Se (+)	
	Young (<12 months)	Old (<18 months)	Young (<12 months)	Old (<18 months)
Selenium Conc. (ng/ml)	156.8 ± 10.4^{a}	131 ± 18.4^{a}	372.2 ± 16^{b}	394.4 ± 13.7^{b}

Source of Variation	P value summary	Significant?
Interaction	ns	No
Selenium	***	Yes
Time	ns	No

Table 4.1 Plasma selenium levels in the mice with Se-deficient or Se-adequate diets at younger than 12 months or than 18 months of age. The effects of age and dietary selenium on the level of plasma selenium were analyzed by two-way ANOVA.

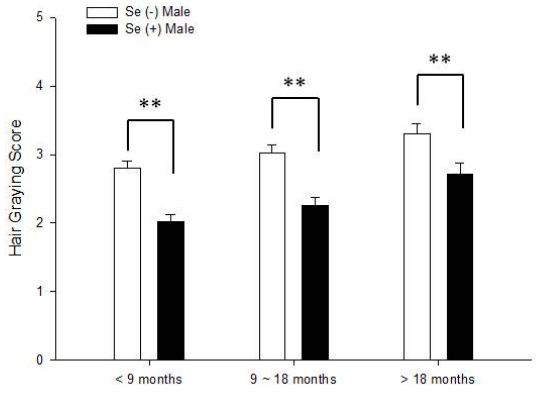
(A)



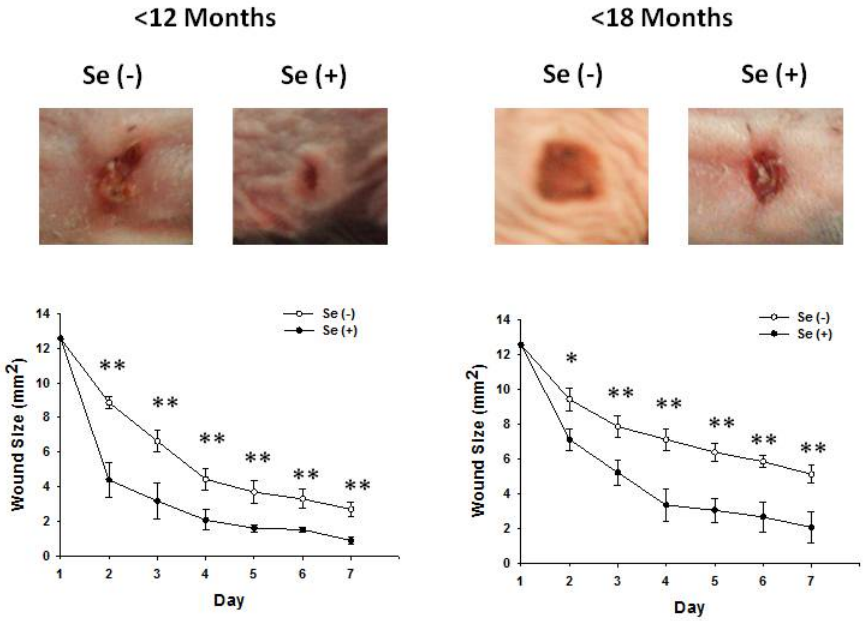
	Age (mo)	score 5	4	3	2	1	0	*n*
Se (-)	7-9	1	2	24	5	0	0	29
	9-18	4	12	26	7	1	0	34
	> 18	2	8	9	1	0	0	10
Se (+)	7-9	0	1	7	20	3	0	30
	9-18	0	4	18	16	6	0	40
	> 18	0	3	10	3	1	0	14

*, $P < 0.01$.

(B)



(C)



(D)

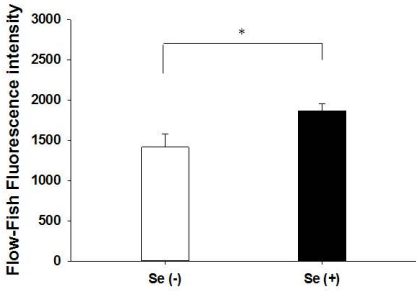


Figure 4.1 Se-deficient G3 *Terc* $^{-/-}$ mice had early onset of aging phenotypes and faster telomere attrition. The scores of hair graying and skin abnormality were recorded in different age, and the references for scoring hair graying and skin abnormality were also showed here (A). The average scores of hair graying and skin abnormality were showed in different ages (*, $p<0.05$, compared to Se-deficient mice) (B). The wound healing experiment was performed on Se-deficient and Se-adequate mice at age of 12 months or 18 months. The wound was generated by 5-mm biopsy, and the size of the wound was measured in the following 7 days. The representative pictures were listed. (*, $p<0.05$; **, $p<0.01$, compared to Se-adequate mice) (C). The length of telomere was measured by Flow-FISH. The isolated colonocytes from 24 month-old mice were subjected to perform FISH with FITC-telomere-PNA probe, which was used to hybridize with telomere. The stronger FITC intensity represents longer telomere with more FITC-telomere-PNA probes on it (*, $p<0.05$, compared to Se-deficient mice) (D).

4.2 Dietary selenium deprivation causes age-related abnormities in Short telomere mice

Markers for the alteration of health span

In humans, aging is associated with numerous pathophysiological conditions including increased glucose and body weight leading to diabetes and cardiovascular disease. The role of Se in diabetes is complicated and awaits further investigation. These Se-deficient G3 *Terc*$^{-/-}$ mice had alterations in plasma levels of markers that predict the onset of diabetes, liver damages and muscular dysfunction, including blood glucose and lactate dehydrogenase (**Table 4.1**). These plasma markers were measured by AniLytics Inc. (Gaithersburg, MD) as described previously (Baur et al., 2006). Plasma glucose level was greater in fasting Se-deficient than Se-adequate G3 *Terc*$^{-/-}$ mice at both 12 and 18 months of age, and was lowered in 18 than 12 months of age. Plasma lactate dehydrogenase activity was greater in fasting Se-deficient than Se-adequate G3 *Terc*$^{-/-}$ mice at 12 but not 18 months of age. Plasma amylase activity or albumin level was not affected by dietary Se status or age of the mice.

Parameter	Young (<12 months)		Old (~18 months)		
	Se (-)	Se (+)	Se (-)	Se (+)	
Trigelycerides (mg dl^{-1})	44.6 (5.9)	66.2 (6.0)	42.8 (3.3)	74.2 (4.7)	Fasting
	49.4 (5.7)	62.0 (4.3)	29.0 (1.3)	39.0 (3.3)	Fed
Cholestrol (mg dl^{-1})	96.6 (2.6)	87.4 (7.2)	101.4 (6.8)	107.4 (9.7)	Fasting
	130.8 (5.9)	118.8 (7.9)	126.8 (3.0)	127.4 (8.5)	Fed
Glucose (mg dl^{-1})	167.4 (8.0)	129.4 (13.2)	128.0 (11.1)	97.4 (5.2)	Fasting
	192.6 (5.6)	196.8 (13.5)	175.2 (7.0)	206.2 (10.3)	Fed
Amylase (Ul^{-1})	2594.8 (173.0)	2614.6 (156.8)	2516.4 (249.4)	2351.6 (124.9)	Fasting
	3040.4 (175.9)	3136.0 (205.9)	2607.8 (132.9)	2807.0 (102.1)	Fed
Asp aminotransferase (Ul^{-1})	59.6 (5.3)	87.8 (14.4)	62.0 (2.8)	124.8 (8.0)	Fasting
	46.0 (3.0)	48.8 (4.3)	47.0 (2.5)	42.4 (1.6)	Fed
Ala aminotransferase (Ul^{-1})	14.4 (2.0)	18.0 (4.5)	13.6 (2.1)	18.4 (2.9)	Fasting
	9.4 (2.3)	14.6 (5.3)	10.2 (1.3)	9.4 (2.5)	Fed
Creatine phosphokinase (Ul^{-1})	126.0 (21.4)	68.2 (29.6)	96.4 (26.4)	131.4 (34.3)	Fasting
	113.6 (27.3)	67.0 (24.5)	57.8 (11.9)	70.4 (20.0)	Fed
Lactate Dehydrogenase (Ul^{-1})	243.6 (42.7)	161.2 (8.4)	284.4 (24.1)	296.0 (33.1)	Fasting
	194.0 (11.0)	190.2 (11.7)	207.4 (36.0)	159.4 (17.3)	Fed
Alkline Phosphatase (Ul^{-1})	45.0 (5.0)	34.6 (4.0)	33.0 (3.4)	30.8 (3.5)	Fasting
	51.8 (3.3)	46.8 (4.4)	42.4 (1.8)	45.6 (2.3)	Fed
Bilirubin (mg dl^{-1})	0.58 (0.10)	0.54 (0.06)	0.48 (0.17)	0.28 (0.08)	Fasting
	0.16 (0.02)	0.24 (0.07)	0.14 (0.02)	0.26 (0.09)	Fed
Albumin (g dl^{-1})	3.72 (0.18)	3.56 (0.20)	3.08 (0.19)	3.38 (0.15)	Fasting
	4.02 (0.16)	3.58 (0.12)	3.54 (0.14)	3.60 (0.11)	Fed
Creatine (mg dl^{-1})	0.34 (0.02)	0.30 (0.03)	0.28 (0.02)	0.24 (0.02)	Fasting
	0.34 (0.02)	0.34 (0.02)	0.28 (0.02)	0.30 (0.0)	Fed

