ROLE OF CELLULAR SENESCENCE IN AGE-RELATED INTERVERTEBRAL DISC DEGENERATION

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Aging is one of the major risk factors for intervertebral disc degeneration (IDD). IDD is characterized by loss of disc matrix homeostasis due to decreased matrix synthesis and increased matrix breakdown. Elevated oxidative burden is closely associated with age-related IDD, and cellular senescence has been suggested as the mechanism by which oxidative stress perturbs the balance between disc anabolic and catabolic matrix homeostasis. Cellular senescence is characterized by a state of irreversible growth arrest and chronic secretion of increased inflammatory cytokines and matrix proteases. Increased number of senescent cells have been recorded with advancing age in discs of rodents and humans. In vitro studies have confirmed the catabolic nature of stress-induced senescent disc cells in promoting matrix homeostatic imbalance. However, the underlying molecular mechanism of senescent disc cells which enable their secretory phenotype and whether cellular senescence is causal in driving age-related IDD is yet to be ascertained. Herein, we examined the metabolic changes in oxidative stress-induced senescent (SIS) disc cells. Compared to non-senescent disc cells, SIS disc cells, in addition to acquiring a catabolic phenotype characterized by elevated fragmentation of aggrecan and collagen II and expression of IL-6 and IL-8, had upregulated mitochondrial content and ATP-linked respiration.

This increase in mitochondrial ATP-linked respiration was driven by increased protein secretion in SIS disc cells because abrogation of protein synthesis using cycloheximide suppressed the mitochondrial ATP-linked respiration. As disc cells are known to rely on glycolysis, this result revealed heretofore unknown metabolic flexibility in disc cells to adapt to meet the underlying energy demand of their secretory phenotype. Next, we elucidated if a causal relationship existed between senescence and age-related IDD by examining the discs of p16-3MR transgenic mice following selective clearance of $p16^{INK4a*}$ senescent cells by the drug ganciclovir (GCV). In aged p16-3MR mice, treatment with GCV lowered the levels of disc MMP13, a major matrix protease, and reduced disc fragmentation of aggrecan. GCV-treated old mice also exhibited increased disc aggrecan and improved histological disc features. Altogether, these results suggest that cellular senescence adversely impacts the disc tissue and can serve as a therapeutic target to attenuate the age-related IDD.

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PREFACE

The past five and half years of graduate school have had a tremendous impact on my professional growth as well as personal life. First and foremost, I would like to thank my mentor, Dr Nam Vo, for giving me the opportunity to join his lab. It is because of his encouragement and support that I have been able to bear the multiple ups and downs that are part and parcel of graduate school. The numerous opportunities to attend and present at conferences have helped me become a better scientist. I am truly grateful to him for teaching me good science and fostering a desire for excellence in research.

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1.0 INTRODUCTION

1.1 BIOLOGY OF INTERVERTEBRAL DISC

Intervertebral discs are situated between adjacent vertebrae and consists of an outer fibrosus annulus fibrosus (AF) that enclose a gelatinous nucleus pulposus (NP). The discs are present between vertebrae and connected to them by cartilaginous endplates (CEP)[1]. The AF possesses highly organized lamellae of collagen type I fibrils that run parallel to one another. The AF functions primarily to resist the tensile forces generated during bending or twisting and to bear the circumferential stresses required to restrain NP swelling. Unlike the AF, the NP is composed of proteoglycan aggregates encased in randomly organized loosely organized networks of collagen type II and elastin. The NP functions mainly to counteract the axial compression with large swelling pressure [1], [2].

