Cellular Senescence: A Nuanced Mechanism and Its Contemporary Implications

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Abigail Goldberger

Mentor: Dr. Alyssa Schuck, Biology

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I. Abstract

Cellular senescence is a unique molecular mechanism with characteristics and consequences that vary greatly across distinct physiological contexts. Definitionally, senescence represents a retreat from cellular division to a viable limbo state with altered cellular metabolism and secretory patterns. Immune clearance of the senescent unit typically follows suit. This pattern would seem to contribute to appropriate tissue turnover, a phenomenon favorable with regard to wound healing, tissue regeneration, and tumor suppression. It is therefore intriguing to consider the contributions of senescence to various disease states including age-related degenerations and even cancer itself, the very condition against which senescence is thought to be protective. To better elucidate the apparent contradictions within the conversation of senescence, this paper highlights key features of senescence as it relates to healthy embryonic development, cancer, and age-related physiological deteriorations. With a developing understanding of the outcomes that may be attributed to senescence, contemporary research seeks novel techniques to harness the positive potential of senescence and mitigate its negative effects. Since spinal cord injury (SCI) is sometimes considered to be a model of accelerated aging, an investigation was launched to consider the possible relationship between excessive cellular senescence and the long-term negative effects of spinal cord injury. Levels of p53, p27, and p16, three protein regulators of senescence, were assessed by Western immunoblotting in rat SCI models. Gene expression of IL-6, IL-1α, IL-1β, TNFα, and CXCL1, coding for proinflammatory cytokines, was measured via mRNA analyses. No significant association was ultimately achieved between SCI and altered markers of cellular senescence, indicating the low probability of a relationship between cellular senescence and spinal cord injury outcomes.

II. Introduction

Normal cellular development, cancer, and aging may seem at first blush to be three distinct biological concepts, but the phenomenon of cellular senescence may be an essential player in all of their stories. Cellular senescence is a programmed withdrawal from the mitotic, or cell-division, cycle to a viable but non-proliferative state. In other words, the cells in question cease to divide, yet do not immediately self-destruct; as such, senescence is a phenomenon distinct from the related process of apoptosis by which a cell would systematically deconstruct and auto-digest. In its ideal form, senescence is primarily thought to be a tumor-suppressive mechanism, encouraging tissue regeneration by reducing the burden of outdated and/or damaged cells. A senescent cell demonstrates a senescence-associated secretory phenotype (SASP), a chemical profile that recruits immune clearance of the senescent unit (Muñoz-Espin, *et al*. 2014).

The SASP, however, may evolve and include the release of other potentially harmful molecules that can impair surrounding cells or elicit their 'secondary' senescence via paracrine regulation, inter-cellular communication by which one cell induces another nearby cell to undergo change. Excessive cellular senescence has been linked to various pathologies, including, but not limited to, degenerations related to the regular aging process (Muñoz-Espin, *et al*. 2014). Both a failure to senesce when appropriate and, conversely, an inappropriate prevalence of senescence may even contribute to the etiology of cancer. This is due to the fact that cells persisting in undue proliferative activity are in greater danger of giving rise to tumorigenic progeny, and cells emitting prolonged SASPs can negatively influence the cellular environment in favor of cancer development (Zeng, *et al*. 2018).

Senescence evidently encompasses potential positive implications as well as, alternatively, potential damages. A distinction must therefore be drawn between acute and chronic senescence. Targeted stresses, such as a specific wound or injury, initiate acute senescence with efficient immune clearance that promotes tissue regeneration (Muñoz-Espin, *et al.* 2014). Likewise, acute senescence is also relevant during normal embryogenesis, when cellular development is carefully controlled and tissue patterning is highly coordinated (Storer and Keyes, 2014). The overall process of acute senescence is critical for healthy tissue homeostasis. In contrast, chronic senescence results from stresses of prolonged duration, gradual onset, and/or slow progression; for instance, DNA damage or reactive oxygen species (ROS) may elicit such a response. With chronic senescence, the senescent cells accumulate over time, leading to an exaggerated senescence profile and an SASP that ultimately evolves into a more damaging form. The enlisted immune clearance is thus ineffective, and the net effect can be harmful (Muñoz-Espin, *et al*. 2014).

Several discrete categories of stimuli can induce pathways of damage-induced senescence and funnel down into a number of common cellular mediators (Figure 1). DNA damage or ROS are perhaps most intuitive in that they can initiate cellular distress resulting in a proliferative halt. Even epigenetic modifications, heritable changes to gene expression, may lead to a senescence response. For instance, when the promoter of the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene is derepressed, corresponding cell cycle inhibitors will be produced in excess quantities (Muñoz-Espin, *et al*. 2014). Distinct within the umbrella of DNA damage, both the activation of an oncogene or the mutation of a tumor suppressor gene can promote senescence as well. These genetic events are cancer-promoting, and

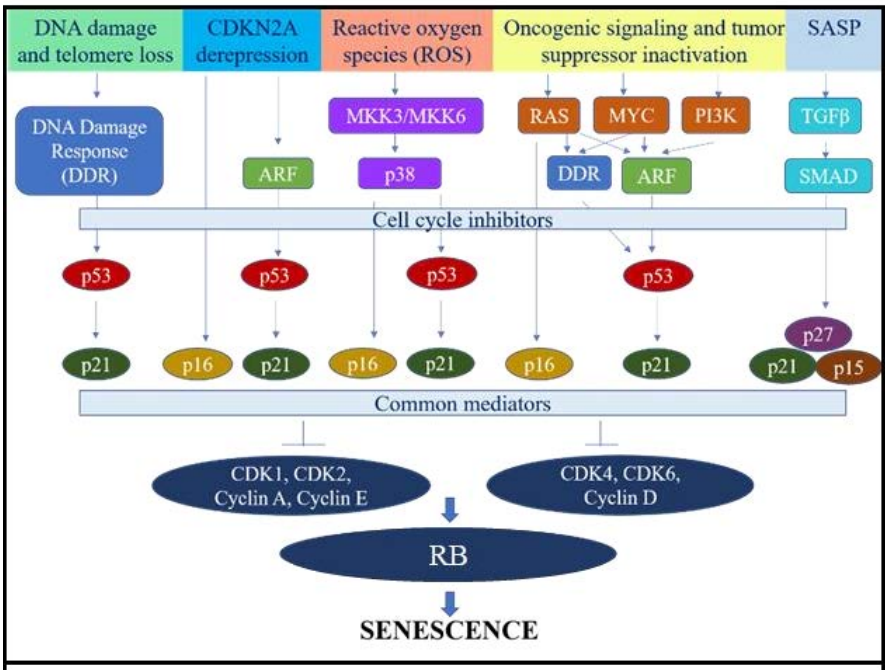

Figure 1: Pathways of damage-induced senescence. Reconstructed based on figure from Muñoz-Espin, Daniel, *et al.* Nature Reviews.

senescence is a cellular effort to curtail unrestrained proliferation (Chandeck and Mooi, 2010).