Parameter		Age	Selenium	Interaction
Trigelycerides (mg dl^{-1})	Fasting	ns	**	ns
	Fed	**	*	ns
Cholestrol (mg dl^{-1})	Fasting	ns	ns	ns
	Fed	ns	ns	ns
Glucose (mg dl^{-1})	Fasting	**	**	ns
	Fed	ns	ns	ns
Amylase (Ul^{-1})	Fasting	ns	ns	ns
	Fed	*	ns	ns
Asp aminotransferase (Ul^{-1})	Fasting	*	**	ns
	Fed	ns	ns	ns
Ala aminotransferase (Ul^{-1})	Fasting	ns	ns	ns
	Fed	ns	ns	ns
Creatine phosphokinase (Ul^{-1})	Fasting	ns	ns	ns
	Fed	ns	ns	ns
Lactate Dehydrogenase (Ul^{-1})	Fasting	**	ns	ns
	Fed	ns	ns	ns
Alkline Phosphatase (Ul^{-1})	Fasting	ns	ns	ns
	Fed	ns	ns	ns
Bilirubin (mg dl^{-1})	Fasting	ns	ns	ns
	Fed	ns	ns	ns
Albumin (g dl^{-1})	Fasting	*	ns	ns
	Fed	ns	ns	ns
Creatine (mg dl^{-1})	Fasting	*	ns	ns
	Fed	*	ns	ns

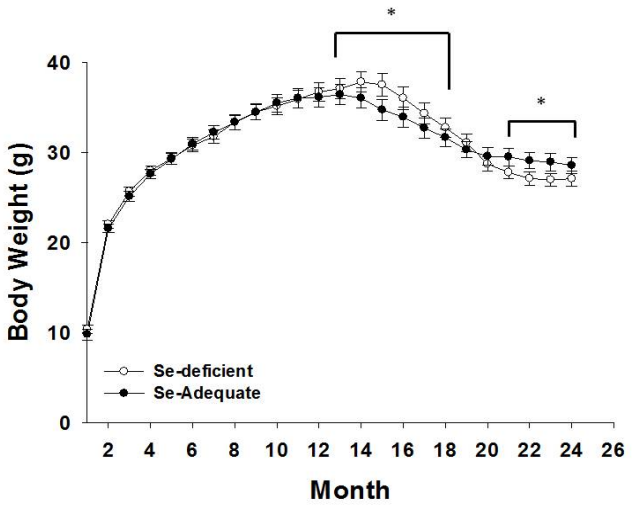
Table 4.2 Chemical factors in Se-deficient and Se-adequate mice at the age of 12 months or 18 months (*, $p<0.05$; **, $p<0.01$; ns, non-significance)

Diabetes-like symptoms

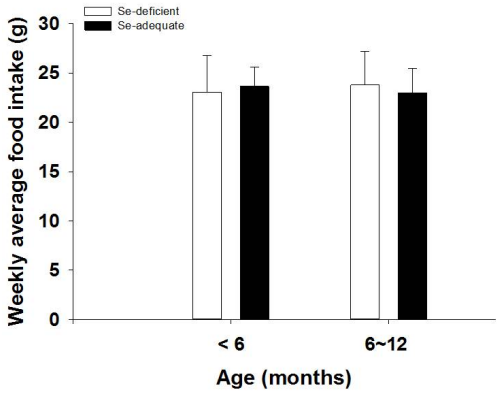
Based on the analysis of plasma markers, the fasting blood glucose in Se-deficient G3 *Terc*$^{-/-}$ mice was higher than Se-adequate G3 *Terc*$^{-/-}$ mice at both 12 and 18 months of age. Additionally, Se-deficient G3 *Terc*$^{-/-}$ mice have significantly heavier average body weight than Se-adequate G3 *Terc*$^{-/-}$ mice between 13 and 18 months of age. (**Fig. 4.2A**), but there was no significant difference on weekly food intake either at younger than 6 months or between 6 to 12 months of age **(Fig. 4.2B)**. These results suggested defective glucose regulation in Se-deficient G3 *Terc*$^{-/-}$ mice. To explore this further, the glucose tolerance test, insulin tolerance test were performed, and the level of plasma insulin was determined during glucose tolerance test. The result of glucose tolerance test indicated glucose tolerance was similar in Se-deficient and Se-adequate G3 *Terc*$^{-/-}$ mice at 9 month-old (**Fig. 4.2C**). However, Se-deficient G3 *Terc*$^{-/-}$ mice had gradually developed glucose intolerance at both 12 and 18 month-old, and the symptom was aggravated as the mice age (**Fig. 4.2E and F**). Interestingly, the glucose intolerance symptom didn't exist in either Se-deficient or Se-adequate G1 *Terc*$^{+/+}$ mice **(Fig. 4.2C)**. On the other hand, the onset of diabetes is usually preceded by insulin resistance. To test if Se deficiency affects insulin sensitivity during the aging process, the whole-body insulin sensitivity test was performed by insulin tolerance test (0.25 U/kg body weight, i.p. injection). The Se-deficient mice demonstrated insulin resistance compared with Se-adequate G3 *Terc*$^{-/-}$ mice at both 12 and 18 months of age. After the insulin injection, the reductions of whole-blood glucose, relative to their initial levels, were ~30% less ($P < 0.05$) in the Se-deficient than in the Se-adequate mice during the time course (**Fig. 4.2G and H**). To determine whether glucose intolerance in the Se-deficient mice was affected by blood

insulin level, the plasma insulin concentrations were determined in glucose tolerance test (as described in **Fig. 4.2**) by using a mouse insulin ELISA kit (Alpco Diagnostics, Windham, NH). At 12 months of age, plasma insulin level was lower in Se-deficient than in Se-adequate G3 *Terc*$^{-/-}$ mice after glucose injection (**Fig. 4.2I**). Strikingly, glucose administration did not elicit the elevation of plasma insulin in Se-deficient G3 *Terc*$^{-/-}$ mice at 18 months of age, although there was clearly an age-dependent decline in the insulin induction upon glucose administration in Se-adequate G3 *Terc*$^{-/-}$ mice (**Fig. 4.2J**). Altogether, these results strongly suggest that dietary Se deficiency deteriorated both insulin sensitivity on peripheral cells and insulin production in the pancreas, resulting in blood glucose intolerance and the rising of fasting blood glucose during the aging process.

