Abstract

Title of Thesis:	Selenium-Induced Senescence Involves Heterochromatin Formation
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We have recently shown that selenium compounds can induce a senescence response in a manner depending on ataxia-telangiectasia mutated (ATM), DNA-dependent protein kinase (DNA-PK), p53 and reactive oxygen species (ROS). To test the hypothesis that the selenium-induced senescence response involves epigenetic changes in senescence-associated heterochromatin foci (SAHF), we determined the expression of histone H3 Lysine 9 trimethylation (H3K9me3), a marker of SAHF, in human primary MRC-5 cells treated with methylseleninic acid (MSeA, 2 μM) for 2 days, followed by a 7-day recovery, in the presence or absence of KU55933 (10 μM), an ATM kinase inhibitor, and NU7026 (10 μM), a DNA-PK kinase inhibitor. Our results showed that MSeA treatment induced the formation of SAHF and H3K9me3 foci. Pre-treatment of the cells with KU55955 or NU7026 resulted in numerous and smaller foci, and they did not co-localize with the MSeA-induced SAHF. These results suggest that the MSeA-induced senescence response involves epigenetic changes of H3K9me3 in a manner depending on ATM and DNA-PK.

Selenium-induced Senescence Involves Heterochromatin Formation

by

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Chapter 1: Selenium, epigenetic regulation and DNA damage response

Introduction

Recent interest in the epigenetic changes modulated by selenium has grown due to the role of this essential nutrient in chemoprevention, DNA damage and epigenetic reglation[1,2]. The role of selenium and the selenoproteins that are developed from the mineral are widely studied and it is known that they reduce oxidative stress in mammalian cells[3,4]. Although selenium's role in general health is accepted today, at one time selenium was a mysterious substance and seen only as a dangerous chemical.

Selenium: Historical perspective and nutrition

Selenium is an essential nutrient vital to proper cellular metabolism. Once strictly considered a toxin, it has been shown to have implications in various aspects of optimal cellular and organismal health including DNA maintenance, protection against oxidative stress, and the prevention of cancer.

Selenium's history as a toxin is more extensive than its tenure as an essential trace mineral. The Swedish chemist Jons Jakob Berzelius first identified it as he searched for tellurium in paint scrapings[5]. He named the element after the Greek moon goddess, Selene, since he was looking for tellurium, which is named after the Greek Earth goddess Tellus. In fact, the medieval adventurer Marco Polo may have been the first historical

record of the mineral's effects on mammalian health. During his expedition through China, Marco Polo observed that horses that consumed plants in the mountainous regions of the Shaan-Xi Province would experience hoof fractures that would eventually detach from the animal. These plants could have stored selenium from the soil since what Marco Polo described in his horses is indicative of selenium toxicity. Selenium's toxicity was confirmed in the early 20th century when KW Franke, an American chemist, identified the mineral as the culprit in alkali disease, a condition that affected cattle who grazed on plants grown in selenium rich soil[5].

Although selenium in high amounts can have toxic consequences, a selenium deficiency is similarly detrimental and has been associated with two diseases: Keshan disease and Kashin-Beck disease. Keshan disease was first characterized in China in 1935 as an infectious illness[6]. Interestingly, the illness has only been diagnosed in populations that live in certain regions of China where the soil with particularly low selenium levels[7]. This condition can result in an enlarged heart, cardiac arrhythmias, and congestive heart failure. Kashin-Beck disease is similar in that it only occurs in certain regions of China[7]. However, the illness is characterized by osteoarthritis, muscle degeneration[7,8]. Research shows that these diseases are linked to low levels of the proteins that selenium is used to create, selenoproteins[9].

Selenoproteins

Selenium is the 34th element on the periodic table and is in the same group as oxygen and sulfur. Although selenium shares some attributes with some elements in the same group, it is nutritionally unique in terms of its sources. This trace mineral is found in a variety of foods including animal organs, animal muscle, and cereals; however, the most concentrated levels of selenium are found in that of the Brazil nut. A single Brazil nut contains the US adult RDA for selenium – 55 μg. Yellow tuna also contains a considerably higher amount of selenium than other foods – 92 mcg per 3 ounce serving. Some of the selenium from these foods can be metabolized into selenoproteins

There are over 24 selenoproteins and many have been shown to be essential as antioxidants in different parts of the cell. Dietary selenium compounds, such as selenomethionine, selenocysteine, or selenite, are converted to selenoproteins to carry out metabolic functions. Before the selenium compounds are incorporated into selenoproteins, they must first metabolize to hydrogen selenide (H_2Se), which can be used to create selenoproteins. H_2Se can also be methylated, which has epigenetic implications. Selenoproteins are unique because they have their own mRNA codon that allows selenium's insertion into endogenous selenoproteins as selenocysteine co-translationally. Selenocysteine insertion is designated by the UGA that is otherwise a stop codon if the mRNA does not include a stem-loop structure in the 3'-UTR of

selenoprotein mRNA called the selenocysteine insertion sequence (SECIS) element[10]. This prevents UGA from carrying out its usual role to stop translation and instead to read as selenocysteine. The SECIS element allows for the production of all selenoproteins including glutathione peroxidase 1-4, thioredoxin reductase, and selenoprotein P. These selenoproteins are particularly important in terms of reducing oxidative stress.

The mitochondria produce copious amounts of reactive oxygen species (ROS) within cells as a result of the oxidative phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) during cellular respiration. These ROS compounds typically manifest as H_2O_2. The accumulation of ROS results in a toxic cellular environment that can have devastating consequences on the organelles and more importantly on the genetic material housed within the mitochondria and the nucleus. This cytotoxicity can also lead to apoptosis in some instances. However, the cellular machinery so diligently developed by the process evolution has a system that codes for the production of protective enzymes that can counteract the devastating effects of cytotoxicity. Many of the crucial enzymes involved in this counterattack are selenoproteins, GPX1 being the most abundant redox selenoprotein.

