The Effect of Stress Induced Premature Senescence on the Expression of Heterogeneous Ribonucleoprotein

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THE EFFECT OF STRESS INDUCED PREMATURE SENESCENCE ON THE EXPRESSION OF HETEROGENEOUS RIBONUCLEOPROTEINS.

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A thesis submitted in partial fulfillment of the requirements for the Masters degree.

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Table of Contents

ACKNOWLEDGEMENTS ........................................................................................................ III

ABSTRACT .............................................................................................................................. IV

ABBREVIATIONS: ...................................................................................................................... V

INTRODUCTION ...................................................................................................................... 1

CELLULAR SENESCENCE AND AGING ................................................................................ 1
OXIDATIVE DAMAGE .................................................................................................................. 6
HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEINS (hnRNPs) ........................................... 7
CELL CYCLE REGULATORS AND THE ROLE OF hnRNPs ......................................................... 9

METHODS ................................................................................................................................... 15

CELL CULTURE ........................................................................................................................ 15
IMR-90 Cell Culture ................................................................................................................ 15
Premature senescent cell preparation ..................................................................................... 15

SENESCENCE ASSOCIATED B-GALACTOSIDASE ASSAY (SA-B-GAL) ...................................... 16

CELL CYCLE PROFILE ............................................................................................................. 16

PROTEIN SAMPLE ISOLATION ................................................................................................. 16

WESTERN IMMUNOBLOCKING ............................................................................................... 17

Li-Cor Odyssey detection ........................................................................................................ 17

Enhanced Chemiluminescence detection .............................................................................. 18

IMMUNOCYTOCHEMISTRY ..................................................................................................... 18

ANTIBODIES .......................................................................................................................... 19

RESULTS .................................................................................................................................... 20

DISCUSSION .............................................................................................................................. 31

CONCLUSIONS ....................................................................................................................... 34

WORKS CITED .......................................................................................................................... 38
Acknowledgements

This research was supported by NIH/NCI U54CA137788/U54CA132378.

Foremost, I would like to extend my gratitude to Dr. Karen Hubbard, who gave me the tools to grow as a researcher and a person. I am also thankful for the amazing people who assisted me on my path, such as Dr. Ciara Bagnall, Benelita Tina Elie, Fathema Uddin, and Herman Kucharavy,

I would also like to thank my master’s thesis supervisory committee, Dr. Mark Pezzano and Dr. Tadmiri Venkatesh, for donating their valuable time to guide me through my defense.

In addition, I would like to thank everyone in the City College Biology Department for their support. Special thanks goes to Mrs. Christine Klusko, who goes above and beyond to support every staff member and student that comes her way.
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Chairperson of the Supervisory Committee: Dr. Karen Hubbard

Abstract

The role of heterogeneous nuclear ribonucleoproteins (hnRNP) in cellular senescence is yet to be defined. Cellular senescence is a terminal growth arrest in somatic cells. It is thought to be the consequence of telomeric shortening that acts as a DNA damage signal. Conversely, cells induced into premature senescence (SIPS) by oxidative stress, is independent of telomere attrition. Premature senescence has been proposed to be physiologically relevant as it can be induced by treatment with chemotherapeutic agents. In particular, we are studying the roles of hnRNP A1 and A2 in the maintenance of the senescence phenotype. hnRNPs are a family of RNA binding proteins that play key roles in various metabolic functions of the RNA such as splicing. hnRNP A1 in addition to its biochemical functions in RNA metabolism can bind to mRNAs for transport through the nuclear envelope. Our studies have shown that the protein level of hnRNP A1 is lower in senescent cells
than in young cells. Thus, we have hypothesized that the altered expression of hnRNP A1 potentially modulates gene expression profiles and may maintain the senescent phenotype. We have observed that varying the levels of hnRNP A1 alters the expression levels of p21, p16 and ARF, all cell cycle regulators. To determine if this is the case during stress induced premature senescence (SIPS), IMR-90 fibroblast diploid normal lung cells were treated with a sub-lethal concentration of hydrogen peroxide that has been shown to induce premature senescence. To determine if hnRNPs may play a role in vivo, we measured their expression in rat hippocampal lysates isolated from rats treated with a combination of the chemotherapeutic drugs, doxorubicin and cyclophosphamide. We have shown here that stress induced premature senescence is not modulated in the same hnRNP dependent fashion as replicative senescence and possibly independent of p38MAPK.

**Abreviations:**

- **SASP:** Senescence associated secretory phenotype.
- **SIPS:** Stress Induced pre-mature senescence
- **hnRNP:** Heterogeneous ribonucleoprotein
- **CDKN2A:** Cyclin Dependent Kinase Inhibitor 2A
- **PDL:** Population doubling level
- **ROS:** Reactive oxygen species
- **CICD:** Chemotherapy induced cognitive decline
Introduction

Cellular senescence and aging.

As humans age, we become increasingly prone to developing many different chronic multi system diseases. It is hypothesized that there are basic aging processes that drive all of these different diseases and cellular senescence is believed to be one of the major basic aging processes (Balducci and Ershler 2005). Cellular senescence has been implicated in aging and degenerative age diseases. Such diseases are neurodegeneration, muscular degeneration, osteoporosis, sarcopenia as well as decreased kidney and lung function (Balducci and Ershler 2005, Valko, Leibfritz et al. 2007).

Cellular senescence is the halt of replication and the cell cycle in somatic cells. Once a cell becomes senescent it can remain active for prolonged periods of time, however, they cease to respond to growth factors. Replicative senescence is the result of telomeric shortening and the resulting DNA damage. In contrast, premature senescent cells can be induced via various stress stimuli that can be accomplished by the exposure to producers of reactive oxygen species (ROS) such as hydrogen peroxide or doxorubicin (Kim, Kim et al. 2006).