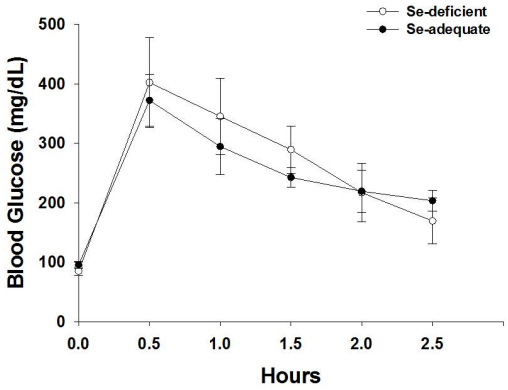
(A)


Body Weight (g)
Month
Se-deficient
Se-Adequate
*
*

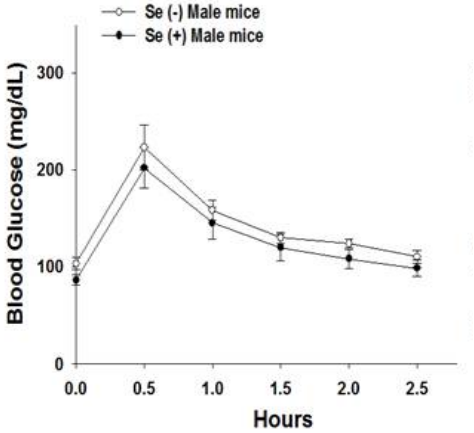
(B)


Weekly average food intake (g)
Se-deficient
Se-adequate
< 6
6~12
Age (months)

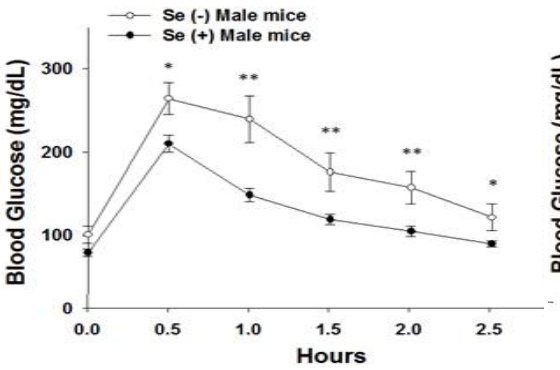
(C)


Blood Glucose (mg/dL)
Hours
Se-deficient
Se-adequate

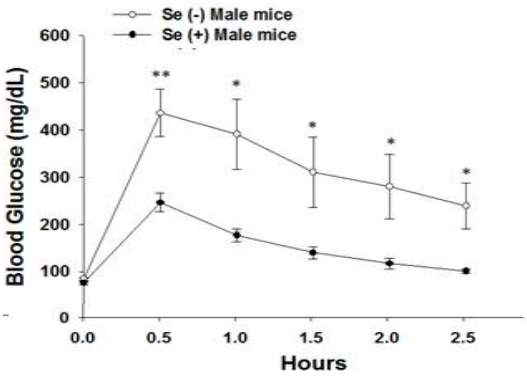
(D)


Blood Glucose (mg/dL)
Hours
Se (-) Male mice
Se (+) Male mice

(E)


Blood Glucose (mg/dL)
Hours
Se (-) Male mice
Se (+) Male mice

(F)


Blood Glucose (mg/dL)
Hours
Se (-) Male mice
Se (+) Male mice

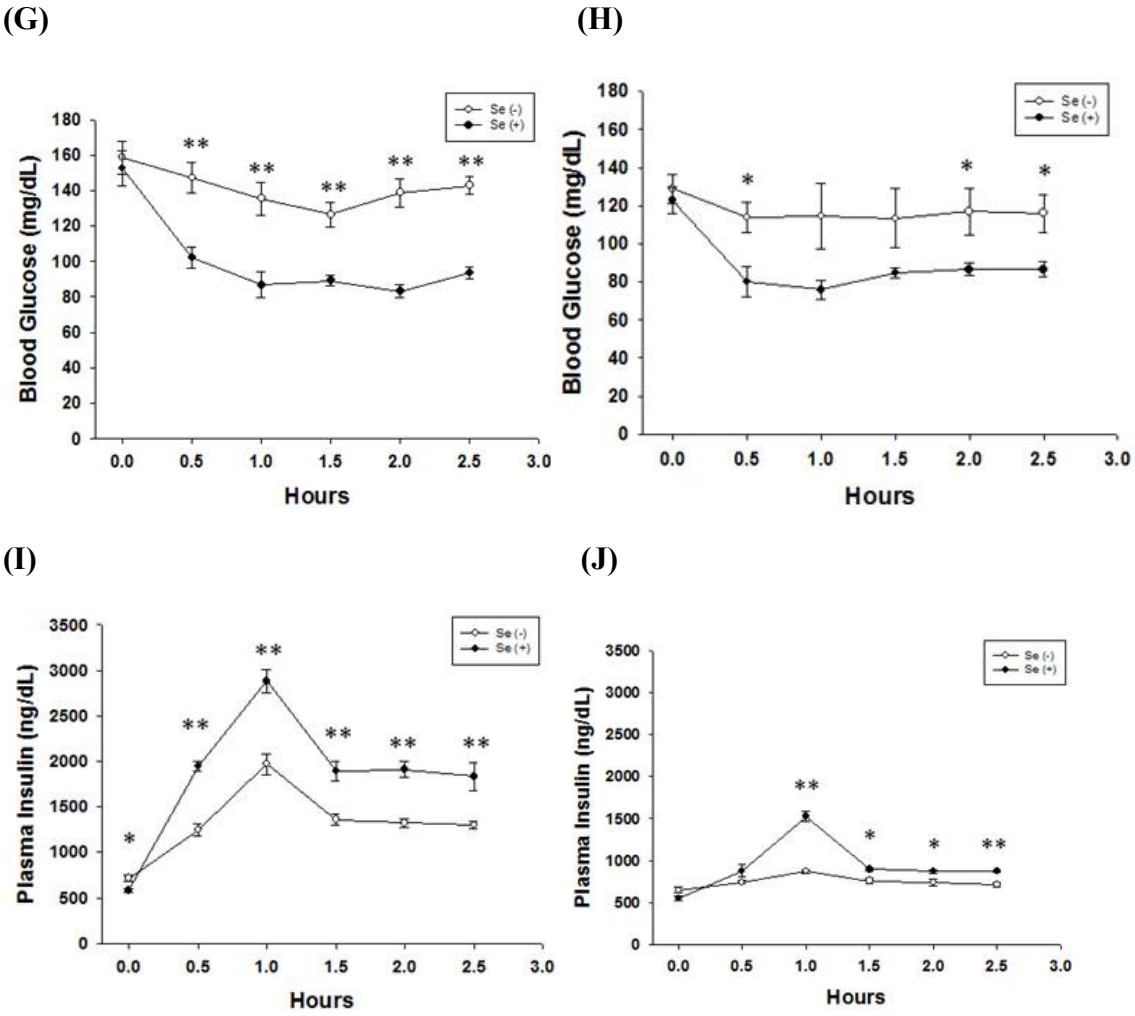
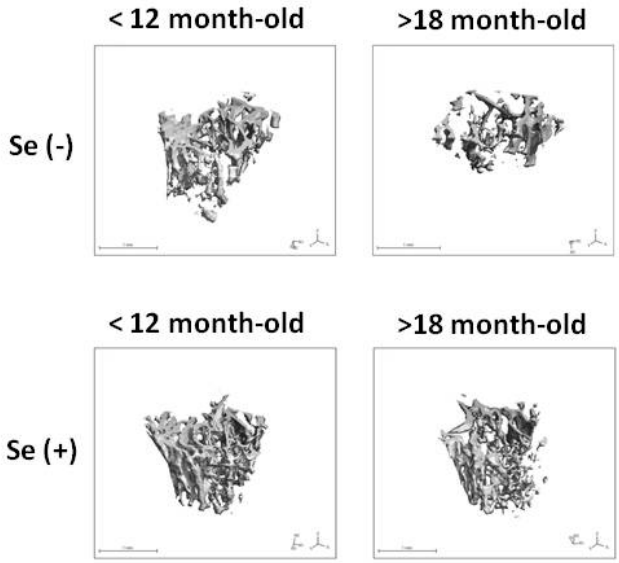