The GPX Family

GPX1 was the first selenoprotein discovered and is found in the cytosol and mitochondria. The enzyme was first found in red blood cells where it is involved in reducing H_2O_2 produced as a result of hemoglobin metabolism [11]. The active site of

GPX1 is the selenocysteine residue and its substrate is H_2O_2. In this redox reaction, $H_2O_2$is reduced to water when the selenocysteine of glutathione peroxidase reacts with H_2O_2 to form a 2 water molecules and glutathione disulfide [12]. This redox reaction is an essential mechanism for reducing H_2O_2 molecules that can react with superoxide (O_2-) and become the hydroxyl radical (OH) capable of irreversible cell damage. The implication of GPX1 in reducing H_2O_2 in all cells is supported by its implications in various diseases, including cancer, diabetes, and cardiovascular disease.

Due to the aspect of cancer being a disease promoted by genomic instability as a result of oxidative stress, GPX1 polymorphisms and dysfunction have been associated with various cancers. Epidemiological evidence suggests that a low dietary selenium level is associated with an increased risk for some cancers[13,14] . Levels of GPX1 are strongly determined by dietary selenium levels and thus GPX1 has been implicated with various cancers. However, it is not conclusive as to the role of GPX1 in some kinds of cancer. A classic study showed a protective effect for selenium supplementation in prostate cancer[15]. These findings are controversial when the Selenium and Vitamin E Cancer Prevention Trial (SELECT) trial found that there was no significant difference for decreased prostate cancer risk with selenium supplementation [16]. Interestingly, a 2010 European study also found an inverse relationship between selenium status and prostate cancer [17]. In addition, it has been shown that there are decreased levels of GPX1 in pancreatic cancer patients when compared to chronic pancreatitis patients, providing some evidence for a role of GPX1 in pancreatic cancer [18]. More evidence is needed to provide concrete answers as to the role of selenium and GPX1 in prostate cancer. Lung

cancer also has implications with selenium deficiency, including GPX1 expression levels. A recent Polish study showed that a decreased selenium status resulting in decreased GPX1 expression is associated with an increased risk for lung cancer [14].

GPX1 expression may also be involved in metabolic syndromes including type 2-diabetes, and cardiovascular disease. A Japanese cross-sectional study provided evidence for a link between metabolic syndrome and GPX1. It was found that men with a GPX1 polymorphism were more at risk for metabolic syndrome [19]. Murine models suggest that a GPX1 deficiency and increased oxidative stress could result in insulin sensitivity, suggesting that decreased GPX1 expression promotes type 2-diabetes [20]. The expression of GPX1 has also been shown to impact blood pressure. A recent study found that hypertension was increased in GPX1 knockout mice [21].

The remaining GPX family members of interest have a similar role as GPX1, but they are expressed in other tissues and have slightly different functions. GPX2, also called gastrointestinal GPX, is expressed in the epithelial cells of the intestinal tract and reduces H_2O_2 in these cells in a manner similar to that of GPX1 [22]. GPX2 has also been shown to protect against intestinal inflammation and colorectal cancer [23]. GPX3 has a catalytic role that parallels GPX1, but is secreted from certain cells and performs its H_2O_2-eliminating function in the blood plasma[24]. GPX3 has been implicated in preventing platelet dependent blood clot formation [25] as well as having a regulatory role in normal and leukemia stem cell development [26]. GPX4 is also called phospholipid hydroperoxide glutathione peroxidase due to its role of reducing peroxidized phospholipids [27]. This GPX

also has an interesting developmental role that has been shown in murine models. Using a GPX4 knock out mouse model, it was demonstrated that a lack of GPX4 results in disrupted organogenesis resulting in embryonic lethality [28].

The above shows the importance of the GPX selenoproteins in normal cell homeostasis. However, there are other selenoproteins that are important to normal cell function, including the thioredoxins and selenoprotein P.

Thioredoxin Reductase Family

The thioredoxin system is essential to the maintenance of proper cell development and defense against oxidative stress and was recently reviewed [29]. The system includes the reducing agents thioredoxin, thioredoxin reductase, and NADPH. The array of enzymes in this system help to maintain many cellular redox systems and are therefore associated with the pathology of many cellular malfunctions. The thioredoxin reductases are selenoproteins and play a critical part in the redox reaction that characterizes the thioredoxin system. There are three thioredoxin reductases: TRxR1, TRxR2, and TRxR3. These enzymes are similar to the GPX enzymes in that they also carry a SEC residue in their catalytically active site.

TRxR1 is involved in the cytosolic reduction of oxidized thioredoxin by NADPH. It is an important enzyme due to its effect of reducing ubiquinone to its anti-oxidant form, ubiquinone-10, [30] and its involvement in the mitigation of oxidative agents [31]. More

importantly, thioredoxin reducatase has also been found to have an essential role in proper embryogenesis [32] [33]. Interestingly, TRxR1 is also overexpressed in some forms of malignant cancer. One study showed that TRxR1 deficiency reversed the growth of tumors in an animal model, suggesting that TRxR1 is essential for the progression of tumorigensis [34]. TRxR1 has also been implicated in the prevention of neurodegenerative disease [35], as well as in the development of breast cancer [36], lung cancer [37], and may have a role in chemically induced hepatocarcinogenesis [38].

The other two thioredoxin reductases have functions analogous to TRxR1, but occur in different parts of the cell or in different cell types. Mitochondrial thioredoxin reductase is the second thioredoxin of interest. In addition to reducing thioredoxin, it also reduces selenite, alloxan, and 5,5 '-thiobis (2-nitrobenzoic acid) [39]. Reminiscent of TRxR1, TRxR2 is also upregulated in some carcinomas, indicating that the overexpression of this enzyme promotes cancer development [40]. However, TRxR2 has also been shown to be involved in the protection of cells following an ischemic event [41]. Also, TRxR2 has been shown to have a more influence in preventing mitochondrial dysfuction in sepsis than mitochondrial glutathione peroxidase [42]. The third thioredoxin reductase, TRxR3, occurs in the testis and has a key role in mammalian spermatogenesis [43].