Biologically speaking, senescence is elusive to define and identify due to the absence of any uniform, conclusive markers. Most of the damage-induced senescence pathways are united upstream by the frequent cell-cycle inhibitor p53, but p53 is also common to apoptotic pathways. Levels of the inhibitors p21 and p16 are typically upregulated within the senescence profile; p21, at least, is considered to be downstream of p53. Less prevalently, p19 and p27 may be elevated as well. The overall activation of these cell-cycle inhibitors results in a net repressive effect on the cyclin-dependent kinase (CDK) / cyclin complexes that would normally contribute to cellular proliferation. Activation of the tumor suppressor

retinoblastoma (RB) gene product is also implicated downstream as a critical mediator in multiple senescence pathways (Muñoz-Espín, *et al*., 2013).

Histochemical staining for senescence-associated β-galactosidase (SAβGAL) is often conducted as an additional relevant assay because SAβGAL is a lysosomal enzyme that is traditionally overexpressed in senescent cells (Muñoz-Espín, *et al*., 2013). In morphological terms, senescent cells tend to be larger than average cells and appear to have a slightly flattened shape (Campisi, 2013). Even beyond the senescent cell itself, the SASP it emits is associated with a host of cytokines, most often proinflammatory, including but not limited to: IL-6, IL-1α, IL-1β, TNFα, and CXCL1 (Graham, *et al.*, 2020). None of these identifying factors, however, is satisfactory in isolation; senescence should only be determined on the basis of a constellation of cellular flags. Of note, the patterns of senescent markers may even differ in the realm of acute senescence as compared to those of chronic damage-induced senescence, although many parallels do remain (Muñoz-Espín, *et al*., 2013).

Understanding the progression of cellular senescence on a both a molecular and macroscopic physiological level stands to yield therapeutic benefit in the realms of emerging cancer and aging research. Because senescence is, first and foremost, a normal phenomenon in healthy cellular development, investigating the standard mechanism of senescence in development is the gateway to studying the consequences of its irregularities or dysfunctions. If the contributions of senescence to its related pathologies can be further elucidated and concretized, therapeutic strategies designed to target components of senescent pathways could be employed in an effort to ameliorate negative effects.

III. Senescence: A Comprehensive Overview

1. Senescence and Normal Development

Apoptosis has long been recognized for its role in embryogenesis, particularly in tissue-patterning and in the degeneration of unnecessary transient structures such as the interdigital webbing between developing figures and toes (Brill, *et al*., 1999). Even though senescence was first identified in the context of aging and disease, the question has become whether senescence, like apoptosis, may be similarly implicated in embryonic development (Storer and Keyes, 2014). A 2013 report (Muñoz-Espín, *et al.*) used SAβGAL staining to identify structures of interest during mouse development where senescence was predicted to be implicated. Among these structures were the endolymphatic sac of the inner ear and the mesonephros, an excretory organ that is largely transient in many higher-level vertebrates. The study demonstrated that the SASP emitted by the senescent cells recruited macrophages to enter the respective regions and effect clearance, serving in the mesonephros to aid in necessary regression and in the endolymphatic sac to assist in the balancing of different cell populations. While this developmentally programmed senescence appeared to be dependent on upregulation of p21, as is consistent with the literature regarding markers of damage-induced senescence, the developmental p21 upregulation was unexpectedly found to be independent of p53. Many other general senescence markers in the developmental context were still found to be comparable to those of damage-induced, but this distinction with regard to p21 alone is enough to suggest that the different senescence pathways may possess unique nuances in the patterns of their molecular markers. It should be noted that when p21 was knocked out, the developmental senescence was not able to proceed; apoptosis did ‘fill-

in', so to speak, as a compensatory developmental mechanism, though the effects were delayed.

A 2013 report by Storer and colleagues further expands the conversation of developmental senescence by proposing the novel theory that oncogene-induced senescence (OIS) and, presumably, other pathways of damage-induced senescence have actually evolved from the normal pathway of developmental senescence. In other words, cells that encounter damage or threat can reactivate this innate process to shut down their own proliferation. Interestingly, the study identified several key sites of senescence at particular timepoints throughout mouse embryogenesis – of note, at certain known signaling centers. The apical ectodermal ridge (AER), a structure important in signaling for limb patterning, and the neural roof plate, relevant in the direction of the developing central nervous system (CNS), were both positive for known senescent markers. Many of the proteins secreted as signals from these regions were found to be among those secreted as part of the SASP in the adult damage-induced senescence response, reinforcing a connection between the developmental and damage-induced senescence processes. There were, however, evident differences between them as well. p21 was found to be a necessary mediator of embryonic senescence, but p16 appears to be the key player when it comes to OIS. In OIS, p21 levels demonstrate initial elevation and subsequently return to normal limits, at which point p16 elevation, absent by embryonic senescence, becomes measurable. To address these distinctions in keeping with the evolutionary theory of OIS, it is possible to suggest that the senescence response has evolved and advanced in complexity since its developmental origin.

Beyond its role in the patterning of embryonic development, senescence likely plays its part in the coordination of cell plasticity in the grown organism. Recent evidence suggests that the SASP emitted by a senescent cell can induce stem-like qualities in nearby cells, a critical property that addresses the contribution of senescence to tissue regeneration and wound repair. This is, however, only true when the exposure to the SASP is temporary and within reasonable limits. In excess, the effect of the SASP may inappropriately amplify the number of cells existing in undefined states. Such cells, left underutilized for concrete physiological purposes, would be at greater risk for mutation or other forms of damaging dysregulation. Alternatively, neighboring cells may interpret the exaggerated regenerative signals of the SASP to be protumorigenic and respond with initiation of their own senescence in order to limit the effect. This will result in a continually compounded SASP presence and perpetuation of the same cycle, ultimately limiting regenerative capacity (Ritschka, *et al*., 2017).