Figure 4.2 Gradually developed glucose metabolism disorder in Se-deficient mice The monthly average body weights of Se-deficient and Se-adequate mice (A). The weekly food consumption of the mice in Se-deficient or Se-adequate diets at younger than 6 months or between 6 to 12 months of age (B). Glucose tolerance test was performed in Se-deficient and Se-adequate G1 $Terc^{+/+}$ mice at 12 months of age (C), G3 $Terc^{-/-}$ mice at 9 (D), 12 (E) or 18 (F) months of age. Mice were fasted 8 hours prior to start the test. Glucose was injected by i.p. (1 μg/Kg), and blood glucose was measured by glucose meter in every 30 minutes till 2.5 hours after injection. Insulin tolerance test was performed in Se-deficient and Se-adequate G3 $Terc^{-/-}$ mice at age of 12 (G) or 18 (H) months. Insulin was injected by i.p. (0.75 units/Kg), and blood glucose was measured by glucose meter in every 30 minutes till 2.5 hours after injection. Plasma insulin was determined by insulin ELISA kit in Se-deficient and Se-adequate G3 $Terc^{-/-}$ mice at age of 12 (I) or 18 (J) months. The plasma was collected at each time point in the glucose tolerance test, and subjected to measurement. (*, $p<0.05$; **, $p<0.01$, compared to Se-deficient mice)

Bone integrity

Losing the integrity of bone is a classic age-related disorder (Pietschmann et al., 2009). Interestingly, dietary selenium was found to play an important role in bone integrity as evidenced by mouse studies fed with a Se-deficient diet for 4 months (Cao et al., 2012) or with *Trsp* (the gene coded for selenocysteine tRNA) conditional knockout in osteo-chondroprogenitors (Downey et al., 2009) and by human epidemiological studies (Suetens et al., 2001). However, how Se deficiency affects bone integrity later in health span is not known. To explore the alterations of bone integrity in terms of selenium status and aging, in each group, 5 femoral bones per group had been analyzed by μCT scanning. The data showed that the connectivity density was decreased by dietary Se deficiency, the extent of which was significantly greater in mice at 18 months (51%) than in 12 months (22%) of age (**Figure 4.3A and B**). Although changes of all other femoral bone structural indices are not statistically significant, the number of trabecular fiber and separation appeared to be decreased and increased, respectively. Altogether, these results suggest a protective role of nutritional Se against the onset of age-dependent osteoporosis.

(A)



(B)

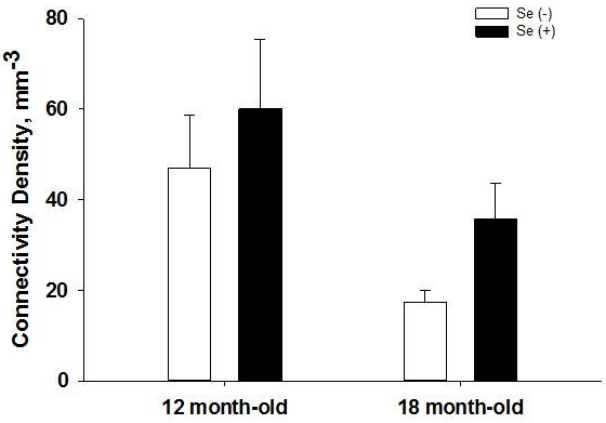


Figure 4.3 Bone integrity decreased dramatically in Se-deficient mice. The femoral bones were collected from Se-deficient and Se-adequate mice at age of 12 or 18 months, and subjected to detect by μCT scanning. The representative reconstitutive bone structure is showed in (A). The connectivity density of Se-deficient and Se-adequate mice at age of 12 or 18 months is showed to indicate the change of bone integrity (B).

4.3 Identification of circulating miRNA signature by openarray and qPCR analyses in Se-deficient, old short telomere mice

miRNAs (microRNA) are regulators of messenger RNA stability and translation and have been proposed as biomarkers for a variety of diseases and physiological conditions (Mendell and Olson, 2012), including aging (Gorospe and Abdelmohsen, 2011; Grillari and Grillari-Voglauer, 2010; Weilner et al., 2013). circulating miRNAs may also participate in intercellular communications when carried by extracellular vesicles in blood as extracellular shuttle RNA (Valadi et al., 2007). Specific nutrients can affect circulating miRNA profiles (Ross and Davis, 2011; Ryu et al., 2011), but the intersection of Se intake, small RNAs, and aging has not been examined. To maximize confidence, a high-throughput platform, TaqMan low density array, was used, and the expression of circulating miRNAs was determined by using individual quantitative polymerase chain reaction (qPCR) assays (Witwer et al., 2012). To this end, the results from the array had profiled over 800 circulating miRNAs in the plasma of Se-deficient and Se-adequate G3 *Terc*$^{-/-}$ mice at 12 and 18 months of age. The quality was assessed by individual quantitative qPCR assays for spiked cel-miR-39 in samples, and the high throughput data were quantile normalized prior to analysis of the datasets. Of the 131 circulating miRNAs that were detectable, the following circulating miRNAs showed the most significant changes ($p < 0.01$): miR-30a, miR-30e, miR-128a, miR-130a, miR-191, miR-328, miR-375 and miR-1937B increased with dietary Se-deficiency and miR-26b, miR-29b, miR-30d, miR-138, miR-191, miR-214 and miR-335 increased with age. Thus, plasma miR-24, miR-30d, miR-191, miR-375, miR-1937B and miR-let-7 defined the unique molecular signature common to both dietary Se deficiency and aging based on the

pilot study. To validate the result, the individual qPCR assays of the top ranked miRNAs ($p < 0.05$) were performed, as well as additional miRNAs reported to be aging-associated (Hamrick et al., 2010; Park et al., 2012; Takahashi et al., 2012). The results (**Figure 4.4A**) confirmed that plasma miR-130a expression was induced by both dietary Se deficiency and aging in G3 *Terc*$^{-/-}$ mice, miR-21, miR-29a, miR-29c and miR-34a expression were induced by dietary Se deficiency but not aging (**Figure 4.4B, C, D and E**), and miR-30d expression was induced by aging but not dietary Se deficiency (**Figure 4.4F**). Altogether, results from the circulating miRNA study suggested that plasma miR-130a was the most significant one to be induced both by dietary Se deficiency and aging.