Hayflick and Moorhead described replicative senescence as the point at which cells reach their terminal cell division and undergo morphological changes. This morphology is irreversible, and the cells cease to respond to growth factors, although they may maintain metabolic activity for a long time (Hayflick 1976).
The lifespan of the cells in culture is dependent on the population doubling (PD) and not the time the cells have been cultured. This phenomenon has been coined the Hayflick limit. Hayflick's work has correlated with other studies that have shown that as cells age and enter senescence, their telomeres are significantly shortened (Hayflick 1976, Rizvi, Raza et al. 2014). Telomeres act as a buffer zone that protects the chromosomes from degradation and fusion to other chromosomes, after multiple replications. Interestingly enough, many cancer cells do not exhibit this decrease in telomere length, which has been attributed to a highly active enzyme called telomerase (Shay and Wright 2011, Falandry, Bonnefoy et al. 2014).

Senescent cell accumulation has been observed in almost all vertebrates as the organism ages (Falandry, Bonnefoy et al. 2014). Interestingly, age related pathologies also correlate with a local increase of senescent cells. Some examples are increased senescence associated markers present in endothelial cells at the base of atherosclerotic plaque and senescent chondrocytes in arthritic joints (Coppe, Desprez et al. 2010). Accumulation is also present in degenerative diseases and benign prostatic hyperplasia (Castro, Giri et al. 2003).

Senescence is seen as a response to stressors that result in a bipartite phenotype. The two distinct models of this response are the irreversible growth arrest and the presence of senescence associated secretory phenotype (SASP) (Balducci and Ershler 2005). The irreversible cell growth arrest is the factor that brings positive protections at young age, such as prevention of malignant cancer development and tissue remodeling. The SASP on the other hand can actually become maladaptive by driving the aging process, which can modulate chronic age
related diseases (Acosta, O’Loghlen et al. 2008). The stressors that can induce these two phenotypes are vast and include genomic damage, metabolic stress and organelle stress (Campisi 2005). Despite there being no specific cellular senescence marker and we rely on a number of different marker together such as increased ROS production, SA-β-gal, increased p16ink4a, and SASP, which are discussed in more detail later on in this paper.

Most of the common chronic age related pathologies have an increased amount of inflammation and are thought to be the result of senescent cell accumulation (Cevenini, Caruso et al. 2010, Freund, Orjalo et al. 2010). The senescent associated secretory phenotype is a collection of forty different chemokines, cytokines and proteases that are the main modulators of inflammation (Coppe, Desprez et al. 2010). In addition, HMGB1, a protein that is usually only found in the nucleus is excreted by senescent cells. In the nucleus, HMGB1 organizes DNA and prepares it for transcription but in SASP and immune cells it can act as a cytokine mediator of inflammation (Wang, Bloom et al. 1999).

Cells possessing SASP have potent paracrine activity, which can influence the differentiation and organization of neighboring cells. This was shown in 3D mammary epithelial organoid β-casein producing culture. When senescent cells were introduced into the culture with normal mammary cells, cells lost their previously observed alveoli structure and the production of the β-casein significantly decreased (Parrinello, Coppe et al. 2005).

Senescent cells also have the ability to induce proliferation and angiogenesis in preneoplastic cells in culture (Krtolica, Parrinello et al. 2001). This has also been
observed *in vivo* with mice that were injected with preneoplastic cells do not develop a tumor (Krtolica, Parrinello et al. 2001). However, once mice were injected with senescent cells the preneoplastic cells transformed into large vascularized tumors.

Interestingly, senescent cells are important for wound healing. The creation of a wound senescent cells increase locally for a short period of time and over time die resulting in undetectable number, usually after 8 days (Laberge, Adler et al. 2013). The cells are primarily endothelial and fibroblast. Ganciclovir is a drug that can kill non-dividing senescent cells by damaging mitochondrial DNA via apoptosis (Laberge, Adler et al. 2013). When mice were treated with Ganciclovir, the wound healing response was drastically hindered Demaria, Ohtani et al. (2014) identified a growth factor secreted by senescent cells called PDGF-A that was present during wound healing, and most importantly this growth factor was able to rescue the wound healing response in Ganciclovir treated cells.

Given this a paradox arises: why does wound healing become worse with age when the amount of senescent cells increases with age (Keyes, Liu et al. 2016)? Interestingly, with age the innate antioxidant capabilities of cells decreases resulting in oxidative stress and therefore induces a chronic presence of senescent cells, instead of a transient presence as we see in young wound healing (Velarde, Flynn et al. 2012).

Senescence also proves to be relevant in post chemotherapy treatment patient outcomes. Chemotherapeutic regiments with ionizing radiation (IR), doxorubicin and phosphomide, to name a few, are all genotoxic and will result in an
increased amount of cells positive for senescent markers. (Demaria, Ohtani et al. 2014).

In a longitudinal study that tracked childhood cancer survivor's health records, has shown that once these patients reach the age of forty five they have a statistically significant prevalence of age related degenerative diseases usually seen in much older populations (Hudson, Ness et al. 2013). These diseases included musculoskeletal, reproductive, hepatic, renal and neurocognitive dysfunctions, to name a few. We have also shown that a chemotherapy cocktail of doxorubicin and cyclophosphamide cause neurodegenerative phenotype. Female rats treated with this regiment exhibited a significantly impaired working and special memory (Salas-Ramirez, Bagnall et al. 2015).

Baker, Wijshake et al. (2011) has shown that transgenic mice lacking p16Ink4a-positive senescent cells have an increased resistance to previously mentioned age associated disorders. Senescent cells have the ability to suppress tumor proliferation (Burton and Krizhanovsky 2014). An example are a hyperplastic legions such as melanocytic nevi are melanocytes that have undergone senescence in-vivo due to activation of the BRAF oncogene (Joselow, Lynn et al. 2017).

Sub lethal concentration of oxidative stress causing agents can be used to induce a SIPS phenotype (Frippiat, Dewelle et al. 2002, Wang, Wei et al. 2013), and therefore possibly a useful model to study replicative senescence. Both SIPS cells and replicative senescent cells exhibit a unique morphological state; they become large and flat, and have an increase SA ß-galactosidase activity (Dimri, Lee et al. 1995, Krishna, Sperker et al. 1999). SIPS fibroblast cells produce a high amount of
reactive oxygen species like H$_2$O$_2$ and contain 30% more 8-oxo-2-deoxyguanosine DNA (Fleming, Muller et al. 2011). Furthermore they accumulate oxidative DNA damage based on the increased amount of free guanosine bases (Kino, Hirao-Suzuki et al. 2017).