Another selenoprotein of interest is selenoprotein P. It is a glycoprotein postulated to be involved in the transport of Se to the tissues and is therefore essential to Se homeostasis.

Selenoprotein P

Selenoprotein P was first identified in the plasma and was the second selenoprotein to be identified after GPX1 [44]. At the time of discovery, its function was unknown, but it opened up the possibility of their being more selenoproteins in mammalian systems. Selenoprotein P is unique in that it contains 10 selenocysteine residues, whereas the other selenoproteins contain only one [45]. Its elusive functions have become more apparent in the past 10 years. Since it is found in the plasma and responds to higher dosages of selenium supplementation, Selenoprotein P acts as a Se nutritional status indicator (along with GPX3)[46]. However, it is better at indicating low selenium status rather than excessive selenium status [47]. The most important role of selenoprotein P identified is its transport of Se from the liver to the tissues to use for selenoprotein production. This feature was identified when the gene encoding selenoprotein P was knocked out it mouse models. It was found that the concentration of Se in the plasma and other tissues was reduced while the concentration of selenium in the liver was increased [48]. However, it has been shown that the brain's supply of Se is regulated by a mechanism separate from the liver to tissue system described above [49].

Selenium and Epigenetics

Several micronutrients, including selenium, are implicated in epigenetic modifications. Epigenetic changes occur when the cytosine/guanine rich regions of DNA known as CpG islands are methylated. Normal methylation is usually regulated and is dispersed evenly

on the DNA, which is important for the many cellular functions that occur within the diverse mosaic of cells that work in concert to maintain homeostasis. Abnormal methylation occurs under at least three conditions: when methylation is not evenly dispersed; when DNA is hypomethylated; when DNA is hypermethylated. These abnormal methylation patterns may lead to genome instability, which is the hallmark of carcinogenesis. In addition to methylation, DNA acetylation and histone modifications are also epigenetic changes that have an impact on genome maintenance. There is a growing body of evidence indicating the role of selenium in these epigenetic alterations and how they impact carcinogenesis and possibly other metabolic disorders.

DNA Methylation

The DNA methylation of certain genes can lead to tumor growth promotion. DNA methylation usually occurs in cytosine-guanine rich (CpG-rich) regions called CpG-islands. These epigenetic modifications occur on CpG islands which are usually promoter associated[50,51]. This process can lead to the silencing of genes, an important aspect of DNA methylation[52]. Many studies have shown that some compounds can inhibit the methylation of these genes by deactivating DNA methyltransferase (DNMT), an enzyme that has the ability to maintain a normal methylation pattern on DNA [53]. The three DNA methyltransferases are DNMT1, DNMT3A, and DNMT3B.

DNMT1 is the most studied of the DNA methyltransferases due to its high concentration relative to the other DNMTs in mammals. One important role of this enzyme is to

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maintain the methylation pattern during the cell cycle [54]. More importantly, some studies have shown that DNMT1 is crucial for embryonic development in mouse models [55]. Another interesting feature of DNMT1 is that it is responsible for the proliferation of human cancer cells. In one study, HTC116 cells underwent apoptosis when the DNMT1 gene was inactivated [56].

The DNMT3s are also required for proper embryonic development [57]. However, there are slight differences in the function of each DNMT3. One study employing a mouse model found that DNMT3A deficient mice are able to thrive to early adulthood but are smaller than usual and the males are rendered infertile, thus allowing for the speculation that DNMT3A is important to spermatogenesis [58]. Mutations in the gene for DNMT3B result in an immunodeficiency disease that can lead to susceptibility of rare diseases [58]. However, double knockout of DNMT3A and DNMT3B is lethal, as shown by mouse models where mouse embryos die during early embryonic development [58].

Selenium and DNMT

Selenium has demonstrated the ability to inhibit the DNMTs. In one study, selenium deficient rats were treated with 5-aza-2'-deoxycytidine, a DNMT inhibitor. It was found that these mice exhibited less abnormal crypt formations than the rats fed a diet containing normal amounts of selenium. In addition, HT-29 cells cultured in the absence of selenium supplementation showed hypermethylated DNA but more DNMT1 expression, supporting the notion that 5-aza-2'-deoxycytidine may protect selenium deficient mice from carcinogen-induced abnormal crypt formation [59].

Histone Modifications

In addition to DNA methylation, histone modifications represent another mechanism for epigenetic changes to occur on the genome. Histones can also be modified by methylation, acetylation, phosphorylation, ubiquitination; however, methylation and acetylation mainly occur during development and have a long lasting consequences on the developing organism[60] These changes occur after translation and can lead to changes in the chromatin structure, which ultimately has an effect on gene expression [61].

An acetyl group neutralizes the charge between the DNA and the histone, which leads to the unraveling of the complex [62]. This unraveling allows for a number of proteins to be recruited on to the DNA, thus activating certain genes. Selenium has been implicated in the acetylation of histones and has been found to affect prostate cancer cells. Increased acetylation of some histones are postulated to be an indicator for genome instability and thus the onset of tumorigenesis. Selenium treatment decreased deacetylation of histone H3, which suggests a possible mechanism for selenium in cancer prevention [63].