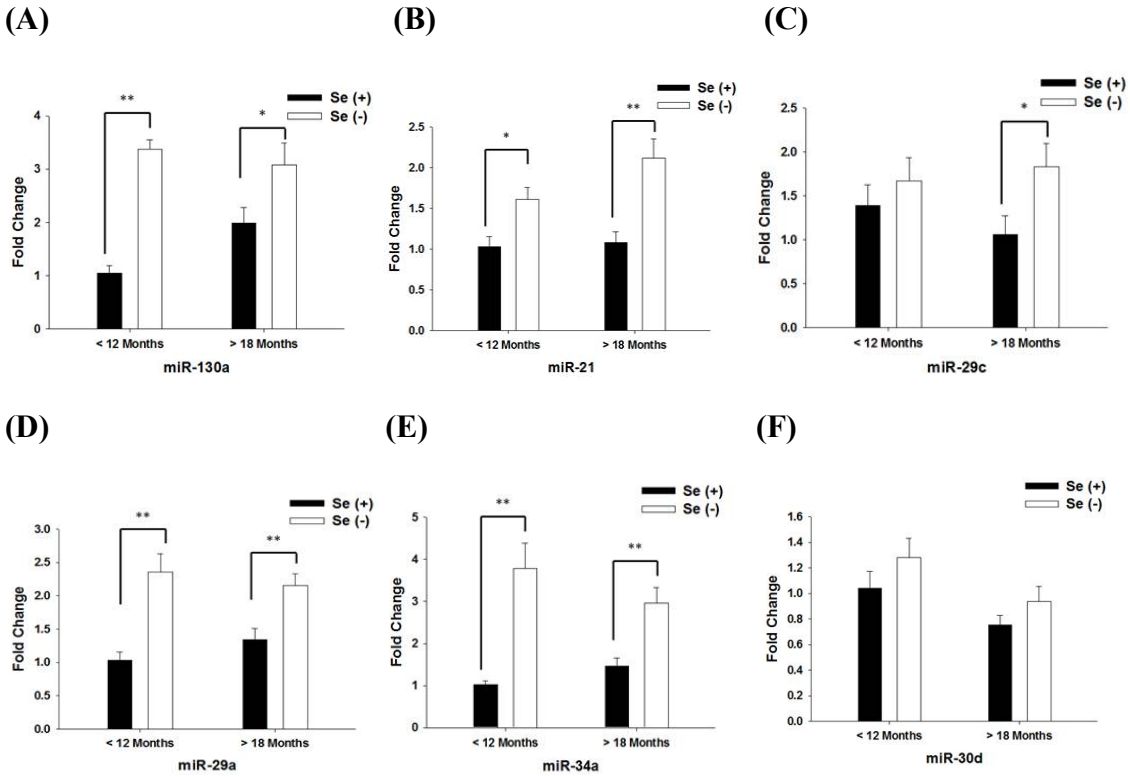


Figure 4.4 Real time PCR confirms the expression of age-related, selenium-related and age- and selenium-related circulating miRNAs. The potential differential expressed miRNAs are reconfirmed by real time PCR. miR-130a is age- and selenium-related miRNA (A). miR-21, miR-29c, miR-29a and miR-34a are selenium-related miRNA (B, C, D and E). miR-30d is age-related miRNA (F). (*, $p<0.05$; **, $p<0.01$, compared to Se-deficient mice)

4.4 Discussion

Mouse models are ideal to test theories of human organismal aging, but one caveat is that they have relatively long telomeres, which in human is much shorter. Due to the end-of-replication problem, telomere length is shortened after each round of cell division, which eventually elicits cellular senescence. Available lines of evidence suggest roles of nutritional Se and selenoproteins during the aging process (McCann and Ames, 2011), but current mouse models of dietary Se or selenoprotein deficiency do not recapitulate sufficient and comprehensive features of normal aging. Similarly, Werner syndrome, due to inactivating mutations of the *WRN* gene, develops a spectrum of age-related disorders, but knockout of *Wrn* in mice does not accelerate the onset of the aging phenotypes as shown in human Werner syndrome unless they are under a short telomere background (Chang et al., 2004). The same is true to recap ataxia telangiectasia in $Atm^{-/-}$ mice (Wong et al., 2003). Telomere attrition to critical length is now understood to be able to induce replicative senescence and to explain the "Hayflick" limit of culturing primary cells. Fibroblasts from individuals with Werner syndrome have accelerated telomere attrition and undergo premature senescence that can be rescued by enforced expression of *Tert* (Wyllie et al., 2000), the telomerase catalytic subunit. Interestingly, embryonic fibroblasts derived from $GPX1^{-/-}$ mice show senescence-like features in a ROS-dependent manner (de Haan et al., 2004). Here, the results had showed that deficiency in dietary selenium accelerated the phenotypes of human normal aging in short telomere mice, including hair graying, wound healing deficiency, faster telomere attrition, diabetes-like symptoms, losing bone integrity and altering the expression of circulating miRNA. Altogether, the data has provided the first evidence that dietary Se at nutritional level of intake attenuates aging and age-related disorders in mice carrying humanized telomeres.

The long-term dietary selenium deficiency in short telomere mice had shown accelerated aging and age-related disorders, such as skin abnormality, hair graying, diabetes-like phenotype, loss of bone integrity and faster telomere attrition. Interestingly, the similar phenotypes of age-related disorders in selenium deficient short telomere mice were also reported previously in the other mouse models of selenoprotein deficiency, selenocysteine (Sec) $tRNA^{[Ser]Sec}$ gene (*Trsp*) knockout and isopentenyladenosine-deficient ($i6A^-$) $tRNA^{[Ser]Sec}$ overexpression. The conditional knockout of *Trsp* in keratinocytes showed skin and hair abnormalities (Sengupta et al., 2010), and in Osteo-Chondroprogenitor resulted in the defects of bone development (Downey et al., 2009). Additionally, the $i6A^-$ overexpression mice had increased glucose intolerance and diabetes-like phenotypes (Labunskyy et al., 2011). Interestingly, the clinical observed mutations in humans on selenocysteine insertion sequence–binding protein 2 (SECISBP2 or SBP2) impair selenocysteine insertion, which leads to selenoprotein deficiency. The patients with SBP2 mutation showed the retardation of bone development, the increase of ROS and DNA damage, and faster telomere attrition (Di Cosmo et al., 2009; Schoenmakers et al., 2010). With these consistencies of the observed phenotypes in genetically modified selenoprotein deficiency and the long-term dietary selenium deficiency induced selenoprotein deficiency in short telomere mice, selenoprotein deficiency is a potential main factors to accelerate aging and age-related disorders in short telomere mice. Noteworthy, *Trsp* mutated, conditional knockout and complete knockout mice have severe survival problems, from shortened lifespan to embryonic lethal (Bosl et al., 1997; Carlson et al., 2009; Downey et al., 2009; Sengupta et al., 2010; Shrimali et al., 2007), so these animal models with severe selenoprotein deficiency had

pointed out the biological functions of selenoproteins may be involved in cell differentiation, development and proliferation, which are critical to maintain health span and lifespan. However, to study the role of selenoproteins in the pathophysiology of normal aging, short telomere mice provides an useful tool to study the biological significance of selenoproteins in aging and age-related disorders, because the phenotypic changes are gradual but not acute and lethal change, which is not normal aging. On the other hand, it is possible to develop intervention for delaying aging and rescuing age-related disorders by using this dietary selenium deficiency induced accelerated aging in short telomere mice.

Diabetes-like phenotype induced by dietary selenium deficiency may lead to unsuccessful aging. Dysfunction on glucose metabolism and developing diabetes are the classic age-related disorders. Based on the pathophysiology of type 2 diabetes, impaired glucose tolerance and insulin resistance are compensated by hyperinsulinemia, and the impaired glucose tolerance and insulin resistance will develop later with the failure of β cells in pancreas (Rao et al., 2004). The data also suggested the Se-deficient G3 *Terc*$^{-/-}$ mice had the prone to develop diabetes-like symptoms. At 9 month of age, Se-deficient G3 *Terc*$^{-/-}$ mice didn't develop glucose intolerance. Then, the impaired glucose tolerance and insulin resistance were developed at 12 moth of age, and the phenotypes were exacerbated at 18 month of age. In the meantime, the ability on regulating plasma insulin was gradually lost in Se-deficient G3 *Terc*$^{-/-}$ mice. Noteworthy, the average body weights are not discernible between Se-deficient and Se-adequate G3 *Terc*$^{-/-}$ mice before 12 month-old, but there is a significant heavier average body weight of Se-deficient G3 *Terc*$^{-/-}$ mice from 14~18 month-old. However, the cause of glucose and insulin intolerance was unlikely to be

induced by obesity. The plasma insulin data suggested that the glucose intolerance and insulin resistance were associated with the limitation on insulin secretion. This may be resulted from aging induced β cell dysfunction in pancreas (Gu et al., 2012; Halvorsen et al., 2000; Lee et al., 2013). Importantly, the activity of telomerase is still detectable in mice but not in human (Liew et al., 2009), so the effect of aging induced β cell dysfunction may not appear in wild type mice. As a consequence, *Terc*$^{-/-}$ mice is a good model to study the disorder on glucose metabolism, especially on the aspect of human normal aging (Kuhlow et al., 2010). Moreover, the observed significant lower average body weight in Se-deficient G3 *Terc*$^{-/-}$ mice between 21~24 month is another evidence to support the possible beta-cell dysfunction, which is one of the typical symptoms of diabetes, because the body uses stored fat instead of blood glucose (Durham et al., 2009). The data suggested that dietary selenium deficiency may exacerbate the effects of aging, which dampening the normal function of β cells and resulting in glucose intolerance, insulin resistance and diabetes.