**Oxidative damage**

It has been proposed that normal aging is the result of accumulation of oxidative stress from reactive oxygen species (ROS) (Squier 2001). ROS are produced during normal cellular processes and metabolism. ROS production can be the result of exposure to sub-lethal amounts of heat, ultraviolet light, chemotherapeutic drugs, radiation and even phagocytic leukocytes (Robinson 2008). The accumulation of tissue damage, specifically due to ROS, is considered to be the root cause of the aging process. As previously mentioned, DNA is negatively impacted by ROS resulting in many different lesions such as base modifications, strand breaks and cross-linking. Similar to what occurs in tissue culture, the accumulation of of 8-oxo-2-deoxyguanosine DNA in aging individuals’ increases in part due to a decreased ability to repair the damage (Kaneko, Tahara et al. 2003). It is worth mentioning that ROS does not only affect DNA, but also causes oxidative damage to proteins and lipids which result in pathologies such as cardiovascular disease (Panth, Paudel et al. 2016).

ROS containing superoxide radical anion, O$_2^-$, and hydroxyl radical, OH', are produced during normal metabolic activity in the mitochondria of living cells (Cadenas and Davies 2000). During cellular respiration Cytochromes P450 readily convert diatomic oxygen to O$_2^-$, which is then converted to hydrogen peroxide
The amount of hydrogen peroxide is generally kept at low levels, and is efficiently converted to non-toxic compounds like water. However, certain conditions such as the presence of transition metals, resulting in the Fenton reaction and the exposure to UV radiation causing photolysis of hydrogen peroxide can increase the amount of hydroxyl radicals significantly (Panth, Paudel et al. 2016). The resulting DNA damage accumulation in the cells may induce them to become prematurely senescent independent of telomere shortening through intracellular signaling molecules regulating kinase-driven pathways (Chang, Broude et al. 1999). Also, naturally produced one-electron free radicals can react with certain parts of the DNA, often guanine, and cause errors and modifications (Panth, Paudel et al. 2016). It has also been shown that levels of mitochondria derived hydrogen peroxide are increased in aging fly models (Sohal and Sohal 1991).

**Heterogeneous nuclear ribonucleoproteins (hnRNPs)**

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a vast family of RNA binding proteins (RBPs) that are the most abundant of all eukaryotic nuclear proteins and ranging in size from 34 to 120 kDA (Pinol-Roma, Choi et al. 1988). Post-transcriptional modifications directed by RNA binding proteins (RBPs) play a key role in the maturation of nascent RNA. hnRNPs process the hnRNA through capping, polyadenilation, splicing, and shuttling the pre-mRNA between the nucleus and cytoplasm (Pinol-Roma, Choi et al. 1988, Krecic and Swanson 1999). Despite the diversity of hnRNPs they contain many highly conserved RNA recognition motifs (RRM). These motifs, such as the UP1 region in hnRNP A1, play a crucial role in RNA
binding that is responsible for telomere regulation and splicing (Xu, Jokhan et al. 1997).

Currently over twenty different hnRNPs are known to exist in the nucleus, including hnRNP A1, A2/B1, C1/C2 and K (Krecic and Swanson 1999). In eukaryotic cells, hnRNPs are part of the RNA polymerase II machinery and the nascent RNA binds them immediately (McCloskey, Taniguchi et al. 2012). This RNA – hnRNP complex remains bound through the maturation of the RNA and up to the point the RNA is transported out of the nucleus (Dreyfuss, Matunis et al. 1993). hnRNP A1, A2/B1 and K are known to shuttle between the nucleus and the cytoplasm, and are involved in different cell processes. hnRNP A1 and A2/B1 in particular, are thought to play a key role in cell proliferation and survival (He, Brown et al. 2005).

The hnRNP A2/B1 protein plays an important role in the correct localization of transcripts that contain the A2 response element or A2RE (Shan, Moran-Jones et al. 2000). This interaction modulates the correct localization of transcripts that contain the A2RE in oligodendrocytic and neuronal mRNA (Shan, Munro et al. 2003). Increased levels of intracellular amount of Ca\(^{2+}\) have been shown to promote the A2RE interaction with hnRNP A2/B1 (Muslimov, Tuzhilin et al. 2014).

Interestingly, it has been shown that stress induced senescence and oxidative stress increases the level calcium present in the cell derived from the ER through the phospholipase C, IP3, and IP3R pathway (Borodkina, Shatrova et al. 2016). The increase in calcium ions may affect neuronal activity by altering the hnRNP A2/B1 interaction with A2RE. It has been suggested that oxidative stress may lead to various neurodegenerative pathologies (Valko, Leibfritz et al. 2007).
Cell Cycle regulators and the role of hnRNPs

During normal cellular growth and division, the cell goes through four key regulatory stages: preparation for DNA synthesis and checking for errors, G1; DNA synthesis, S; the final check of the DNA for damage and preparation for mitosis, G2; and finally the completion of the cell cycle resulting in production of two daughter cells via mitosis (Selvarajah, Elia et al. 2015). With necessary growth factors available to the cell, it may continue to undergo mitosis until the terminal division or exit the cell cycle and enter a non-dividing state called G0 (Marcotte and Wang 2002). Cells can also be in a sub G0 pseudo stage where DNA is becoming fragmented due to the onset of apoptosis (Newbold, Martin et al. 2014).

In contrast to actively dividing cells, senescent cells do not enter the S phase in order to prevent possible faulty or incomplete genetic information from replicating, usually arresting in G1 or G2 after reaching their terminal division (Mao, Ke et al. 2012). In addition, senescent cells become phenotypically different from their younger counterparts. Senescent cells become larger in size with a visibly flat and spread-out appearance (Marcotte and Wang 2002). During this transition, a host of molecular changes occur, many of which are yet to be elucidated. Some key molecular changes can be seen in the cell cycle regulatory proteins such as cyclins and cyclin dependent kinases (cdK's) (Capparelli, Chiavarina et al. 2012). The levels of these cells cycle regulators, specifically those present at G1 and G2, are severely down regulated or nonexistent in senescent cells (Zhu et al., 2002).