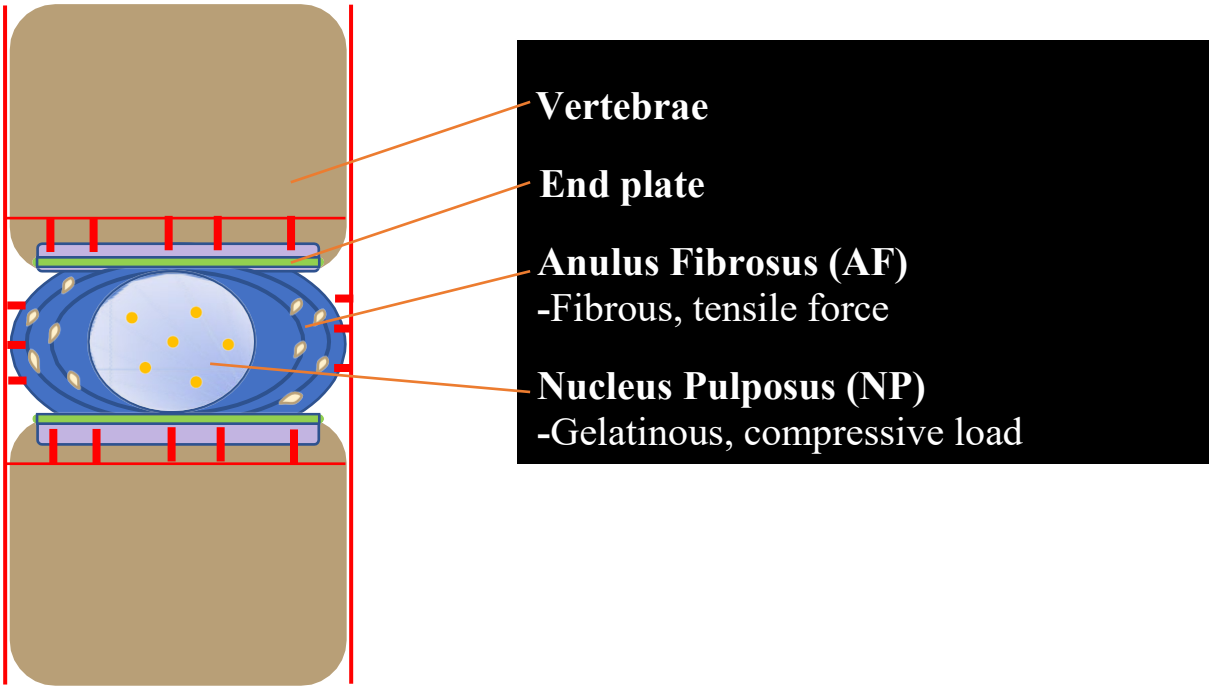

Figure 1. Anatomy of intervertebral disc (IVD) and surrounding structures [3]
This sketch represents a functional spinal unit consisting of vertebrae, IVD, and endplates. The IVD consists of the inner gelatinous nucleus pulposus (NP) and the outer fibrous annulus fibrosus (AF). While the NP serves to counteract the compressive forces and distribute them evenly throughout the disc, the AF functions to resist the tensile forces generated during bending and stretching (adapted from [3]).

The swelling pressure of the discs emanates from the residing proteoglycans. Although both NP and AF possess proteoglycans, NP has the highest proteoglycan content [4], [5]. NP possesses a variety of proteoglycans in its extracellular matrix, with aggrecan being by far the most abundant proteoglycan. Aggrecan possess a large core protein with over 2000 amino acids. It contains three globular domains – G1, G2, and G3. The G1 domain binds to the hyaluronan chain. The link protein stabilizes the interaction between G1 and hyaluronan. The interglobular domain (IGD) separates G1 and G2 and contains sites for proteolytic cleavage. There are several chondroitin and keratan sulphate glycosaminoglycan (GAG) chains between G2 and G3. These GAG chains are negatively charged and can attract counterions and imbibe water, and can

provide the high osmotic pressure needed in resisting compressive loads [6] [7]. Several aggrecan monomers are found to bind to the hyluronan chain and form aggregates.