The identified circulating miRNAs are the potential molecular markers to evaluate the molecular age and the risk of age-related disorders. The role of circulating miRNA had been identified as a molecular marker and a new species of endocrine signaling molecules (Rottiers and Naar, 2012). In the study, the up-regulation of selenium-related miRNA (miR-29a/c, miR-21 and miR-34a) and selenium/aging-related miRNA (miR-130a) had been identified. Although there is only miR-130a related to both age and selenium, the other selenium-related miRNAs may also participate in the pathway of promoting aging. miR-29 had been proposed to involve in senescence (Martinez et al., 2011; Takahashi et al., 2012), and miR-29 activates p53 to promote aging (Ugalde et al.,

2011); miR-34 is also related to stabilize p53 for the activation of senescence (Yamakuchi and Lowenstein, 2009); miR-21 limited the replicative lifespan, and increased endothelial progenitor cells senescence through suppressing high-mobility group A2 (Dellago et al., 2013; Zhu et al., 2013b). The emerging evidence indicates that the identified selenium-related miRNA in my study may play a critical role in the observed accelerated aging in Se-deficient G3 *Terc*$^{-/-}$ mice. On the other hand, the age-related disorders had also been reported to be associated with some miRNAs. It had been found that the up-regulation of miR-21, miR-29a/c or miR-130a interferes the ability of wound healing (Pastar et al., 2012; Wang et al., 2012; Yang et al., 2013). On the aspect of glucose metabolism, the up-regulation of miR-34a or miR-29a/c is highly correlated to diabetes (Choi et al., 2013; Kong et al., 2011; Locke et al., 2013; Nesca et al., 2013; Roggli et al., 2012); miR-130a was highly expressed at hyperglycemia condition (Esguerra et al., 2011); miR-21 and miR-34a promoted the pro-inflammatory cytokine induced β cell failure (Roggli et al., 2010); the up-regulation of miRN-29a promotes impaired glucose tolerance (Bagge et al., 2012). Moreover, miR-21 may induce osteoporosis (Yang et al., 2013). The data suggested that the selenium-related and selenium and aging-related miRNAs are not only playing as molecular markers for aging and age-related disorders, but also provides the link of pathological progressions of aging and age-related disorder and dietary selenium.

The selenium-related miRNAs are associated with the promotion of aging, but they also suppress tumorigenesis. Based on openarray and real-time PCR identified miRNA, I subjected the identified miRNAs to DIANA miR PATH V. 2.0. for the analysis of miRNA targeting pathways (Vlachos et al., 2012). Interestingly, selenium-related

miRNAs (miR-34a, miR-29a/c and miR-21) significantly interfere main cancer pathways, small cell lung cancer, non-small cell lung cancer, colorectal cancer and pancreatic cancer. The major impacts are interfering proliferation, angiogenesis and metastasis. Meanwhile, the current evidence suggested miR-29a (Park et al., 2009; Zhu et al., 2013a; Zhu et al., 2012), miR-29c (Fan et al., 2013; Wang et al., 2013a; Wang et al., 2013b) and miR-34a (Chen et al., 2013; Chiyomaru et al., 2013; Garofalo et al., 2013) have the role on anti-tumorigenesis. On the other hand, although miR-21 was proposed to be an oncomir to promote tumorigenesis and to be highly expressed in cancer cells (Hong et al., 2013; Nouraee et al., 2013), the higher expression level of miR-21 was found to activate senescence instead of tumorigenesis in human normal cells (Dellago et al., 2013). Normal cells are not like cancer cells, which still retain the functional tumorigenesis barrier, oncogene-induced senescence, to prevent the development of malignant cells (Bartkova et al., 2006). As a result, Se-deficiency activated miRNAs may reinforce proliferative inhibition and senescence to promote aging and also to suppress tumorigenesis. The recent report from Kasaikina et al. supported my findings that both selenium deficiency and supra-nutritional selenium intake suppressed chemically induced hepatocarcinogenesis in FVB mice, and the potential of proliferation was also lower in these two conditions (Kasaikina et al., 2013). Thus, even though cancer is considered as one of the age-related disorders, the human normal aging model, selenium deprivation in Short telomere mice, may not be a good tool for the pathophysiological study of cancer.

The recent evidence on SELECT trial, GPx1 overexpression and the increasing level of Sepp1 points out the potential adverse health effects of supra-nutritional selenium consumption, especially the increased risk of diabetes. However, in the study, the results

demonstrated that Se-deficient G3 *Terc*$^{-/-}$ mice showed accelerated aging and age-related phenotypes compared with Se-adequate mice. The results shed lights on the importance of maintaining adequate dietary selenium is important to prevent selenium deficiency induced aging and age-related disorders. Because dietary selenium regulates the expression of selenoproteins, which at least have 11 out of 25 selenoproteins related to aging (McCann and Ames, 2011), the function of individual selenoprotein on anti-aging is required to be identified. Although the known functions of selenoproteins, antioxidation, redox signaling and protein folding, are reported to potentially protect against aging, there are still some dietary selenium sensitive selenoproteins with unknown functions (Kipp et al., 2009; Sunde, 2010), which may involve in the prevention of accelerated aging. On the other hand, the new proposed direction that the role of selenium or selenoprotein on regulating the expression of miRNA will be the key regulator to maintain optimal health.

Chapter 5: Conclusion and Prospects

In this dissertation, cell-based and animal-based approaches have been employed to determine the cellular and physiological roles of selenium in replicative senescence and organismal aging. The novel finding is that deprivation of selenium at nutritional levels of intake accelerates aging and age-related disorders in a mouse model of human normal aging, short telomere *Terc*$^{-/-}$ mouse. Interestingly, previous studies have shown that dietary selenium fluctuation alters the expression of selenoproteins to a various degree, and SelH is one of the selenoproteins most sensitive to dietary selenium deficiency. By employing the SelH stable knockdown human diploid fibroblast, the results demonstrate that SelH is critical for the prevention of early onset of replicative senescence in mortal cells through the protection of genome stability. These findings advance the field of selenium and aging, and offer several novel prospectives for future recommendations of dietary selenium consumption towards healthy aging.

5.1 The improved recommendation for dietary selenium consumption

The recommendation of dietary selenium consumption has beenproposed to consider the basal level of body selenium status and the form of dietary selenium. Current reports based on NPC and SELECT trials provide inconsistent results on selenium being a chemoprevention agent. Interestingly, the discrepancy may be attributed to the different basal level of body selenium in participants and the form of selenium (Hurst and Fairweather-Tait, 2009; Rayman et al., 2009). If the basal body selenium status is high prior to entering the trials, individuals do not benefit from selenium supplementation; however, in the population with lower body selenium status, selenium supplementation

indeed confers cancer chemoprevention. Additionally, the forms of selenium supplementation are different in the two clinical trials. Different selenium compounds target their specific pathways of metabolism, but the best form of selenium for selenium chemoprevention is still little known. Supported by results from animal studies, one thing clear is that selenomethione is not an ideal selenium speciation for chemoprevention. Moreover, the risk of diabetes after selenomethionine supplementation was found to be increased in those who have high basal level of body selenium. Thus, only those with sub-optimal body selenium status benefit from supplementation of certain forms of selenium. The beneficial effect occurs only within a narrow range surrounding the level of nutritional adequacy.