This is most likely attributed to the over expression of cdK inhibitors in senescent cells, such as p21, p14, p16 and p53 (Capparelli et al., 2012). Senescent
cells have an increased expression of various lysozomal enzymes, particularly a pH neutral senesce associated biomarker β-galactosidase (SAb-Gal) (Krishna et al., 1999). This marker is expressed by senescent and prematurely senescent cells, but is not present in pre-senescent quiescent cells. SAb-Gal assay can therefore be used to verify the presence of senescent cells (Dimri, Lee et al. 1995). When normal human diploid fibroblast cells are exposed to oxidative stress they begin to exhibit a stress induced premature senescence (SIPS) (Chen, Bartholomew et al. 1998).

One of the most important cell cycle regulators that controls tumor suppression is p53 (Vogelstein, Lane et al. 2000). Normal healthy cells maintain low levels of p53 through negative regulation of MDM2, which forms a complex with p53 at the transcription activation domain. When the MDM2-p53 complex forms, p53 loses its transcription factor function while simultaneously being marked for degradation (Vogelstein, Lane et al. 2000). When cells experience stressors such as DNA damage via the activation of cell cycle inhibitors, the MDM2-p53 complex is severed. Now free p53 is not being actively degraded and its transcription activation site is available (Wei, Hemmer et al. 2001). The increase in p53 levels and activity causes multiple different phenotypes such as cell cycle arrest and apoptosis. p53 can cause the upregulation of both upstream and downstream cell cycle and survival signals.

Figure 1. Summary of p53-p21 and p16-Rb stress induced senescence pathways in mammalian fibroblasts models.
The full mechanism of this regulation is yet to be understood, but it is known to regulate the biological response and the fate of the cell.

Both p14\textsuperscript{ARF} and p16\textsuperscript{INK4a} play key roles in modulating replicative senescent phenotype and are key cell cycle regulators (Sharpless and DePinho 1999). As seen in Figure 1, p14\textsuperscript{ARF} works by inhibiting MDMD2, resulting in an activation of p53, which can then induce p21 expression, and therefore inactivating CDK complexes responsible for transcription of genes active during G1 stage of the cell cycle (O’Connor 1997). Alternatively, cells that exhibit phosphorylation of cdc2 mediated cellular which result in a G2 cell cycle arrest (Poon, Jiang et al. 1996).

When hnRNP A1 and A2 were overexpressed, this resulted in a change in splicing (Zhu, Xu et al. 2002). The CDKN2A gene contains two mRNA targets, p14\textsuperscript{ARF} and p16\textsuperscript{INK4a}, that are important for senescence. The overexpression of hnRNP A1 and A2 promoted the use of distal splice sites and loss of certain exons (Mayeda, Helfman et al. 1993). This resulted in the production of more p14\textsuperscript{ARF} splice variants of the CDKN2A gene. The over expression of both hnRNP A1 and A2 increased the ratio of p14\textsuperscript{ARF} to p16\textsuperscript{INK4a}. These observations indicate that hnRNP A1 and A2 binding might be moderating the expression of the above mentioned cell cycle regulators. During replicative senescence hnRNP A1 and A2 and C1/C2 protein expression decreases in normal human fibroblast cells and an increased amount of p16\textsuperscript{INK4a} has been observed (Hubbard, Dhanaraj et al. 1995, Wang, Wu et al. 2001).

In addition, we have shown that the ratio of p14\textsuperscript{ARF} to p16\textsuperscript{INK4a} mRNA is directly related to the expression of hnRNP A1 and A2 (Zhu, Xu et al. 2002). Given that senescent cells exhibit lower levels of hnRNP A1 and A2 (Hubbard, Dhanaraj et
al. 1995), it is thought that p16\textsuperscript{INK4a} levels will be higher than p14\textsuperscript{ARF}. Therefore, it was hypothesized that hnRNP A1 and A2 were able to modulate the gene expression through alternative splicing of cell cycle regulators that maintain the replicative senescence phenotype.

In addition to the decreased expression of hnRNPs in senescent fibroblast cells, we have shown that hnRNP A1 and A2 accumulates in the cytoplasm of senescent (Shimada, Rios et al. 2009, Capparelli, Chiavarina et al. 2012). In contrast, hnRNP A1 and A2 are localized in the nucleus in proliferative cells and G0 growth arrested cells. It has been suggested that accumulation in the cytoplasm is the result of hnNRPs being phosphorylated (van der Houven van Oordt, Diaz-Meco et al. 2000). Senescent cells have been shown to have an increase in p-38 MAPK expression which interacts with hnRNP A1 (Shimada, Rios et al. 2009, Ziaei, Shimada et al. 2012).

Inhibition of p-38 MAPK activity resulted in an increase in hnRNP A1 levels, the same however was not observed for hnRNP A2. The accumulation in the cytoplasm of these crucial RBPs may in part impact the stability of various mRNAs (Shimada, Rios et al. 2009, Ziaei, Shimada et al. 2012). It has also been shown that when hnRNP A1 and A2 were knocked out via siRNA, this induced the senescent phenotype (increased F-actin and larger cell morphology). Therefore, it was proposed that hnRNP A1 is regulated in part via p-38 MAPK phosphorylation to partially maintain the senescent phenotype (Hubbard, Dhanaraj et al. 1995, Zhu, Xu et al. 2002, Shimada, Rios et al. 2009).
Another major RBP, hnRNP-K, binds to cysteine rich regions, plays a key role in p53 DNA damage response and cellular arrest in the G1/G2 stages. hnRNP K is inducted by the inhibition of the Ubiquitin E3 ligase MDM2 (or HDM2). It has been shown that hnRNP K siRNA results in a decreased expression of p53 target genes and a resulting defective DNA damage response (Moumen, Masterson et al. 2005). DNA damage via UV and IR radiation has shown to increase the levels of hnRNP K in fibroblast cells (Moumen, Masterson et al. 2005). It was also shown that levels of hnRNP-K are low in normal fibroblast cells and are increased in tumorigenic cells (Gao, Yu et al. 2013). This RNA binding protein may have a role in modulating premature senescence and senescent like phenotypes in a novel way different from hnRNP A1 and A2.