Stress-induced senescence

The cells of most organisms do not replicate indefinitely. Although there are some cells that can replicate for an innumerate amount of times, such as cancer cells, and some organisms that are essentially immortal, like Tetrahymena thermophile [64] most cells do not have the capability to forgo cell death after a certain amount of replications. Most cells go through a torrent of damage as they replicate, which are repaired through various

mechanisms as the entire genome replicates. However, as the telomeres on the ends of chromosomes shorten with each replication, there comes a time in the life cycle of a cell when it stops dividing. This permanent arrest of the cell cycle is called senescence. Senescence is the result of a complicated cascade of cellular events[65]. It can be induced via two distinct pathways: after a specific number or replications or from environmental stimuli that affect the DNA and lead to the formation of reactive oxygen species. These two pathways are respectively known as replicative and stress induced senescence.

The role of replicative senescence is a part of the life cycle of most cell types. In 1961, Leonard Hayflick first observed this phenomenon in human embryonic tissue[66]. Before his discovery, it was believed that cells were essentially able to replicate indefinitely[67]. Hayflick observed that cells from embryonic tissues were only capable of dividing a certain number of times after they were sub-cultured[66]. The cells would eventually start dividing slower and ultimately die. Further studies showed that specific markers were able to indicate whether a cell had undergone replicative senescence. The shape of the cell is one such indicator. Cells undergoing replicative senescence may appear stretched and flattened when compared to their non-senescent counterpart[68]. Another marker is the increased activity of the enzyme senescence-associated β-Galactosidase. Ultimately, these markers are the result of the shortening of telomeres that occur with each cell replication. With each round of cell replication, the telomeres, the guanine-rich ends of chromosomal DNA, shorten. This is mainly due to the absence of telomerase activity, the enzyme that maintains the integrity of telomeres, in most cells. Telomerase is highly expressed in immortal cells, such as those of many cancer cells[69].

That stress induced senescence is more intricate than replicative senescence is how it induced. Stress induced senescence occurs when DNA is damaged and cells experience certain forms of stress. It can be induced by a variety of stressors, including reactive oxygen species, various trace minerals when in excess, and radiation. These stressors can lead to DNA double strand breaks, which are sensed by the MRN complex and in turn causes the ATM protein pathway to be activated [70]. The ATM pathway initiates a cascade of downstream protein activation, prompting for the mediation of the DNA damage response. The downstream proteins affected by ATM include the proteins p53 and Chk2, which are phosphorylated by the ATM protein. The Chk2 protein ultimately leads to the p53 protein, which is a tumor suppressor. The p53 protein can then activate the p21 protein pathway that will eventually activate the Rb protein, leading to cellular senescence [71]. This has been established based on evidence from knocking out the genes for ATM and p53 in mice, which inactivates the senescent response.

Stress induced senescence is often the saving grace that leads to the non-proliferation of cancer cells. An overexpression of the RAS oncogene causes normal cells to go into a senescent state [72]. The RAS oncogene is required for the MAPK cascade, which upregulates the proteins $p16^{INK4a}$ and $p19^{ARF}$, the mediators for the RAS oncogenic senescence.

Other features of senescence include senescence associated heterochromatin foci (SAHF). SAHF are structures that appear when cells are in a senescent state. Although they appear in senescent cells, they only appear in stress-induced senescence. The

structure is characterized by a large nucleus and points of DNA, and can be visualized under a fluorescence microscope after being stained with 4',6-diamidino-2-phenylindole (DAPI). Each point of DNA when stained with DAPI is believed to be a single chromosome [73]. This single chromosome has intertwined within it proliferation-promoting genes that consequently lead to the senescent state.

There are a couple of markers of that can be employed to show the SAHF state more completely. These include histone variants and heterochromatin proteins, as well as certain chromatin regulators.

Histones are proteins that condense DNA into the compact form when it is structured as chromosomes. These proteins are very rich in the positively charged amino acids lysine and arginine. Histone H3 is of particular interest because its amino acid sequence is nearly the same in all eukaryotic cells [74], suggesting that histone H3 is an important protein conserved during evolution Histone H3 is also a protein that is involved in the formation of SAHF. When the chromosomes that are the foci condense, which are visible by use of DAPI staining, the position 9 Lysine of histone H3 can be tri-methylated [75]. This tri-methylated state of histone H3 allows for the recruitment of proteins, specifically HP1-γ [76], to heterochromatin

Heterochromatin proteins are involved in a variety of epigenetic activities, including gene transcription and chromatin structural organization [77]. There are three isoforms of this protein found within mammalian cells: HP1-α, HP1-β, and HP1-γ. The structure of these

proteins consists of an amino-terminal chromodomain, a hinge region, and a carboxyl-terminal chromo-shadow domain [77]. They have the ability to associate with chromatin by being recruited to sites where a di-methylated or tri-methylated histone H3 occurs, where they have the function of repressing or activating genes [77]. All three are a part of a DNA damage response that can be induced by ultraviolet induced lesions and DNA double strand breaks. The three proteins are recruited to sites of UV induced lesions where they play a critical role in the repair of these lesions. It has been observed that these proteins are essential in their role in mediating UV induced damage to DNA [75]. The protein HP1-γ is especially important in the formation of SAHF. HP1-γ becomes phosphorylated at the position 93 serine residue in senescent cells. This allows HP1 to become deposited into SAHF and might have the propensity to maintain the structure of these foci.

The chromatin regulators involved in the formation of SAHF are HIRA and ASF1a. HIRA and ASF1a are proteins involved in depositing variants of histone H3 into nucleosomes [78]. These two proteins mediate the formation of the histone variant μH2A, which is another marker for SAHF [78].

The process of SAHF forming begins with the senescence-inducing event. The formation of SAHF forms is a process that involves various steps eventually leading to permanent cell cycle exit. Many of the steps that lead to the formation of these structures is poorly defined, but there exists a general chain of events that typically take place as SAHF form. The first event that takes place is the condensation of the chromosomes in the cell. These are visible by using immunofluorescence when stained with DAPI. The proteins involved

in this event include HIRA and ASF1a [76]. As described above, these proteins mediate the formation of μH2A. One of the events that follow is the tri-methylation of the position 9 lysine on Histone H3 (H3K9Me3). Once histone H3 is methylated, HP1 proteins, specifically HP1γ, can bind to it. These proteins, along with the condensed chromosomes, help to elucidate the SAHF structure.