However, the stage of life cycle should also be considered concerning selenium supplementation. In human normal aging, accumulating errors in the body contribute to the symptoms of aging and age-related disorders. Optimal body selenium status mitigates disadvantages but maximizes advantages regarding healthy aging. Early in life, supranutritional selenium is beneficial to secure the expression of fully functional selenoproteins for maintenance of physiological functions and cancer prevention by activating tumorigenesis barrier in pre-malignant cells. At mid stage, the intake of dietary selenium should be cut down to an adequate level. Since genome instability is induced by reactive oxygen species and accumulates with age, the oxidative stress generated from excessive amount of selenium may exacerbate the aging process. On the other hand, previous studies have shown that increased selenium consumption may promote diabetes-like symptoms. As such, lowering dietary selenium intake to an adequate level may maintain functional glucose homeostasis and thus healthy aging. Later in life, dietary

selenium deficiency may be beneficial. This is supported by our findings in that selenium deficiency activates anti-carcinogenesis miRNAs and SelH deficiency promotes early onset of cellular senescence, a tumorigenesis barrier, in pre-malignant cells. Altogether, it is equally important to adjust the recommendation of dietary selenium consumption according to the stage of life cycle, body selenium status and selenium speciation, towards optimized health span and lifespan.

5.2 The role of rDNA damage on replicative senescence and aging

The nucleolar protein SelH plays a role in the protection against genome instability and early onset of replicative senescence. Nucleolus, the region for ribosomal RNA (rRNA) transcription, has been proposed to serve as an oxidative stress sensor in the cell (Lewinska et al., 2010). When cells experience severe stress, rRNA transcription is stopped through JNK2 inactivating transcription factor, TIF-1A (Mayer et al., 2005), which allows the cells to overcome the stress. Interestingly, the accumulation of extrachromosomal rDNA circles promotes enlargement and fragmentation of the nucleoli in aging yeast, which might be further accelerated by rDNA damage (Oberdoerffer and Sinclair, 2007; Sinclair and Guarente, 1997). Persistent rDNA damage may constitutively trigger DNA damage responses and activate replicative senescence (Kobayashi, 2011). Moreover, the formation of heterochromatin in rDNA has been found to be beneficial for extending longevity in yeast (Larson et al., 2012). These results point to the possibility that rDNA integrity is a critical factor in favor of longevity. Additionally, HR is needed for rDNA repair, so the functional HR is required to stabilize rDNA. Furthermore, the HR protein, Rad52, has been found to maintain the stability of rDNA copy number together with Shu complex (Bernstein et al., 2013). On the other hand, Rad52 facilitates

OGG1 for the repair of 8-oxoG, but the helicase activity of Rad52 is blocked by OGG1 (de Souza-Pinto et al., 2009). As a result, the increased oxidative rDNA damage may require BER pathway to fix the lesion but concomitantly suppresses HR on rDNA. Ultimately, rDNA instability can promote replicative senescence. As shown in the current study, the loss of SelH in the nucleoli may induce intensive DNA damage that dampens genome stability, although the hot spot of DNA damage under this condition is still unknown. Thus, the critical question is whether SelH can maintain nucleolar integrity in the context of oxidative stress sensing and rDNA stability. To address this question, it is of future interest to analyze the hotspots of rDNA damage and to evaluate the efficacy of different types of DNA repair pathways in SelH knockdown cells.

5.3 The control of miRNA expression for optimizing health span and lifespan

The control of miRNA expression is a potential strategy optimizing health span and lifespan. Results from the animal study indicate that circulating miRNAs are useful biomarkers and provide "hormone-like" features for long distance regulation. On the aspect of optimizing health span and lifespan, it is of future interest to elucidate the regulation of the miRNAs expression. miRNAs are powerful regulators. The flexibility of sequence specificity enables miRNAs to regulate various signaling pathways. Furthermore, the species of miRNAs are too numerous. With these characters in mind, miRNAs are indeed powerful yet complicated regulators in the body. Thus, it is required to gather further evidence before starting manipulating the expression of specific miRNA(s). Reconfirming the role of individual miRNA is necessary to verify the biological significance in the body. Based on the differentially expressed miRNAs by selenium deficiency and aging, it is possible to study the biological function of specific

miRNAs by overexpressing them in cells or in mice. Furthermore, genomic and proteomic studies can determine the cross-talk between different miRNAs. The expression profiles of miRNAs at different stages of life or genders are important to comprehensively realize the roles of miRNAs, which may help to identify the desired end point of the intervention. On the other hand, circulating miRNAs are secreted or released by various types of tissues and organs. Thus, the increase in circulating miRNAs may also indicate the pathophysiological change in specific tissue or organ. To determine the main tissue or organ that is affected or secretes the specific miRNA(s) is equally important in monitoring the whole expression profile change of circulating miRNAs. Furthermore, one should consider to evaluate the pathophysiological condition in the body and provide proper ways to optimize the miRNA profile for optimizing health span and lifespan.

Selenium continues to shine in supporting optimal health span and lifespan through cancer prevention and other health benefits, regardless of the debating results from the two major selenium clinical trials (NPC and SELECT). Currently, the application of selenium chemoprevention seems underappreciated; however, re-analysis of the SELECT results indicates a role of selenium in cancer prevention. Body selenium status and the form of selenium decisively impact on the success of selenium chemoprevention. Therefore, the notion of selenium chemoprevention in fact is supported by both NPC and SELECT trials. Altogether, exciting lines of evidence provided in this dissertation have provided a new chapter in that selenium is involved in aging and age-related disorders through selenium at nutritional level of intake or selenoproteins. At this point, we may use a different angle to study the biological functions of selenium and selenoproteins in

aging and age-related disorders. Although the clinical trials had established that selenium is not a panacea for cancer, little is known about the physiological functions of selenium and selenoproteins during organismal aging. Finally, Selenium intervention is not a closed case. Herein, the novel approach employing a mouse model of aging carrying "humanized" telomere has enabled to demonstrate a physiological role of selenium at nutritional levels of intake in the attenuation of aging and age-related disorders, for the first time. In conclusion, it is a great opportunity for selenium scientists to jump out the original circle, and to explore the new avenues towards understanding selenium functions in the biology of aging.

Abbreviation List

1. ROS: Reactive oxygen species
2. BER: Base excision repair
3. NER: Nucleotide excision repair
4. MMR: Mismatch repair
5. HR: Homologous recombination
6. NHEJ: Non-homologous end-joining
7. DSB: Double strand break
8. T-loop: Telomeric-loop
9. D-loop: Displacement-loop
10. ATM: Ataxia telangiectasia mutated
11. ATR: Ataxia telangiectasia mutated rad3 related
12. DDR: DNA damage response
13. SSB: Single strand break
14. HAT: Histone acetyltransferase
15. HDAC: Histone deacetylase
16. MAPK: Mitogen-activated protein kinase
17. DNA-SCARS: DNA segments with chromatin alterations reinforcing senescence
18. SAHF: Senescence-associated heterochromatin formation
19. SA-β-gal: Senescence-associated beta-galactosidase
20. Rb: Retinoblastoma
21. HMGA: High-mobility group A
22. SASP: Senescence-associated secretary phenotype