Since we have shown that the levels of some hnRNPs in replicative senescent cells are diminished it is of interest to know whether the same trend holds true with pre-mature senescent cells. This is relevant because some chemotherapeutic agents such as doxorubicin induce oxidative stress to control tumor proliferation (Kim, Kim et al. 2006). Others and we have shown that chemotherapeutics drugs such as doxorubicin and cyclophosphamide induce cognitive dysfunction or CICD (Salas-Ramirez, Bagnall et al. 2015). It has been proposed that CICD is the result of oxidative stress induced by chemotherapeutic agents (Ahles and Root 2018, Moruno-Manchon, Uzor et al. 2018). Therefore, we have examined hnRNP levels in pre-mature senescent cells and in the hippocampus brain region of rats treated with doxorubicin and cyclophosphamide chemotherapy cocktail. Given these drugs are meant to halt the progression of tumorigenesis partly through oxidative stress, it is
possible that hnRNP A1, A2 and K may modulate the induction of premature senescence in cancer cells.
Methods

Cell culture

**IMR-90 Cell Culture**

Early passage IMR 90 human diploid fibroblast cells were sub-cultured in complete media consisting of Dulbecco’s Modification of Eagles Medium (DMEM) and F12 in a 1:1 mixture (Gibco). The media was supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin streptomycin (Gibco).

The cultures were incubated and maintained at 37ºC, in 5% CO₂ with the media being changed three times per week. When the cells became above 80% confluent they would be harvested using 0.25% trypsin-EDTA (Life Technologies) and seeded on polystyrene tissue treated culture plates (Fisher Scientific) at a density of 1x10⁶ cells per 100mm².

**Premature senescent cell preparation**

A stock solution of ACS 30% hydrogen peroxide (Fisher Scientific) was diluted to an initial stock dilution of 1M using sterile deionized water. Working solutions of 150uM hydrogen peroxide were prepared using growth media immediately before treatment of cells under a biological hood and sterile conditions. Cells were incubated with fresh media and 150uM of hydrogen peroxide for 2 hours and then the media was changed. Cells were then treated again the next day to ensure cell cycle arrest. Cells were then allowed to recover for 48 hours and the cell morphology and population growth was observed.
**Senescence Associated β -Galactosidase assay (SA- β -Gal)**

The culture media was removed from IMR 90 cell cultures. The cultures were washed with PBS, fixed with 4% paraformaldehyde for 10 minutes at room temperature and washed three times in PBS to remove all fixative. Cells were then incubated with a staining solution (0.2M citric acid sodium phosphate buffer (pH 6), 100 mM potassium ferrocyanide, 100mM potassium ferricyanide, 5M NaCl, 1M mgCl₂ with 20 mg/mL of X-gal dissolved in dimethylformamide [DMF]) for 24 hours at 37ºC without CO₂. Staining observed confirms induction of senescence. Ten fields were counted to quantify the senescent positive cells.

**Cell Cycle profile**

The cell cycle profile of IMR 90 normal human fibroblast cells cultured in complete DMEM medium containing hydrogen peroxide for 4 hours was analyzed using a flow cytometer. Total DNA was stained using FxCycle Violet dye (Invitrogen) and the DNA staining intensity was detected with a DB LSR II flow cytometer excited with a 405 nm laser. Data analyses were conducted using BD FACSDiva 8.0.2.

**Protein sample isolation**

Culture media was aspirated from IMR 90 cultures, and then the cells were washed with phosphate buffered saline (PBS pH 7.4). Protein lysates were also isolated from the hippocampus of rats treated with either chemotherapy or saline. The description of how the rats were treated is described in detail in Salas-Ramirez, Bagnall et al. (2015). Then ice cold RIPA Lysis buffer (Thermo Scientific) was added to the plate. Cells were harvested using a cell scraper and transferred into a micro
centrifuge tube. Sample lysates were homogenized using a 25-gauge needle and then centrifuged at 14,000 x g for 20 minutes at 4ºC.

**Western Immunoblotting**

Protein lysates were mixed with a reducing Laemelli sample buffer containing β-mercaptoethanol and subsequently heated to 95ºC for 5 minutes. Protein samples were separated on a 10% polyacrylamide gel using a Mini-Protean II system (BioRad) and then were transferred onto an Immobilon-FL PVDF Membrane 0.45 µm (Millipore) at 100 volts for 45 minutes. Following the transfer the membranes were analyzed using either a Li-Cor Odyssey scanner or ECL film detection.

**Li-Cor Odyssey detection**

Membranes were initially blocked for 1 hour in Odyssey tris buffered saline (TBS) blocking buffer (Li-Cor) according to the manufacturers protocol. Then membranes were incubated in the primary antibody overnight at 4ºC and then washed three times in TBS-T. Primary antibodies were detected using anti-mouse IR Dye680 or anti-rabbit IR Dye800 (Li-Cor) conjugated antibodies. The membranes were incubated in the secondary antibody solution as per manufacturer instructions for 1 hour at room temperature. Following the incubation, membranes were washed as described above and detected using the Odyssey Imaging System (Li-Cor) by quantifying the Integrated Intensities (II K counts). A student t-test was used to analyze the data.
Enhanced Chemiluminescence detection

The membranes initially for 1 hour in 5% skim milk made diluted in TBS-T for 1 hour at room temperature. Then membranes were incubated in the primary antibody overnight at 4ºC. Following primary incubation, membranes were washed three times and incubated for 1 hour in a secondary horseradish peroxidase conjugated anti-mouse and anti-rabbit antibodies (Thermo Fisher). Membranes were washed three times following secondary incubation. Signals were visualized using enhanced chemiluminescence through the use of the SuperSignal™ West Pico PLUS Substrate (Thermo Scientific). The film was scanned and quantification relative to β-actin was preformed using ImageJ software and a student t-test was used to analyze the data.