2. Senescence and Cancer

Cancer typically manifests as a failure relating to the control of normal growth and developmental processes, so it is no wonder that the phenomenon of cellular senescence is a relevant part of the discussion. The role of senescence in cancer, though, is complex and highly context dependent. Senescence may, in turn, be considered either cancer-protective or cancer-promoting. On the most fundamental level, senescence is thought to be tumor-suppressive because of the limit it would seem to impose upon cellular proliferation. In fact, replicative senescence, as it is known, can best be exemplified by the Hayflick limit, the reality that cells will cease to divide after a roughly set number of mitotic divisions (Hayflick

and Moorhead, 1961). These cells remain initially viable yet without further growth, and they exhibit some senescence-associated characteristics such as increased levels of cell cycle inhibitors p21 and p16, among others (Zeng, *et al*., 2018).

The basis for the Hayflick limit is likely rooted in the shortening of chromosomal telomeres with every successive division. Telomeres are regions of non-coding DNA at the ends of the chromosomes that protect against inter-chromosomal fusing as well as the loss of genetic material that would otherwise occur due to DNA polymerase's inability to replicate DNA at the outer chromosomal limits. When the telomeres are eroded past a critical point, the cell will typically initiate senescence and eventually enter a state of cellular crisis that could culminate in apoptosis (Akincilar, *et al*., 2016). Telomere shortening has, in fact, been identified as a good molecular marker for cellular senescence (Bernadotte, *et al.*, 2016). Thus, every cell has a built-in mechanism to curtail its own lifespan, which prevents uncontrolled proliferation and the genetic errors that might otherwise result in cells that are past their prime.

In order for cancer cells to develop and thrive, this proliferative barrier must be overcome. In around 90 percent of human cancers, activation of the human TERT (hTERT) gene is initiated which allows for the production of telomerase, an enzyme that maintains telomere length (Jafri, *et al.*, 2016). The cellular crisis that would follow if the telomeres were to continue shortening with excessive cell proliferation is therefore averted, and the cells can achieve immortality. Essentially, successful cancer cells develop a strategy to

circumvent the limitation imposed by senescence, emphasizing the role of senescence in tumor-suppression.

On the other end of the spectrum, chronic senescence in a given tissue can promote changes in the cellular microenvironment that are actually cancer-favoring. A cell is not an island; rather, it exists in dynamic communication and interaction with its surrounding environment which includes its extracellular matrix (ECM), nearby blood and lymphatic vessels, inflammatory markers, immune cells, and connective tissue cells such as fibroblasts, among other components. In recent years, cancer research has seen a growing focus on the contribution of the tumor microenvironment to the development and progression of cancer cells. Fibroblasts, for instance, can become 'activated' in response to certain cell stresses or other signals, resulting in characteristic gene expression patterns. At this point, they are considered cancer-associated fibroblasts (CAFs) and can aid in angiogenesis (the formation of new blood vessels), remodeling of the ECM to promote potential for invasion, and secretion of growth factors and cytokines (Wang, *et al*., 2017). They also support epithelial-to-mesenchymal transition (EMT), a shift in cellular phenotype often associated with wound healing that permits greater mobility and invasive capacity during the development of cancers such as carcinomas (Roche, 2018).

There has been noted overlap between the secretory phenotypes of CAFs and senescent cells, suggesting that, like CAFs, senescent cells may contribute to a pro-tumorigenic microenvironment. The SASP has also been seen to elicit EMT. Because EMT is favorable within the context of tissue regeneration but negative in the sense of cancer

promotion, it has been proposed that the SASP, or rather senescence as a whole, should be considered to be antagonistically pleiotropic: it is a mechanism with alternatively beneficial and deleterious effects at different stages of life (Schosserer, *et al*., 2017). The theory of antagonistic pleiotropy suggests that many harmful human conditions with genetic bases have no logical right to exist, because natural selection should have selected against them long ago. The fact of their continued persistence implies some selective survival advantage conferred early in life that favors the passage of the trait even though it will yield negative eventual outcomes (Carter and Nguyen, 2011). This framework helps to contextualize the apparently contradictory functions of senescence as it relates to cancer and aging; it is possible that senescence has a protective effect of tissue turnover and wound healing in youth but a cancer-promoting effect in the elderly (Schosserer, *et al*., 2017).

The concept of antagonistic pleiotropy also dovetails with the distinction between acute and chronic senescence. Optimal senescence would be of the acute form exhibited in youth, where the stresses are well-defined, and the senescence response is both contained and well-organized. As an individual ages and various damages and/or exposures accumulate over time, acute senescence may yield to the less effective chronic senescence which can foster general chronic inflammation or other physical degenerations. Cancer incidence certainly rises along with advancing age; while cancer may look different than many other age-related diseases, its rate begins to climb at around the same point in life – roughly the midpoint – with a strikingly similar curve that approximates exponential kinetics. The data support a possible unifying factor between cancer and aging. It is feasible to suggest that chronic senescence could be that link (Campisi, 2013).

This strange duality of senescence in cancer has not precluded some consideration of the use of therapy-induced senescence (TIS) as a means of cancer treatment. Traditionally, cancer treatments consist of cytotoxic agents intended to cause cell death, but these regimens often result in harsh side effects, including immunosuppression, anemia, fatigue, nausea, and other symptoms. Using different agents or even lower doses of these same agents to induce senescence rather than cell death may be able to create the same cancer-fighting influence by halting excessive proliferation without inducing such extreme side-effects. Some cancer cells may have even evolved to escape apoptosis altogether, due to mutations in key apoptotic mediators, yet retain the capacity to undergo senescence; this would mean TIS could target those cells that may otherwise sidestep conventional treatment, thereby mitigating the negative effect of drug resistance (Schosserer, *et al*., 2017).