23. PARP1: Poly(ADP-ribose) polymerase 1

24. MRN complex: Mre11-Rad50-NBS1 complex

25. DRI: Dietary reference intake

26. RDA: Recommended dietary allowance

27. NPC: Nutritional prevention of cancer

28. SELECT: Selenium and vitamin E cancer prevention trial

29. SBP2: SECIS-binding protein 2

30. GPx: Glutathione peroxidases

31. Trx: Thioredoxin

32. TrxR: Thioredoxin reductases

33. DIO: Iodothyronine deiodinase

34. T4: Thyroxine

35. T3: 3,5,3'-triodothyronine

36. rT3: Reverse triiodothyronine

37. Sepp1: Selenoprotein P

38. apoER2: Apolipoprotein E receptor-2

39. ER: Endoplasmic reticulum

40. UGGT: UDP-glucose:glycoprotein glucosyltransferase

41. SelH: Selenoprotein H

42. ChIP: Chromatin immunoprecipitation

43. HSE: Heat shock element

44. STRE: Stress response element

45. MRE: Metal response element

46. SelN: Selenoprotein N

47. SelW: Selenoprotein W

48. MsrB: Methionine sulfoxide reductase B

49. NAC: *N*-acetylcysteine

50. DMSO: Dimethyl sulfoxide

51. shRNA: Short hairpin RNA

52. H_2DCFDA: 2', 7'-dichlorodihydrofluorescein diacetate

53. γH2AX: Phospho-H2AX on Ser-139

54. pATM Ser-1981: Phospho-ATM on Ser-1981

55. PCNA: Proliferating cell nuclear antigen

56. GSH: Glutathione

57. mBCl: Monochlorobimane

58. qPCR: Quantitative polymerase chain reaction

59. ip: Intraperitoneal

60. TMB: 3,3′,5,5′-tetramethylbenzidine

61. Terc: Telomerase RNA component

62. Trsp: The gene coded for selenocysteine tRNA

63. miRNA: MicroRNA

64. SEC: Selenocysteine

65. i6A$^-$: Isopentenyladenosine-deficient $tRNA^{[Ser]Sec}$ overexpression

66. SECIS: Selenocysteine insertion sequence

67. rRNA: Ribosomal RNA

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ANIMAL HANDLER'S MEDICAL SURVEILLANCE PROGRAM

NAME: Tsung-Yu Wu **DATE: January 5, 2010**

DEPARTMENT: NFSC **SUPERVISOR: Dr. Cheng**

The above-named employee works with: Purpose Bred Rodents

This clearance will expire three years from the above date.

☒ **1.** If box is checked : This employee has completed a medical questionnaire in accordance with the University's Animal Handler's Medical Surveillance Program. There does not appear to be any medical deficit that would disqualify him / her from working with laboratory animals.

☐ **2.** If box is checked : This employee has completed a medical questionnaire in accordance with the University's Animal Handler's Medical Surveillance Program. The employee may work with laboratory animals with the following limitations:

☐ **3.** If box is checked : This employee has received three rabies immunizations and/or has a positive rabies titer; he/she is cleared to work with animals that may be infected with rabies.

Rebekah Giannakos, CRNP

Cc: Employee's Supervisor

Wen-Hsing Cheng

From: Janet Peterson [peterson@umd.edu]
Sent: Monday, January 25, 2010 2:22 PM
To: Wen-Hsing Cheng
Cc: Lucy Liangli Yu
Subject: "Selenium, DNA repair, and aging"
Attachments: BSL2 BMBL-5.pdf

MEMORANDUM

TO: Wen-Hsing Cheng
Nutrition and Food Science

FROM: Janet Peterson
Biosafety Officer

SUBJ: DES #10-07
"Selenium, DNA repair, and aging"
Approved 14 January 2010 for a period of 4 years

The UM Institutional Biosafety Committee met on 14 January 2010 to review your rDNA registration, "Selenium, DNA repair, and aging." Experiments were approved at BSL2 containment for experiments involving lentiviral vector systems to suppress gene expression in primary cell cultures, and at ABSL2 containment for inoculation of mice with transformed human cells; animals may be housed at ABSL1 after inoculation. The use of needles with lentiviral vectors should be limited to situations where there is no alternative, such as inoculation of animals. This approval will expire on January 31, 2014, at which time you will need to submit a new registration form if the research is still active.

A copy of BSL2 containment criteria is attached for your review. It is your responsibility to ensure that your rDNA research is conducted in compliance with these criteria and with the NIH Guidelines for Research Involving Recombinant DNA Molecules (http://oba.od.nih.gov/rdna/nih_guidelines_oba.html), and to inform all lab personnel of the risks of the research and measures to take to reduce those risks.

Your approval number is DES#10-07. Please cite this number on the internal routing form of future grant applications that utilize the same recombinant DNA systems. If changes are made in the host/vector/insert system before the end of 4 years, you will need to submit a new registration document. The online biological agents registration form may be accessed at https://des.umd.edu/research/login.cfm. Please contact me at peterson@umd.edu if you have any questions or need further information.

cc: L. Yu

--
Janet S. Peterson, RBP, CBSP
Biosafety Officer, University of Maryland
Assistant Director, Department of Environmental Safety
301.405.3975

UNIVERSITY OF
MARYLAND
DIVISION OF RESEARCH
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

0101 Lee Building
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W. Ray Stricklin
IACUC Chair
wrstrick@umd.edu
Phone: (301)405-7044

November 10, 2010

Dr. Wen-Hsing Cheng
Department: Food & Nutrition Sciences
University of Maryland
whcheng@umd.edu

Dr. Cheng,

This letter is to inform you that on **October 8, 2010**, the members of the Institutional Animal Care & Use Committee (IACUC) reviewed and approved the protocol for:

Selenium and Genome Maintenance

R-10-79

Please note that an approved protocol is valid for three (3) years unless there is a change in the protocol. Thus, this protocol is valid until **October 8, 2013**. Federal laws indicate that protocols must be reviewed yearly. Thus, in order to keep your approved protocol active you **MUST** submit a protocol renewal/update by the first of the month of the anniversary of your approval (October 2011 & October 2012). All work extending beyond the approval date of the protocol must be submitted to the IACUC as a new protocol.

Sincerely,

W. Ray Stricklin
Asst. Dean, College of Ag. & Natural Resources
Chair, IACUC

CC: Doug Powell, Amanda Underwood

UNIVERSITY OF MARYLAND
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

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DATE:	January 8, 2013
TO:	Wen-Hsing Cheng
FROM:	University of Maryland College Park (UMCP) IACUC
PROJECT TITLE:	[359205-3] Selenium and Genome Maintenance
IACUC REFERENCE #:	R-10-79
SUBMISSION TYPE:	Response/Follow-Up
ACTION:	APPROVED
APPROVAL DATE:	January 8, 2013
EXPIRATION DATE:	October 8, 2013

Thank you for your submission of the Animal Study Protocol [R-10-79] Selenium and Genome Maintenance. The University of Maryland College Park (UMCP) IACUC has APPROVED your submission. This approval is based on the committee's review of the appropriate use and care of animals within your research goals.

Research must be conducted in accordance with this approved submission. All changes must be submitted to the University of Maryland College Park (UMCP) IACUC as a revision. Conducting research outside the scope of your approved submission is reportable to federal entities and will be investigated by the University of Maryland College Park (UMCP) IACUC and may require interruptions to the research project.

Any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate revision forms for this procedure which are found on the IRBNet Forms and Templates Page.

All UNANTICIPATED PROBLEMS involving UNEXPECTED MORBIDITY or MORTALITY to animals or study personnel must be reported promptly to this office.

This protocol requires continuing review by this committee on an annual basis. Please use the appropriate forms for this procedure. Your documentation for continuing review must be received with sufficient time for review and continued approval before the expiration date of October 8, 2013.

Please note that all research records must be retained for a minimum of three years after the completion of the project.

If you have any questions, please contact Amanda Underwood at 301-405-5037 or graebel@umd.edu. Please include your project title and reference number in all correspondence with this committee.

W. Ray Stricklin, IACUC Chair

This letter has been electronically signed in accordance with all applicable regulations, and a copy is retained within University of Maryland College Park (UMCP) IACUC's records.