The above described a better-known scenario for SAHF formation. The functions and formation of SAHF are still being discovered. Some theorize that SAHF actually contribute to the aging process, as some of the structures involved in their formation are observed in the aging cells of animals. One example is the presence of the HIRA protein in the dermal fibroblasts of aging baboons [77].

Senescence is the penultimate fate of a cell. They are unable to undergo the process of mitosis and remain stagnant. After the state of senescence, a cell will die. Senescence is seen as a way to abrogate the proliferation of pre-cancer cells, which is an established early tumorigenesis barrier[79,80] In the Cheng Lab, we have previously shown that selenium compounds at sub-lethal doses can activate a senescence response in non-cancerous but not in cancerous cells, consistent with the view that senescence is an early tumorigenesis barrier and providing a new avenue towards the mechanistic understanding of selenium chemoprevention at the early stage of tumorigenesis[81].

Chapter 2: Materials and Methods

Cell culture

We used MRC-5 cells to explore the possibility that selenium-induced senescence involves SAHF. These cells were originally obtained from Coriell Institute (Camden, NJ). Cells were grown in Eagle's minimum essential medium (Mediatech Inc., Herndon, VA) that included a mixture of 15% fetal bovine serum, essential amino acids, non-essential amino acids, vitamins, and a penicillin/streptomycin solution. They were incubated in 5% CO_2 at 37 °C.

Immunofluorescence Microscopy

After cells were grown to confluency, they were trypsinized and seeded onto a 6-well dish. To determine the senescent response, the cells were incubated with 2 uM methylseleninic acid (MSeA). The anhydrous MSeA obtained from Sigma-Aldrich was dissolved in phosphate-buffered saline. To determine if the selenium-induced senescent response involves ATM and DNA-PK, cells were incubated with KU55933, a DNA-PK inhibitor, and NU7026, an ATM inhibitor, both obtained from Tocris and dissolved in dimethyl sulfoxide (DMSO). Cells treated with MSeA only were incubated for 48 hours

followed by a single wash of PBS, while the cells pretreated with KU55933 or NU7026 were incubated for 24 hours, washed once with PBS, followed by a 48 hour MSeA treatment and subsequently washed once again with PBS. Immunofluorescence experiment was performed immediately following the indicated recovery time. Coverslips were washed 3 times with PBS, fixed in 4% paraformaldehyde in PBS for 10 minutes, washed again 3 times with PBS, permeabilized in 90% methanol for 10 minutes in -20°C, and then washed three times with PBS once more. The coverslips were then incubated in 0.3% Triton-X for 10 minutes, washed 3 times with PBS, and then blocked in 3% BSA for 1 hour, followed by a wash with PBS 3 times. Coverslips were subsequently incubated overnight in -4°C with antibodies against heterochromatin marker proteins H3K9me3 (Abcam, ab8898, 1:1000). Coverslips were washed 5 times with PBS-T (1% Tween-20 in TBS), incubated at room temperature in secondary antibodies (Alexafluor 594 anti-rabbit at 1:500) for 1 hour. After washing the coverslips 5 times with PBS-T, the cells were mounted on slides using a drop of ProLong® Gold antifade reagent containing DAPI (Invitrogen). We used a Zeiss AxioObserver 100 fluorescence microscope to obtain images. Fifteen pictures were taken randomly from each slide. We performed each experiment at least three times.

Quantification of SAHF positive cells

I used a positive/negative approach that was used previously in the Cheng Lab to determine whether a cell was positive for SAHF and to determine colocalization [81,82]. Using the 15 randomly taken pictures, I counted the SAHF positive cells and used this number to calculate the percentage of SAHF positive cells on each slide.

The ascertainment of colocalization was determined using the intensity of the DsRed (the secondary antibody against H3K9me3) and DAPI. I counted the cells that showed colocalization as positive and used this number to calculate the percentage of colocalization on each slide.

Statistics

Statistical analysis was completed using Microsoft® Excel for Mac 2011 Version 14.3.2. To ascertain the statistical significance between the treatments and the control, we used a two-tailed Student's t test ($p<0.05$).

Chapter 3: MSeA induces SAHF in a manner depending on the kinase activity of ATM and DNA-PK

MSeA induces SAHF

Prior research in our lab suggested that treatment of non-cancerous MRC-5 cells with MSeA resulted in SAHF (Fig 1) [83]. This piece of evidence gave an indication that the epigenome may be involved in the regulation of selenium-induced senescence. To test the possibility that selenium-induced senescence involves SAHF formation, we treated MRC-5 normal lung cell fibroblasts with 2µM MSeA for 48 hours followed by a 7-day recovery period. We interpreted data from the third day of recovery. Using immunofluorescence, we observed that distinct punctate foci developed when using DAPI (Fig 2a). Upon further observations using a SAHF marker, H3K9me3, we observed that this marker also revealed single spotted structures indicative of SAHF (Fig 2a). Quantification of the SAHF showed a significantly greater amount of SAHF development when compared to the control (Fig 2c). These results suggest that MSeA treatment does induce SAHF. In addition, we observed an overlapping colocaliation pattern when viewing the merged image of the fluorescence-enriched regions of DAPI-stained DNA and H3K9me3 (Fig 2a). This is also reflected in the quantification, where we found that a significantly greater amount of colocalization occurred in cells treated with MSeA when compared to the control (Fig 4). These results intimate that selenium-induced senescence involved SAHF.