The concern remains, though, that the SASP of the newly induced senescent cells could induce secondary senescence in neighboring cells or even distant tissues, thus unduly amplifying the general senescence presence to the point that it becomes chronic and immune clearance becomes ineffective. Counterproductively, the TIS may therefore result in a net cancer-promoting effect due to changes evoked in the cellular microenvironments. Dangers include the potential for relapse or even the development of secondary tumors in satellite locations (Schosserer, *et al*., 2017). It is possible that the senescent cells themselves could lie dormant and have the ability to return to proliferation down the road; though senescence is often stated to be irreversible, this may not be true in cases where critical proteins involved in maintaining senescence are knocked down by other biological processes or therapies (Ewald, *et al.*, 2010), (Khalem, *et al*., 2004).

To address this challenge, TIS may be employed in conjunction with techniques designed to subsequently target and destroy senescent cancer cells, so those cells do not linger indefinitely in the tissue. A 2013 study published by Wiland and colleagues proposed the application of virotherapy, one such technique using viral invasion and replication to kill cancer cells, to that effect. Despite concerns that the altered metabolism of senescent cells might interfere with normal viral ability to hijack cellular infrastructure, experimental use of the measles vaccine virus (MeV) showed more effective replication of MeV in the senescent tumor cells than in non-senescent neighbors. These results imply that the compounded use of TIS and virotherapy could represent a promising way to capitalize upon the anti-cancer properties of senescence while minimizing the long-term risks. It does remain possible, though, that knocking out senescent cells entirely could interfere with appropriate tissue regeneration needed to recover from damages wreaked by the cancer treatment (Schosserer, *et al.*, 2017). The hope, therefore, would be that the secondary effects of TIS will be fewer and less severe than those of conventional chemotherapy such that the body's recovery efforts post-treatment need not be as pronounced.

3. Senescence and Aging

With regard to senescence and cancer treatment, the goal is to maximize the induction of senescence in tumor cells and ideally destroy those cells before negative secondary consequences of senescence may occur. In contrast, aging research increasingly seeks to minimize the occurrence of senescence to ameliorate age-related physiological degenerations such as sarcopenia (loss of muscle tissue), atherosclerosis and heart failure, and neurodegeneration; it is thought that these biological breakdowns may be related to

cumulative effects of senescence (Campisi 2013). As individuals age, cellular damages accumulate over time. These may be due to decades of environmental exposures, buildups of mutations, and declines in the efficiency of repair processes, among other factors. As a response invoked by a variety of damage pathways, senescence is expected to occur with greater incidence in the tissues of older adults. Because the types of damages contributing to aging effects are so diverse and difficult to pinpoint, one approach is to combat the consequences of those damages rather than the causes themselves. Cellular senescence is a prime focal point to confront (Ogrodnik, *et al*., 2019).

Limiting senescence may promote tissue regeneration that could potentially counteract some aging effects. Since senescence itself is classically considered to support positive tissue turnover, this may seem counterintuitive. It is therefore critical to recall the distinction between acute and chronic senescence. The senescence that dominates in aging subjects would be within chronic parameters such that the net negative effects actually undermine the potential benefit (Schosserer, *et al*., 2017). Acute senescence is induced by controlled stimuli for defined purposes, while chronic senescence is a response to one or more of a network of insidious, progressive damages. The impact of cells exhibiting chronic senescence is therefore nowhere near as coordinated, nor is immune clearance of those cells very efficient (Childs, *et al*., 2015). Enabling cells to evade senescence could alleviate the senescent burden and the effects of the SASP on the tissues and cellular microenvironments of older adults. Additionally, when senescence occurs in progenitor cells of any given tissue, it prevents those precursor cells from further differentiating into their appropriate offspring

which is an added blow against tissue regeneration; in this sense, there is benefit to combatting senescence even beyond the effort to minimize the SASP (Childs, *et al*., 2017).

One proposed tactic involves activation of telomerase, the enzyme that allows for the re-extension of chromosomal ends to prevent replicative senescence. Introducing telomerase, though, is by no means a panacea. While it may pose therapeutic possibility in the aging realm, there is danger of accompanying oncogenic influence. Bypassing senescence entirely leads to the creation of cells that are insensitive to normal replicative limitations. As noted, telomerase is indeed reactivated in many human cancers (Jafri, *et al.*, 2016).

To isolate the role of telomerase in aging without concern for associated cancer, Tomas-Loba and colleagues expressed telomerase in cancer-resistant mice in a 2008 study. These mice were bioengineered to carry a triploid gene load of p53, Arf, and p16, three important tumor suppressors that would provide greater-than-average protection from tumor development. The subjects with expressed telomerase showed reductions in aging-associated degenerative inflammation of epithelial tissues, improved performance on various metabolic fitness parameters, and overall extended life expectancies. Positive fitness effects were even identified in several organs that were not expressing telomerase, such as the brain and differentiated muscle; these data suggest that the general anti-aging benefits of the experimentally manipulated telomerase expression in this study were systemic rather than merely local. The potential for employing telomerase expression to minimize replicative senescence in aging subjects is certainly an intriguing avenue of continuing investigation; its promise is, however, limited *in vivo* due to persistent concerns of cancer contribution.

Evidently, any technique designed to turn off or override the molecular mechanism of senescence altogether runs the risk of generating immortal cell lines that will be unresponsive to normal physiological cues. An alternative anti-aging strategy would be to directly target cells that are already senescent and aim to clear them more efficiently. Previously, virotherapy was proposed in accompaniment with therapy induced senescence (TIS) as a means to eliminate senescent cells after the application of cancer treatment (Wiland, *et al*., 2013); this approach of targeted senescent clearance is conceptually similar to what is being sought within the field of aging research, but virotherapy for this use has not been designed for non-cancer-associated context.

The general aim is to identify molecular targets or patterns unique to senescence that may be manipulated to selectively clear senescent units with minimal impact on neighboring cells. Senescent cells demonstrate enhanced apoptotic resistance (Wang, 1995); Zhu and colleagues postulated in a 2015 study that senescent cells could have enhanced gene expression of anti-apoptotic, pro-survival factors compared to non-senescent cells of the same cell type. Looking at fat cell progenitors, a common site of senescent cells in humans, they identified gene transcripts of interest via comparative expression assays and then used small interfering RNAs (siRNAs) to knock down individual elements. 39 transcripts were targeted altogether and, of those, 17 had a greater impact on viability of senescent cells than non-senescent cells, and 6 actually mediated senescent cell death without notable impact on neighbors. Alternatively, a combination of dasatinib and quercetin, two drugs identified to target downstream products of anti-apoptotic genes, were shown to be preferentially

senolytic (able to kill senescent cells). Collectively, aging mice treated with appropriate senolytic agents showed improved cardiac, neurological, and skeletal health.