Immunocytochemistry

Cells were seeded onto chambered cell culture slides (Corning) and grown to semi-confluency. The cells were then washed with sterile room temperature phosphate buffered saline (PBS pH 7.4) for 5 minutes to remove culture media. Cells were then fixed with freshly prepared 4% paraformaldehyde for 10 minutes. The fixative was removed by washing three times using PBS and then the cells were incubated in 0.5% Triton X-100 in PBS at RT for 5 minutes to permeabilize the membranes. Slides were washed once again and incubated in 5% Bovine Serum Albumin [BSA] (Fisher Scientific). Then the cells were incubated overnight at 4ºC in the primary antibody solution using BSA at a working dilution of 1:200 as per manufacturer instructions. Slides were then washed as described previously and incubated in fluorochrome-conjugated secondary antibody solution using 1% BSA at
a 1:1000 dilution for 1 hour at room temperature in the dark. Cells were then washed again and mounted using ProLong™ Gold Antifade with DAPI (Life) to stain the nucleus. Imaging was performed using ZEN 2012 imaging software with a Zeiss LS710 confocal microscope.

**Antibodies**

The antibodies used in these studies were, Monoclonal mouse antibody 4B10 (1:10,000) was used to detect hnRNPA1/A1; polyclonal rabbit antibody _ (1:10,000) was used to detect hnRNP A2/B1; polyclonal mouse antibody _ (1:10,000) was used to detect hnRNP K; Monoclonal mouse antibody BA3R (1:5,000) was used to detect Beta-Actin.
**Results**

Normal diploid cells have a finite number of cellular divisions. Once a cell reaches its terminal doubling time, it will enter senescence at which point all cell division is arrested but the cell maintain its metabolic activity. Through DNA damage it is possible to induce a stress induced premature senescence (SIPS) phenotype in young cells. In the following set of studies, we used 150 uM hydrogen peroxide to induce SIPS *in vitro* (Frippiat, Dewelle et al. 2002).

To confirm the induction of stress induced premature senescence associated β-Galactosidase (SA-β-Gal) in culture we incubated the culture in X-Gal solution (5-bromo-4-chloro-3-indolyl-β-d-galactoside). This assay produces a blue precipitant in senescent and SIPS cells after completion of the reaction due to the increased lysosomal β galactosidase production (Campisi and Warner 2001). Figure 2 below shows (A) young, PDL 19 and (B) SIPS (pre-mature senescent), PDL 19 IMR-90 normal human fibroblast cells after the β-Gal assay. The premature senescent cells show an increase in SA- β -Gal activity as well as a change in morphology. The premature senescent cells are flatter and larger, similar to replicative senescent cells when compared to young cells.
Figure 2. Senescent and SIPS cells exhibit increased β-galactosidase activity. β-galactosidase assay showing young control H2O2, treated SIPS cells, and replicative senescent cells. Panel A shows young IMR-90 at 19 PDL after β-gal assay with almost absent staining. Panel B shows premature senescent cells at 19 PDL after treatment with hydrogen peroxide and assayed with β-Gal. Panel C, shows replicative senescent cells at 65 PDL after β-gal assay. Increased blue staining shows SA-β-Galactosidase activity at neutral pH and was conducted as discussed in materials and methods section.

Next, the effect of hydrogen peroxide treatment on cell cycle arrest was analyzed using single color FACS (Figure 3). This assay allows for analysis of DNA present in the cell using FxCycle Violet dye. Apoptotic cells that have fragmented DNA will have less signal and show a shift to the left. Cells in G0/G1 and G2 appear as peaks on the 1D FACS with cells in S phase seen in the middle. Cells in G0/1 and G2 can be seen as separate populations because before S phase the cell content is 2N.
and after doubling of DNA becomes 4N. This difference in DNA content can be distinguished by the varied intensity of the dye.

The cells treated with hydrogen peroxide had a much larger percentage of cells arrested in G2, 60% compared to 22% in control, and a decrease in cells in G0/G1 step, 21% compared to 66% in control. In addition a new population of sub-G0 apoptotic cells was observed in the premature senescent cells, approximately 8%.

**Figure 3. SIPS cells arrest primarily in G2** (A) Single color FACS analysis of young and premature senescent cells using Fx-Cycle violet stain (Life). Flow cytometry histogram of premature senescent cells increased G2 (60%) and Sub G1 (8%) population accumulation.
Figure 4. p21 expression is increased in senescent and SIPS fibroblast cells. The western blot analysis of p21 (21 kDa) compared to the loading control beta-Actin Loading Control [BA3R] (42 kDa).

Replicative senescent cells are known to have increased levels of cell cycle regulators such as p21 and p14ARF, which are also cell cycle inhibitors (Sharpless and DePinho 1999). Premature senescent cells have also been reported to increase levels of p21. Figure 4 shows premature senescent cells to have an increase in p21. The increased expression of p21 may be the reason for the growth arrest observed in Figure 3.

Previous studies have shown that replicative senescent cells show a change of sub cellular localization of hnRNP A1 to the cytoplasm (Zhu, Xu et al. 2002, Shimada, Rios et al. 2009, Ziaei, Shimada et al. 2012). Immunocytochemistry was performed in young and pre-mature senescent IMR 90 cells with antibodies specific for hnRNP A1 and hnRNP A2 (Figure 5 A & B). As seen by the co-localization with DAPI, hnRNP A1 and A2, remained localized in the nucleus of premature senescent cells as it the young cells.

Figure 6 shows that hnRNP A1 is down regulated in premature senescent cells about 40% (p=.001). This is consistent with our previous studies where
replicative senescent cells show a significant down regulation (Zhu, Xu et al. 2002). Furthermore, we observed a decrease of about 30% in hnRNP A2/B1 levels in premature senescent cells that was significant (p=.04). Replicative senescent cells show a significant down regulation of nearly 90% (p=.004)(Figure 7).

To investigate the in vivo effect of cyclophosphamide and doxorubicin, a known oxidant that causes oxidation of guanosine bases within the DNA, we analyzed the levels of hnRNP-A1 in hippocampal lysates isolated from rats treated with the drug combination of doxorubicin and cyclophosphamide. The expression of hnRNP A1 is down regulated after treatment about 30% on average, however the data (p=.077) was not statistically significant (Figure 8).