Selenium-induced senescence involves ATM and DNA-PK

DNA-damage induced senescence has been shown to involve the ATM kinase[79,80]. The Cheng lab has established that ATM and DNA-PK are involved in selenium-induced senescence[81]. It was from these that we hypothesized that SAHF, when induced by selenium, involves the ATM and DNA-PK.

To test the possibility that selenium-induced SAHF involves DNA-PK and ATM, we pretreated MRC-5 normal lung cell fibroblasts with KU55933, an ATM kinase inhibitor, and NU7026, a DNA-PK kinase inhibitor. This pretreatment was followed by a 2 μM MSeA treatment for 48 hours followed by a 7-day recovery period. As in the first experiment, we interpreted the data from the third day of recovery.

When we observed the cells pretreated with KU55933, we noticed that localization patters of DAPI and H3K9me3 was similar to that appeared when the cells were treated only with MSeA (Figure 3). However, the MSeA-induced co-localization between SAHF and H3K9me3 was significantly hampered when the cells were pre-treated with KU55933, suggesting that selenium-induced SAHF formation involves the kinase activity of ATM.

The observations obtained from the cells pretreated with NU7026 yielded results distinct from the cells pretreated with KU55933. Interestingly, pretreatment with NU7026 did not produce large SAHF structures as seen with the KU55933 pretreated cells. Instead, a

more dispersed pattern of smaller focus formations developed that is reminiscent of DNA damage foci (Figure 2). Interestingly, H3K9me3 also appeared as small foci, although the degree of co-localization with the condensed DNA region was reduced compared to that treated with MSeA only (Figure 4). Since NU7026 pre-treatment results in a MSeA-induced DNA pattern atypical of SAHF, it is difficult to determine the significance of DNA-PK in selenium-induced SAHF formation. Nonetheless, despite the many overlapping roles of ATM and DNA-PK in the cellular response to DNA damage, ATM and DNA-PK play distinctive roles in MSeA-induced SAHF formation in MRC-5 diploid fibroblasts.
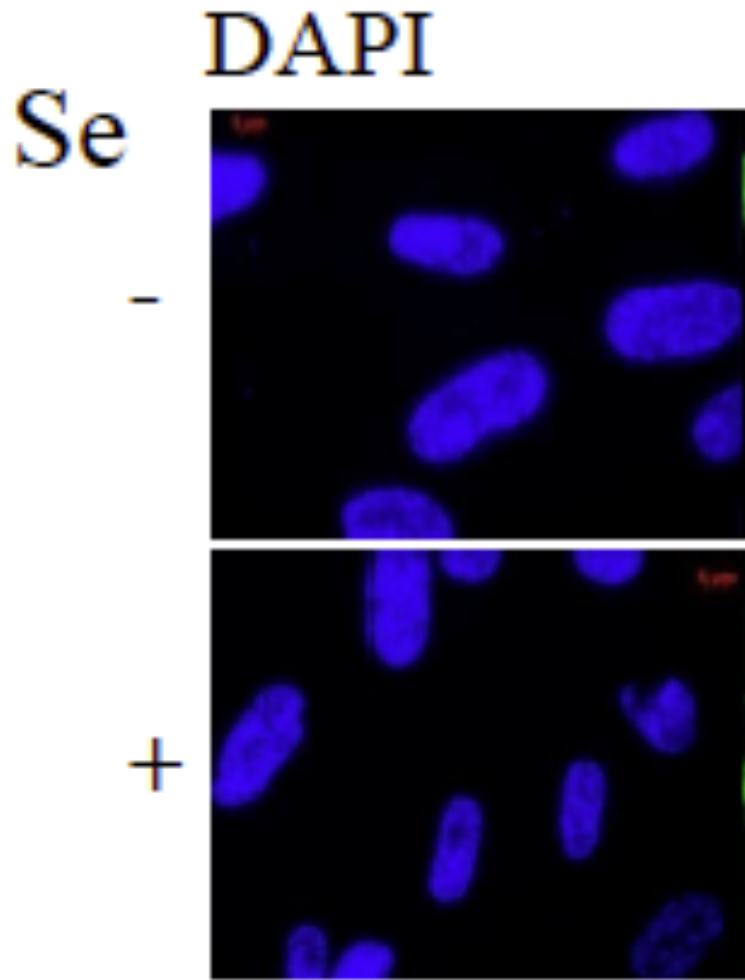
Fig. 1. SAHF-like structures observed in MRC-5 fibroblasts treated with MSeA.
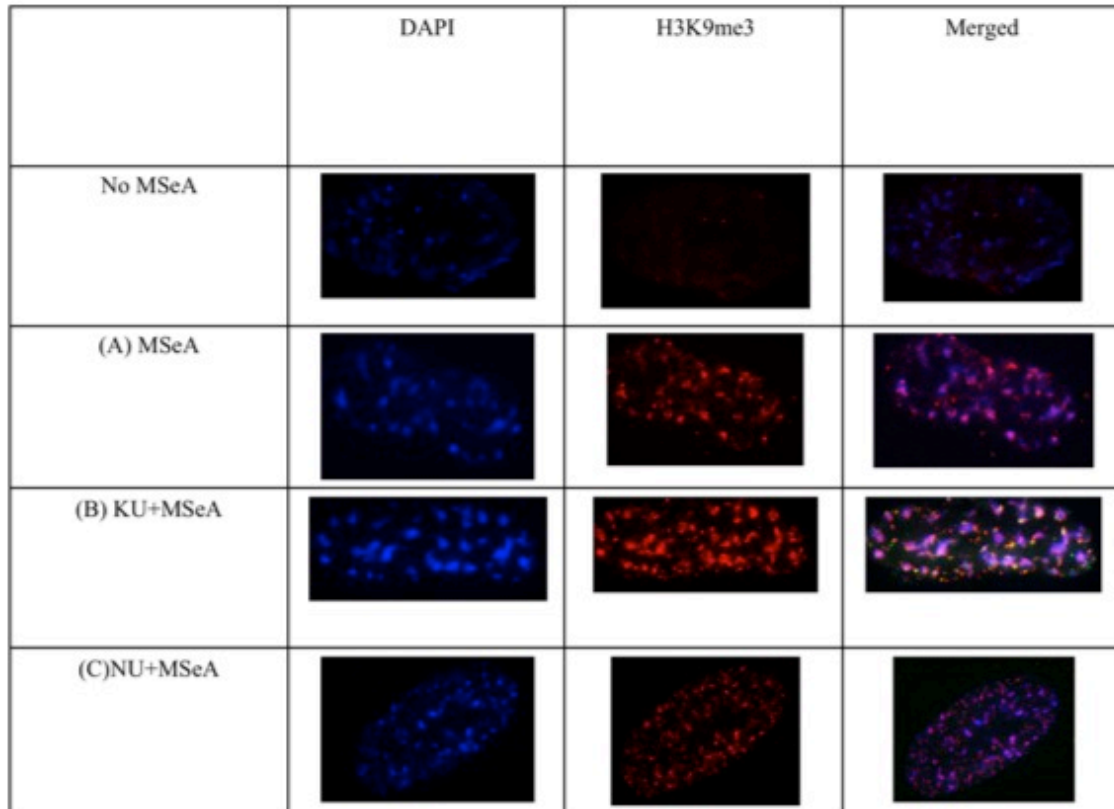
Fig 2. Immunofluorescent analyses of SAHF formation in response to MSeA treatment, KU55933 and MSeA treatment, or NU7026 and MSeA treatment. Cells were (A) treated with MSeA (2 μ M, 48h), (B) pretreated with KU55933 (10 μ M, 24h) and then a treatment of MSeA (2 μ M, 48h), and (C) pretreated with NU7026 (10 μ M, 24h) and then a treatment of MSeA (2 μ M, 48h) was added. All the cells were then grown in a complete medium without the treatment during a 7-day recovery.