Dasatinib and quercetin may have been the first two identified senolytic compounds, but they were not the last. Navitoclax (ABT-263) was soon discovered as a third option, yet another drug designed to inhibit various groups of anti-apoptotic proteins in the hopes of eliminating senescent cells known to upregulate these pathways (Myrianthopoulos, 2018). Besides for inventing novel molecules to remove senescent cells, other studies have focused instead on reducing the effects of the SASP of existing senescent cells. These methods may include the application of RNA interference to mitigate expression of inflammatory factors or the use of steroids such as cortisol and corticosterone to inhibit the SASP burden (Velarde and Demaria, 2016), (Myrianthopoulos, 2018). Overall, the arsenal of senotherapeutic strategies continues to evolve as new drug analogs emerge onto the stage and models of senescent markers are continually refined. It should be noted that any senolytic tactics employed to combat aging effects may have clinical application in cancer treatment as well; they could be as used as alternatives to virotherapy in the accompaniment of therapy induced senescence (TIS), to efficiently clear the senescent cells after the cancer treatment has been applied.

4. Investigating a Possible Connection between Senescence and Spinal Cord Injury

a) Basis for Investigation

It is interesting to consider that spinal cord injuries (SCIs) are sometimes considered to be models of accelerated aging. The initial SCI results in various secondary consequences

that lead to long-term physiological degeneration mirroring some effects of the aging process, reaching the cardiovascular and respiratory systems in addition to the obvious musculoskeletal impact. In an individual with SCI, the aging-like effects may be induced prematurely, but even normal aging may be exacerbated by the presence of the SCI (Cameron, *et al.*, 2014). Since cellular senescence has been implicated as a possible contributing factor in age-related decline, it became clinically relevant to investigate the role of senescence in the pathologies exhibited by those with SCI – for instance, muscle atrophy and general poor muscle performance. If skeletal muscle paralyzed by SCI could be shown to display elevated markers of senescence, it could become a viable target for evolving senotherapeutic approaches already at play in the arena of aging research. Anti-senescent mechanisms could be employed in animal models of SCI to evaluate the molecular outcome on markers of senescence as well as the macroscopic outcome on recovery and prognosis. The study described herein therefore sought to examine the possibility of a relationship between SCI and altered markers of senescence.

b) Materials and Methods

Animals

Our pilot study examined protein and gene expression of several key senescence markers in paralyzed muscle from adult male Sprague-Dawley rats, 4-month old, at sequential timepoints post moderate-severe contusion SCI at thoracic-level 9 (T9). SCI injuries were induced by applying a 250-kilodyne force to the spinal cord at that level using the Infinite Horizons Impactor. The left soleus muscle was obtained from rats at 2 weeks, 1 month, 2 months, and 3 months post-SCI. Six animals were randomly selected from each

group. SCI animals at each timepoint were matched against Sham controls, animals that received T9 laminectomy (removal of a part of the vertebra at that level of the spinal canal) with no injury to or manipulation of the spinal cord itself (Graham, *et al*., 2020).

SDS-PAGE and Western Immunoblotting

Approximately 30 mg of each muscle sample was prepared for SDS-PAGE and Western immunoblotting. Each sample was placed into a 1:10 w/v of 1x RIPA buffer containing phosphatase and protease inhibitors and subsequently homogenized by an automated bead homogenizer. Homogenates were chilled on ice for 10 minutes and then centrifuged at 14,0000g for 15 minutes at 4°C, upon which the supernatant was collected. After determining protein content via microBCA, 60ug of protein was combined with 2x Laemmli sample buffer with 5% β-mercaptoethanol, boiled for 5 minutes, and run through a 4%-20% gradient polyacrylamide gel. Gel content was transferred onto a PVDF membrane, stained initially with Ponceau S., and imaged using a CCD digital camera. A 10-minute rinse in Tris-buffered saline with 0.1% Tween-20 (TBST) was used to destain the PVDF membrane, after which the membranes were blocked with 5% w/v BSA-TBST for 60 minutes. 1% BSA-TBST was prepared containing primary antibodies p53, p27, and p16, important regulators of cellular senescence, at ratios of 1:1,000. The membranes were incubated in these solutions overnight at 4°C. The following day, membranes were rinsed in TBST for three 10-minute intervals and then incubated at room temperature for 60 minutes in 1% skim milk/TBST with an HRP-conjugated secondary antibody at a 1:2,000 ratio. After another TBST wash, an HRP-based chemiluminescent detection solution was added to the

membranes for 5 minutes and they were then imaged using a CCD digital imager (Graham, *et al*., 2020).

RNA isolation and RT-PCR

An miRNeasy Mini Kit (Qiagen) was used according to manufacturer instructions to extract and isolate total RNA from approximately 20 mg of each muscle sample via a Trizol:chloroform extraction and column centrifugation. A Nanodrop 1000 was used to quantify the RNA concentrations in each yield. From 1ug of each sample, corresponding cDNA libraries were created using a High-Capacity RNA-to cDNA kit according to manufacturer instructions. A 1:10 cDNA:nuclease-free water dilution was prepared and loaded into 384 well plates with Taqman primers and probes and 2x Taqman Universal PCR Mastermix. Real-time PCR was conducted using a Quantstudio 12k Flex system. Results were calculated in terms of relative fold change as compared to levels of 18S rRNA. Target genes for the mRNA expression analyses were IL-6, IL-1α, IL-1β, TNFα, and CXCL1, coding for senescence and atrophy-related proinflammatory cytokines (Graham, *et al*., 2020).

c) Results

Expression levels of p53, p27, and p16

The levels of p53, p27, and p16 in the rat muscle extracts were assessed by Western blot analysis at the indicated timepoints (Figures 2 and 3). Total p53 protein levels were demonstrated to be elevated in SCI samples at the 2-week ($p<0.05$), 1-month ($p=0.0510$), and 2-month ($p<0.05$) timepoints. p27, a cell cycle inhibitor elicited as a result of the SASP, was moderately but not statistically elevated at the 2-month and 3-month timepoints (Figure 2).