As a comparison to determine specify, we measured the expression of hnRNP K in cells treated with H₂O₂. We observed an increased expression following hydrogen peroxide treatment in premature senescent cells (p<.05). In addition we observed a slight increase of hnRNP expression in replicative senescent cells (p<.05). We also found there was no statistical difference in the expression level of hnRNP K in hippocampal lysates isolated from rats treated with doxorubicin and cyclophosphamide cocktail as compared to saline controls (Figure 10).
Figure 5. The nuclear accumulation of hnRNP A1 and A2/B1 does not change in fibroblast cells. Young and pre-maturely senescent young IMR-90 cells were subjected to immunocytochemistry and probed for hnRNP A1 expression. DAPI was used to stain the nuclei. Identical fields are shown for DAPI and A. hnRNP A1 (4B10) as well as B. hnRNP A2/B1 fluorescent signals. 40x Magnification.
Figure 6. hnRNP A1 expression is decreased in SIPS fibroblast cells.

A. The western blot analysis of hnRNP A1 [4B10] 34-37 kDa compared to the loading control beta-Actin Loading Control [BA3R] 42 kDa. The lanes are (Y) young PDL 19 cells (N=4), (SIPS) H2O2 treated cells (N=4) and (S) replicative senescent cells (PDL 65). B. Expression of hnRNP A1 controlled for loading using actin and plotted relative to young (PDL 19) control cells. Error bars represent the standard error of means.
Figure 7. hnRNP A2/B1 expression is decreased in SIPS fibroblast cells.

A. The western blot analysis of hnRNP A2/B1 (34-37 kDa) compared to the loading control beta-Actin Loading Control [BA3R] 42 kDa. The lanes are (Y) young PDL 19 cells (N=4), (SIPS) H2O2 treated cells (N=4), and (S) replicative senescent cells (N=2), right to left. B. Expression of hnRNP A2/B1 controlled for loading using beta-Actin and plotted relative to young (PDL 19) control cells. Error bars represent the standard error of means.
Figure 8. hnRNP A1 expression is decreased in the hippocampus region of the brain in chemotherapy treated animals.

A. The western blot analysis of hippocampal lysates taken from rats treated with doxorubicin and cyclophosphamide and control. First lane shows the expression of hnRNP A1 (34-37 kDa) and beta-actin (42 kDa) in the saline control (N=7) and the second lane shows the chemotherapy treated animal (N=8). B. Expression of hnRNP-A1 controlled for loading using actin and plotted relative to saline control samples.
Figure 9. hnRNP K expression is increased in SIPS fibroblast cells.

A. The western blot analysis of hnRNP K (34-37 kDa) compared to the loading control beta-Actin Loading Control [BA3R] 42 kDa. The lanes are young PDL 19 cells (N=4), SIPS H2O2 treated cells (N=4), and replicative senescent cells (N=2), right to left. 

B. Expression of hnRNP A2/B1 controlled for loading using beta-Actin and plotted relative to young (PDL 19) control cells. Error bars represent the standard error of means.
**Figure 10.** A. The western blot analysis of hippocampal lysates taken from rats treated with doxorubicin and cyclophosphamide and control. First two lanes show the expression of hnRNP-K (66 kDa) and beta-actin (42 kDa) in the saline control animal (N=7) and the second two lanes show the chemotherapy treated animal (N=9), left to right. Bands shown are representative of the population. B. Expression of hnRNP-K controlled for loading using actin and plotted relative to saline control samples. Error bars represent the standard error of means.
Discussion

The multitude of genes responsible for replicative cell senescence modulation via various forms of mRNA processing is yet to be fully elucidated. The expression of hnRNP A1 and A2 has been previously shown to be important during replicative senescence. hnRNPA1 and A2 show a decreased expression in replicative senescent normal human diploid fibroblast cells (Zhu, Xu et al. 2002). In addition, the overexpression of hnRNPA1 and A2 resulted in a higher ratio of p14^{ARF} compared to p16^{INK4a} due to a change in the alternative splice decisions and exon skipping (Zhu, Xu et al. 2002). This indicated that the replicative senescent phenotype is potentially maintained by alterations in the posttranscriptional processing of the mRNA.

Replicative senescence has been hypothesized to be a result of oxidative stress. While it is known that telomere shortening is one of the main causes for the induction of replicative senescence, it has been hypothesized that oxidative stress may also be a major contributing factor (Colavitti and Finkel 2005). It has been suggested that intracellular reactive oxygen species play a role as signaling molecules and oxidized macromolecules mediate the senescent phenotype. (Colavitti and Finkel 2005)

It is of interest to determine whether the stress induced premature senescence (SIPS) caused by ROS can modulate hnRNPA1 gene expression as this form of senescence has physiological relevance. Here we show that hnRNPA1 is not
regulated by oxidative stress induced premature senescence at the same magnitude as it is in replicative senescence.

We have shown that hydrogen peroxide can induce the pH neutral senescence associated biomarker β-galactosidase (SA β-gal) (Figure 2) as well as growth arrest in human fibroblast; similar to what has been observed by others (Dimri, Lee et al. 1995, Zhou, Chen et al. 2015).

Furthermore, we have shown that H$_2$O$_2$ treated fibroblast cells exhibit a shift towards G2 stage of the cell cycle (Figure 3). ROS mediated DNA damage can cause G2/M cell cycle arrests via the p53 and ChK1 parallel pathways(Guo, Wu et al. 2014). The increase of cells in G2 stage is likely because of the inactivation of Cdc25 and Cdc2 by Chk1, resulting in cellular arrest (Bucher and Britten 2008). A small population of the H$_2$O$_2$ treated cells also transitioned into hypodiploid sub-G0 stage, with less then 2N DNA content. These cells are in the process of apoptosis both mitochondrial and extrinsic pathways after being arrested in G2/M for too long (DiPaola 2002).

hnRNPs shuttle between the nucleus and cytoplasm continuously in young proliferating cells, however during replicative senescence hnRNP A1 and A2 accumulate to the cytoplasm. This change in localization has been attributed to the increase of p-38 MAPK (Shimada, Rios et al. 2009). Here we show that H$_2$O$_2$ SIPS cells do not share the same change in localization pattern of hnRNP A1 and A2 in senescent cells (Figure 5). The two hnRNPs remained at high levels in the nucleus in both SIPS and control cells. Activation of p-38 MAPK can be induced by ROS from
exogenous sources such as H$_2$O$_2$, however hnRNP A1 and A2 localization pattern may not driven by increased levels of p-38 MAPK alone in SIPS cells.