Fig. 3. Quantification of SAHF formation in response to MSeA treatment, KU55933 and MSeA treatment, or NU7026 and MSeA treatment. SAHF positive cells with numerous, small foci was determined by counting the SAHF as indicated by DAPI and H3K9me3 markers and were compared to cells that were not treated with MSeA (p<0.05).
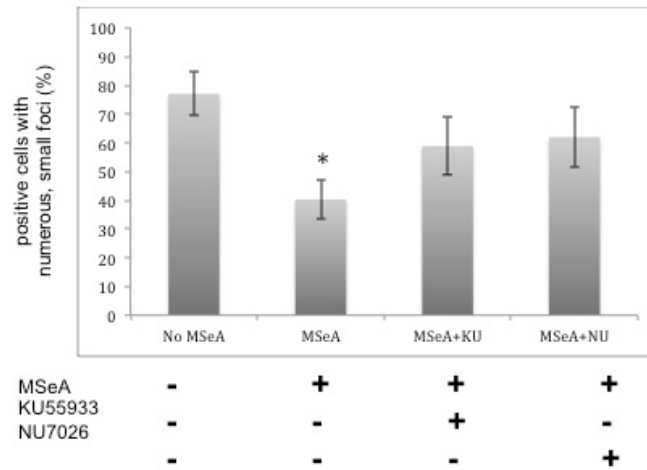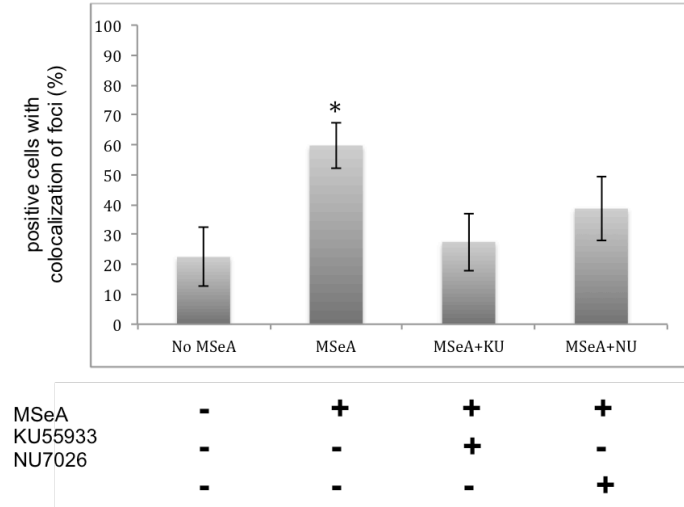