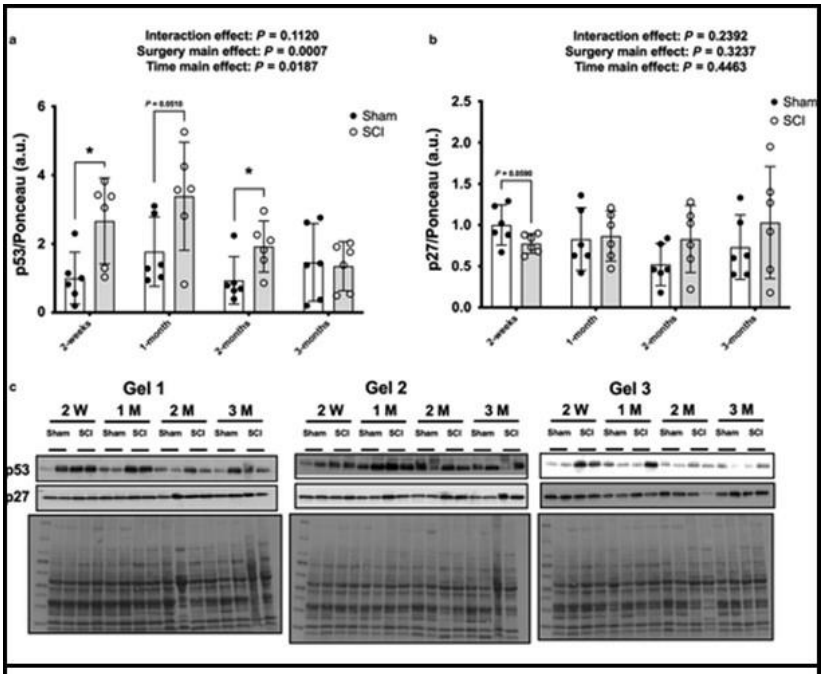

Figure 2: Fold changes in total protein expression of p53 and p27 post-SCI as compared to Sham.
(A) p53 levels and (B) p27 levels at 2 weeks, 1 month, 2 months, and 3 months post-SCI. (C) Representative immunoblots and Ponceau stains for p53 and p27, conducted on the same PVDF membrane and divided according to 3 cohorts.

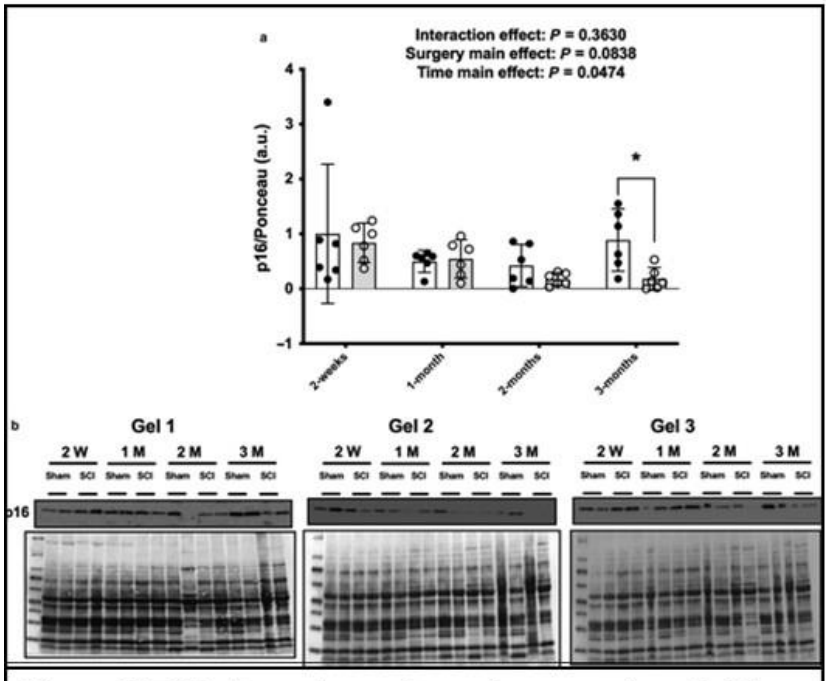

Figure 3: Fold change in total protein expression of p16 post-SCI as compared to Sham.
(A) p16 levels at 2 weeks, 1 month, 2 months, and 3 months post-SCI. (B) Representative immunoblots and Ponceau stains for p16.