We have proposed that the maintenance of replicative senescence is in part regulated by hnRNP A1 and A2 (Zhu, Xu et al. 2002, Ziaei, Shimada et al. 2012). This may be due to a decrease of hnRNP A1 and A2 in senescent cells altering the levels of expression of target genes during senescence. This was supported by the increase of growth inhibitors and f-actin in siRNA known down of hnRNP A and A2 cultures (Zhu, Xu et al. 2002, Shimada, Rios et al. 2009).

Western blot analyses showed that the levels of hnRNP A1 (Figure 6) as well as hnRNP A2/B1 (Figure 7) decreased in SIPS cells, when compared to control (Figure 7). hnRNP A2/B1 are a close cousin to hnRNP A1 with over 80% of conserved of amino acids (Dreyfuss, Matunis et al. 1993). This is especially interesting since hnRNP A2/B1 expression is increased in the dendrites and soma as a result of neuronal activity and brain derived neurotropic factor (BDNF) (Leal, Afonso et al. 2014).

Interestingly, we observed an upregulation of hnRNP K in premature senescent cells (Figure 9). hnRNP-K is a p53 co-activator and plays a role in activating the p21 cell cycle regulator. Other groups have also observed this increase in expression of hnRNP K after DNA damage due to UV radiation (Moumen, Masterson et al. 2005) and also in tumorigenic cells (Gao, Yu et al. 2013). Thus, hnRNP K may play a role in the DNA damage response.

Given that we know that hnRNP A1 can modulate the levels of p21 in hnRNP A1 siRNA knockdown senescent models (Shimada, Rios et al. 2009), it is of interest
to determine whether hnRNP K can also play a role in p21 expression and senescence.

The anthracycline doxorubicin is a known oxidative stress-causing source of ROS that can cause the initiation of apoptosis at sub lethal concentrations. In addition it is a popular antineoplastic agent used in chemotherapy to treat a wide range of cancers (Thorn, Oshiro et al. 2011). Recently, we have shown rodents exhibit a cognitive impairment post-treatment with doxorubicin and cyclophosphamide (Salas-Ramirez et al., 2015). In addition it has been suggested cancer survivors suffer from cognitive dysfunction (Ahles and Root 2018). Despite limited access to the brain, doxorubicin causes a systemic inflammatory response and oxidative stress. Our data has shown that hnRNP A1, is down regulated in chemotherapy treated rats. Despite the low statistical power, we observed a constant decrease across all animals at varied levels. This may suggest a possible mechanism that is yet to be elucidated in which hnRNPs in part modulate the cognitive impairment. It has been shown that synaptic activity increases hnRNPs in the dendrites and synapses via a Brain-derived neurotrophic factor (BDNF) dependent mechanism (Leal, Afonso et al. 2014).

Conclusions

An understanding of cellular responses to ROS from stressors such as hydrogen peroxide, ultra violet radiation and chemotherapeutic agents will provide a better understanding of ageing as well as age-related pathogenesis. Little is known about the molecular mechanisms of SIPS caused by ROS and much still needs to be characterized. We have shown here that hnRNP A1 is not modulated in SIPS
by the same mechanism as replicative senescence. We observed a decrease in expression of hnRNP A1 and A2/B1 in hydrogen peroxide treated fibroblast cells, however the localization pattern has not changed when compared to control. In addition, animals treated with doxorubicin and cyclophosphamide did show a decrease in hnRNP A1 in the hippocampus. This may suggest a function of premature senescence in the brain that may contribute to the chemotherapy induced cognitive dysfunction experienced by the rats.

**Future Direction**

The presence of senescent cells is a double-edged sword, by providing tumor suppression and wound healing early in life and switching roles later in life by accumulating and expressing the secretory phenotype. Currently a perfect marker for cellular senescence does not exist, and we rely on a combination of marker to identify cellular senescence. In this paper we investigated possible hnRNPs that may have a role in modulating stress induced premature senescence that showed promise as modulators of replicative senescence. Premature senescence is potentially a more physiologically relevant phenotype, as it is induced by both extrinsic (e.g. chemotherapy) and intrinsic (e.g. mitochondria metabolism) genotoxic factors. Future studies will focus on identifying the common factors that modulate both premature and replicative senescence in hope to eliminate cells with senescence associated secretory phenotype.
Figure 11. The number of hnRNP A1 and A2 positive cells is lower in the hippocampus region of the brain in chemotherapy treated animals. Doxorubicin and cyclophosphamide treated rats had their brains resected and sliced into 30 micron slices. Immunohistochemistry staining using DAB probed for hnRNP A1 (A) and hnRNP A2/B1 (B) showing the hippocampal regions CA1, DG and CA3 at 100x magnification.
Future studies will investigate the biological markers including hnRNP expression in the senescence-accelerated mouse (SAMP8). SAMP8 exhibits higher levels of senescent cells, increased oxidative stress in the brain, and has been used as a model for a number of neurodegenerative diseases (Butterfield and Poon 2005). In addition the increase in hnRNP K in premature senescent cells may be a novel way in which senescence is modulated. To investigate further we will utilize siRNA and conditional knockdown models to decrease or eliminate hnRNP K.

Furthermore, preliminary immunohistochemistry data using DAB staining shows a decreased expression and hnRNP A1 and A2/B1 positive cells in the hippocampus of doxorubicin and cyclophosphamide treated rats (Figure 11). To investigate further, we will investigate the localization and signal intensity of hnRNP A1, A2/B1 and K in the hippocampus of chemotherapy treated rats to elucidate whether premature senescence in the hippocampus may play a role in the cognitive dysfunction observed by Salas-Ramirez, Bagnall et al. (2015).
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