Fig. 4: Quantification of SAHF formation in response to MSeA treatment, KU55933 and MSeA treatment, or NU7026 and MSeA treatment. SAHF positive cells with numerous, small foci was determined by measuring the co-localization pattern of DAPI and H3K9me3 markers and were compared to cells that were not treated with MSeA (p<0.05).
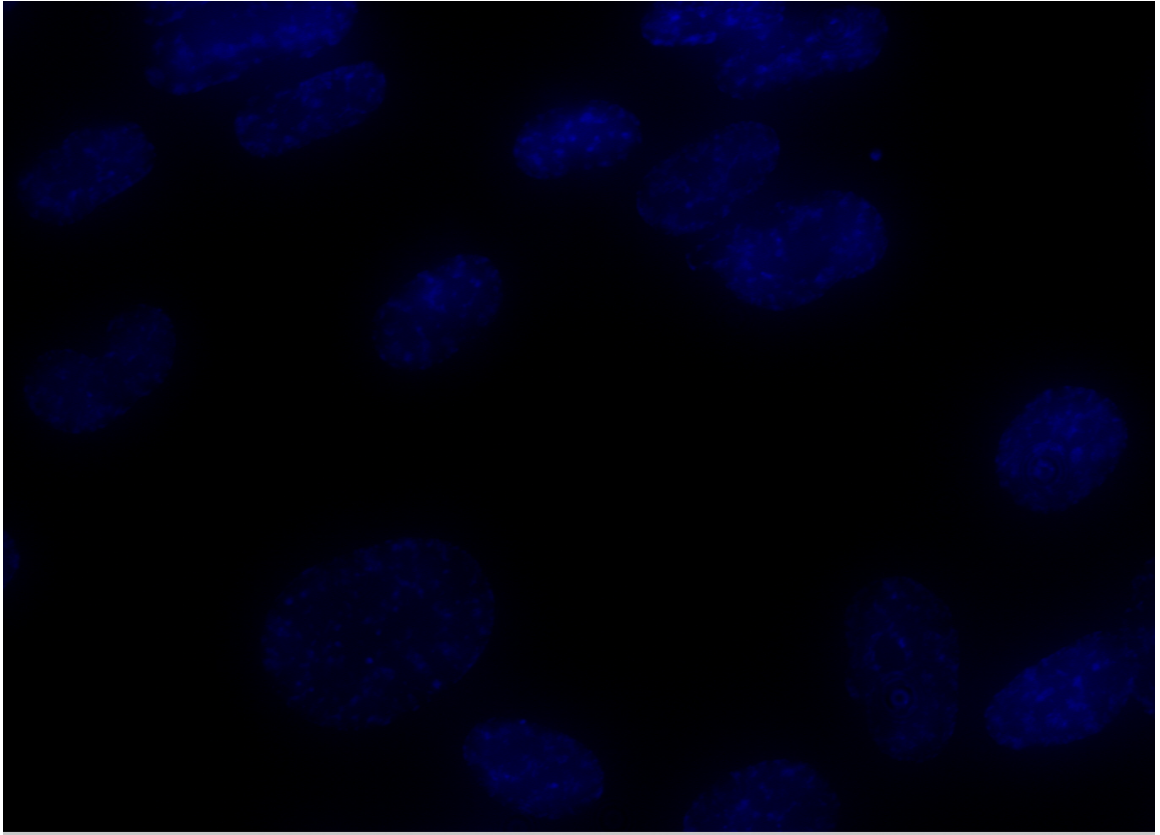
26

Fig. 5: Picture showing field of MRC-5 cells.

Chapter 4: Discussion and Conclusion

Discussion

A cascade of changes occurs when a cell undergoes the process of senescence. SAHF are involved with the maintenance of the senescent state, as well as having involvement with interrupting the recruitment of proliferating promoting genes[84]. Previous research demonstrates that non-cancerous cells treated with selenium compounds at low doses display senescence [8262]. These data led us to speculate a role of SAHF in selenium-induced senescence.

As indicated by the markers that detect SAHF, our results show that these structures do, in fact, after MSeA treatment in MRC-5 fibroblasts. The use of DAPI staining reveals large and distinct formations of DNA enrichment in cells treated with MSeA with or without ATM kinase activity. These structures were more dispersed and widespread in cells pretreated with the DNA-PK kinase inhibitor, NU 7026. One of the markers used to detect SAHF is H3K9me3. The role of H3K9me3 is to repress certain genes in mammalian genomes [85] . Our results show that robust H3K9me3 foci are formed when cells were treated with MSeA with or without DNA-PK kinase inhibition. These data suggest that MSeA treatment has an effect on the methylation of histone H3 independent of DNA-PK kinase activity. However, these robust foci were not seen when cells were pretreated with the ATM inhibitor. As these foci are more reminiscent of DNA damage foci, it is possible that lack of DNA-PK pathway potentiate cells to MSeA-induced DNA

damage. Instead of the less toxic senescence is induced, the same dose of MSeA induces severe DNA damage in the MRC-5 cells with defective DNA-PK kinase activity. Future studies are needed to elucidate the role of DNA-PK and ATM in the DNA damage response during the development of selenium-induced SAHF formation and senescnece.

The colocalization of H3K9me3 with SAHF strongly implicate a senescence state [86]. Cells that were treated with MSeA only and with the DNA-PK inhibitor only showed a robust colocalization pattern (Fig. 1A, 1B). These pieces of evidence further suggest that selenium-induced senescence involves SAHF. Interestingly, pretreatment with the ATM inhibitor also showed a considerable amount of colocalization between the two markers. This suggests that the structures that form when cells are pretreated with the ATM inhibitor also generate SAHF, however, the process may involve other interactions that have yet to be determined.

There are strengths and limitations about this study that are worth mentioning. A strength of this research is that I used reagents that are useful at determining whether or not a structure is SAHF. By using these markers such as DAPI and H3K9me3, I was able to provide evidence for the presence of SAHF. This straightforward approach provides a simple yet complete way to collect data and make conclusions. However, there are some limitations with my experiment. One limitation is that I could have used another marker, heterochromatin protein 1 gamma (HP1 γ). This marker would have yielded more evidence for the structures being SAHF. However, even without this marker, H3K9me3 and DAPI are credible indicators for heterochromatin formation. Another limitation to

my study is that I am unable to understand any mechanistic features of SAHF formation as a result of selenium-induced senescence. Although I observed the structures in my experiments, I am still unclear on what are the mechanisms involved in their creation. More studies are needed to elucidate the pathways that lead to SAHF formation during selenium-induced senescence.

Conclusion

The study of senescence could further the knowledge base of cancer research, thus generating an understanding of this prolific illness and ways to prevent or treat it. From my studies involving selenium, the DNA damage response, and the mechanisms leading to selenium-induced senescence, we can understand how this trace mineral could be a tool to counteract tumorigenesis. Through the study described above, we are closer to understanding the structures involved in creating the senescent state during selenium-induced senescence. This piece of evidence could lead to a better understanding how the structures involved in selenium-induced senescence are able to maintain the senescent state.

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