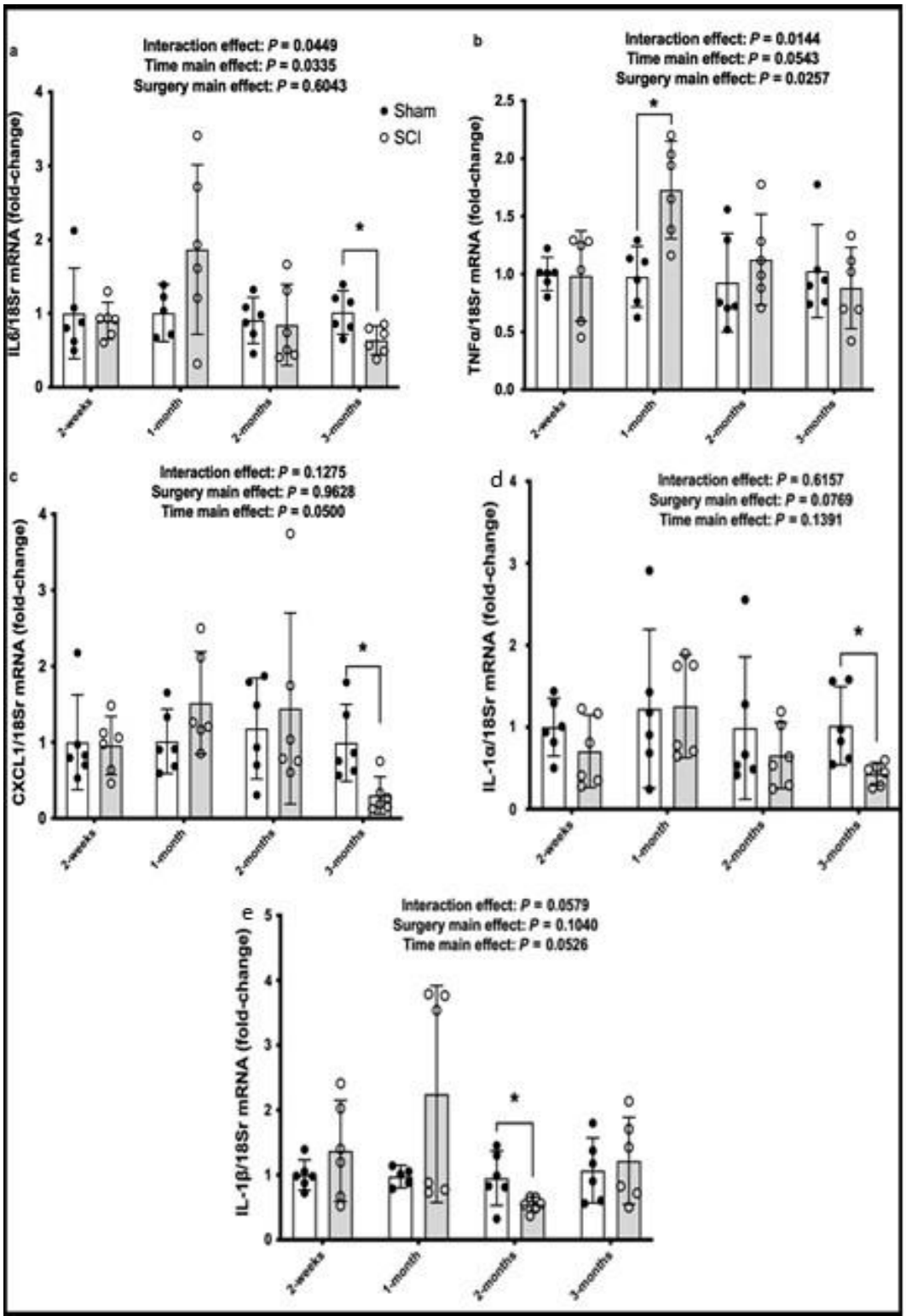

Figure 4: Fold changes in key proinflammatory cytokine expression levels post-SCI as compared to Sham.
Levels of (A) IL6 (B) TNFα (C) CXCL1 (D) IL1-α and (E) IL1-β mRNA levels at 2 weeks, 1 month, 2 months, and 3 months post-SCI as compared to Sham.

Conversely, p16, another cell cycle inhibitor commonly associated with senescence, trended to be lower at 2 months and had a significant decline at 3 months ($p<0.05$) (Figure 3).

Expression levels of cytokines IL-6, IL-1α, IL-1β, TNFα, and CXCL1

The relative levels of cytokines IL-6, IL-1α, IL-1β, TNFα, and CXCL1 were evaluated by mRNA analyses using qPCR at the indicated timepoints (Figure 4). At the 1-month timepoint, IL-6 and IL-1β gene expression exhibited mean trend elevations ($p=0.12$, $p=0.11$ respectively), and TNFα was statistically elevated ($p<0.05$). Other trends of note include a significant reduction ($p<0.05$) in IL-1β at 2 months and a significant reduction ($p<0.05$) in IL-1α, IL6, and CXCL1 at 3 months (Figure 4).

d) Discussion

The preliminary data necessitates further investigation, as some features are consistent with a profile of senescence while others remain either contradictory or inconclusive. p53 is a crucial upstream regulator of many of the senescence pathways, so its significant elevation at the 2-week, 1-month, and 2-month timepoints is certainly of import (Figure 2). Since p53 is, however, a prolific apoptotic marker involved in multiple cell signaling pathways, its elevation cannot be exclusively linked to cellular senescence. There is evidence to suggest that senescence and apoptosis are linked, since both processes are known to be critical during embryonic development and the inhibition of either one triggers comparable compensatory responses (Muñoz-Espin, et al. 2014); p53 is thus reinforced as an intriguing marker related to senescence but is far from a definitive element. The upward

trend observed in p27 levels at the 2 and 3-month timepoints would correlate with the possibility of senescence as well, but its data did not achieve statistical significance.

As a specific marker of several senescence pathways, p16 was expected to be elevated at the acute injury timepoints, so the changes observed in p16 levels demand additional inquiry. Typically, age-related sarcopenia, a decline in muscle mass, is associated with upregulated p16, and targeting the cells expressing p16 can alleviate these observed effects (Muñoz-Espin, *et al*. 2014). It is therefore especially surprising to record no noticeable alterations in p16 at the early timepoints post-SCI but even more puzzling to observe the trend toward decline at the 2-month timepoint and the statistically significant decline at 3 months post injury. Of note, a 2009 study indicated that p53 deficiency can be connected to p16 upregulation; in turn, raising p53 levels back to their normal state was shown to reduce p16 to its original range. Though p53 was not shown to have a definitively repressive effect on p16, a potential relationship between these two tumor-suppressive markers was supported (Leong, *et al.* 2009). As for our data, the significant decline in p16 levels occurs at the 3-month timepoint, precisely when p53 levels return to normal range after their significant elevation throughout the 2-week, 1-month, and 2-month timepoints. The relationship between p53 and p16 in our data remains unclear, but there may be reason to consider the possibility of a time-dependent shift in regulators of different cellular senescence pathways.

TNFα's statistically elevated expression level at the 1-month timepoint does correspond to the general SASP profile of cellular senescence. The elevation trends observed

in IL-1β and IL-6 at the same timepoint are weakly supportive. At 2 months post-SCI, though, these cytokine expression levels would still be expected to be raised; the significant decline in IL-1β at this timepoint is therefore counterintuitive, as well as is the lack of observable trend in any of the other cytokines assessed. The significant reductions in IL-1α, IL6, and CXCL1 at 3 months is similarly problematic. It is possible that there may be interactions between other senescence regulators and the patterns of cytokine expression. Such crosstalk may be elucidated using conditional protein knockdowns in SCI mouse models, to clarify the degree to which it could have influenced the demonstrated results. Regardless, the patterns observed imply that cellular senescence is not predominantly implicated at these acute and subacute timepoints post-SCI.

The markers analyzed in this pilot study were intended to serve as an introductory exploration into the possible connection between senescence and SCI and should not be taken as conclusive determinants. Future areas of study should include more specific markers of senescence, such as p21 which is activated by p53 in multiple senescence pathways and can be considered a more indicative factor than p53 in isolation. Blotting for RB, the protein product of the tumor suppressor retinoblastoma gene, would also be a relevant assay, because activation of RB is downstream of many of the pathways of damage-induced senescence. Staining for SAβGAL (senescence-associated beta-galactosidase) is an important next step as well, because SAβGAL activity is considered to be one of the hallmarks of cellular senescence. Overall, potential interactions between the various markers should always be considered and, as noted, there may even be time-dependent shifts as changes in some early markers influence the levels of later markers (Graham, *et al*., 2020).

IV. Conclusion

Cellular senescence, certainly an enigmatic process, may be more involved in the pathogenesis of various disease states than once would have been anticipated. At the same time, the positive homeostatic function of senescence should not be underestimated. Only a careful balancing of the two apparently opposing perspectives reveals the true utility of senescence and allows modern science to capitalize upon its gains while controlling its detriments. The nascent field of senotherapeutics represents great promise in the management of age-related deficits. Developing research in this area will continue to refine the application of senescence in cancer treatment as well. Next steps must include further delineation of senescent models in vivo and additional determination of associated molecular signatures. These advances will continue to clarify the significance of senescence and sharpen existing senotherapeutic strategies.

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VI. References

Akincilar, S.C., Unal, B., and Tergaonkar, V., 2016, Reactivation of Telomerase in Cancer, Cellular and Molecular Life Sciences, 73:1659–1670.

Bernadotte, A., Mikhelson, V.M., and Spivak, I.M., 2016, Markers of Cellular Senescence. Telomere Shortening as a Marker of Cellular Senescence, Aging, 8:3–11.

Brill, A., *et al*, 1999, The Role of Apoptosis in Normal and Abnormal Embryonic Development, Journal of Assisted Reproduction and Genetics, 16:512–519.

Cameron, I. and Middleton, J., 2014, Ageing with Spinal Cord Injury, *www.aci.health.nsw.gov.au*.

Carter, A. and Nguyen, A.Q., 2011, Antagonistic Pleiotropy as a Widespread Mechanism for the Maintenance of Polymorphic Disease Alleles, BMC Medical Genetics, 12:160.

Childs, B.G., *et al.*, 2015, Cellular Senescence in Aging and Age-Related Disease: from Mechanisms to Therapy, Nature Medicine, 21:1424–1435.

Childs, Bennett G., *et al*., 2017, Senescent Cells: An Emerging Target for Diseases of Ageing, Nature Reviews Drug Discovery, 16:718–735.

Ewald, J.A., *et al*., 2010, Therapy-Induced Senescence in Cancer, JNCI: Journal of the National Cancer Institute, 102:1536–1546.

Graham, Z.A., *et al.*, 2020, Contusion Spinal Cord Injury Upregulates p53 Protein Expression in Rat Soleus Muscle at Multiple Timepoints but Not Key Senescence Cytokines, Physiological Reports, 8:e14357.

Hayflick, L., and Moorhead, P.S., 1961, The Serial Cultivation of Human Diploid Cell Strains, Experimental Cell Research, 25:585–621.

Jafri, M.A., *et al*., 2016, Roles of Telomeres and Telomerase in Cancer, and Advances in Telomerase-Targeted Therapies, Genome Medicine, 8:69.

Kahlem, P., Dorken, B., and Schmitt, C.A, 2004, Cellular Senescence in Cancer Treatment: Friend or Foe?, Journal of Clinical Investigation, 113:169–174.

Leong, W. F., Chau, J.F., and Li, B., 2009, p53 Deficiency Leads to Compensatory Up-Regulation of p16INK4a, Molecular Cancer Research, 7:354–360.

Muñoz-Espín, D. and Serrano, M., 2014, Cellular Senescence: from Physiology to Pathology, Nature Reviews Molecular Cell Biology, 15:482–496.

Myrianthopoulos, V., 2018, The Emerging Field of Senotherapeutic Drugs, Future Medicinal Chemistry, 10:2369–2372.

Ogrodnik, M., Salmonowicz, H., and Gladyshev, V.N, 2018, Integrating Cellular Senescence with the Concept of Damage Accumulation in Aging: Relevance for Clearance of Senescent Cells, Aging Cell, 18:e12841.

Ritschka, B., *et al*., 2017, The Senescence-Associated Secretory Phenotype Induces Cellular Plasticity and Tissue Regeneration, Genes & Development, 31:172–183.

Roche, J., 2018, The Epithelial-to-Mesenchymal Transition in Cancer, Cancers, 10:52.

Schosserer, M., Grillari, J., and Breitenbach, M., 2017, The Dual Role of Cellular Senescence in Developing Tumors and Their Response to Cancer Therapy, Frontiers in Oncology, 7:278.

Storer, M. and Keyes, W.M., 2014, Developing Senescence to Remodel the Embryo, Communicative & Integrative Biology, 7:e970969.

Tomás-Loba, A., *et al*., 2008, Telomerase Reverse Transcriptase Delays Aging in Cancer-Resistant Mice, Cell, 135:609–622.

Velarde, M.C. and Demaria, M., 2016, Targeting Senescent Cells: Possible Implications for Delaying Skin Aging: A Mini-Review, Gerontology, 62:513–518.

Wang, E., 1995, Senescent Human Fibroblasts Resist Programmed Cell Death, and Failure to Suppress Bcl2 Is Involved, Cancer Research*,* 55:2284-92.

Wang, M., *et al*., 2017, Role of Tumor Microenvironment in Tumorigenesis, Journal of Cancer, 8:761–773.

Weiland, T., *et al*, 2013, Enhanced Killing of Therapy-Induced Senescent Tumor Cells by Oncolytic Measles Vaccine Viruses, International Journal of Cancer, 134:235–243.

Zeng, S., Shen, W., and Liu, L., 2018, Senescence and Cancer, Cancer Translational Medicine, 4:70-74.

Zhu, Yi, *et al*., 2015, The Achilles' Heel of Senescent Cells: from Transcriptome to Senolytic Drugs, Aging Cell, 14:644–658.