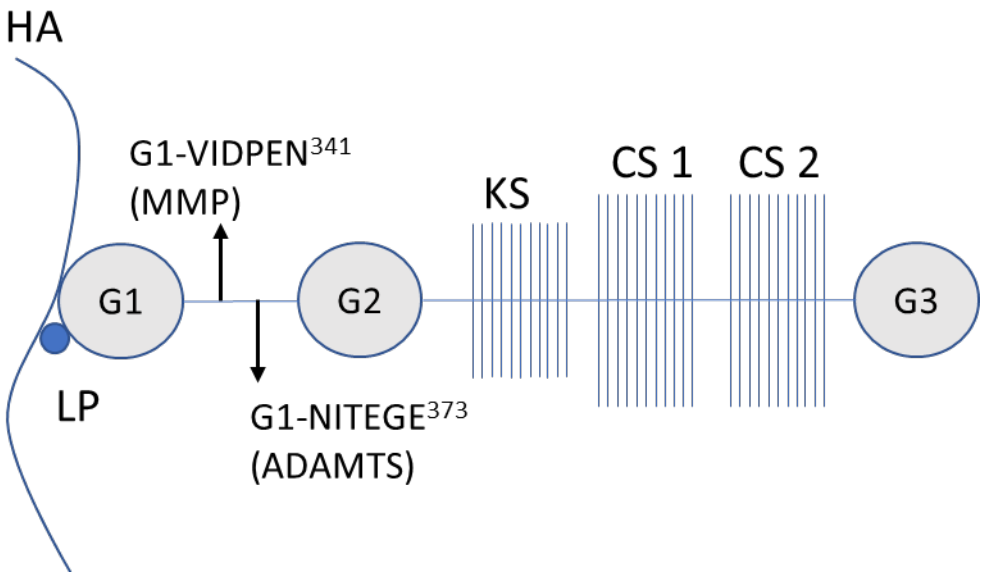

Figure 2. Aggrecan components and organization in the IVD
The aggrecan core protein contains three globular domains- G1, G2, and G3- to which glycosaminoglycans such as chondroitin sulphate (CS) and keratin sulphate (KS) are covalently attached. The aggrecan is present in aggregates that forms via binding of G1 to hyluronic acid (HA). This interaction is mediated by the link protein (LP). Known cleavage sites of proteases MMPs and ADMATSs on aggrecan are indicated (adapted from [3]).

In addition to proteoglycans, the disc contains multiple collagen types. The annulus fibrosus possessing mostly type I collagen and the nucleus pulposus possesses mostly type II collagen [8]. Perturbation in either synthesis or proteolytic cleavage of matrix aggrecan and collagen results in weakened functional ability of disc tissue. Indeed, increased fragmentation of aggrecan and collagen is seen in disc degeneration [9]. Two class of proteases have been shown to play a major role in degradation of matrix proteins in disc: matrix matelloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) [10], [11]. Specifically, MMP -1,-3,-13 and ADAMTS -4, -5 account for much of the degradation of matrix proteins in the disc tissue.

The cells that populate and synthesize the distinct extracellular matrices in disc include chondrocyte-like cells in NP and fibroblast-like cells in AF [12]. This difference is due to the fact that the AF develops from the mesenchyme and the NP develops from the notochord [13], [14]. In the embryonic disc tissue, there is a clear demarcation in the cellular phenotype of NP, which is rich in notochordal cells, and the AF, rich in fibroblasts like cells. However, this difference in phenotype is abolished as the notochordal cells in NP are replaced with cells that are of mesenchymal origin [15]. Consequently, the cells that populate the AF are more fibroblast -like, whereas the NP contain cells that are more spheroidal and chondrocyte-like. The disc tissue does not contain a high cell density and the NP and AF of a mature disc contain $4x10^6$ cells/cm^3 and $9x10^6$ cells/cm^3 cells, respectively [1].

The disc cells require adequate nutrients to stay alive and function. Nutrients travel into disc tissue via two distinct routes- a) diffusion from capillaries that terminate adjacent to the cartilaginous endplate and b) diffusion from capillaries that innervate the outer parts of AF [16]. Consequently, the concentrations of glucose, oxygen, and other nutrients are lowest at center and highest at the periphery of the disc; the cells in the center of a human disc can be 6-8 mm from the nearest blood supply [17]. Disc cells adapt to this hypoxic environment by utilizing the hypoxia signaling governed by the activities of the transcription factor hypoxia-inducible factor-1α (HIF-1α). The disc NP cells are also unique in that they constitutively express HIF-1α [18]. This stabilization of HIF-1α is found to be crucial to the survival and expression of genes that are characteristic of NP cells. These genes include aggrecan, the major proteoglycan in the disc, galectin-3, β-1,3-glucuronyltransferase 1, and VEGF-A [19]. In addition, one of the logical outcomes of HIF-1α stabilization is that the disc cells are completely reliant on glycolysis to generate ATP and rely very little on aerobic respiration even when oxygen is abundant [18],

[20]. Treatment of NP cells with 2-deoxy glucose (2DG), a glycolysis inhibitor, results in significant depression in ATP synthesis, whereas treatment with mitochondrial inhibitor antimycin a does not impact ATP production [18]. Nevertheless, disc NP cells do contain mitochondria with normal architecture which retain the capacity to metabolize fatty acids and perform mitochondria-dependent functions [18], [21].

1.2 INTERVERTEBRAL DISC DEGENERATION AND BACK PAIN

Intervertebral disc degeneration (IDD) contributes to many pathological conditions of spine. One of the major hallmark of disc degeneration is loss of aggrecan content. The reduced aggrecan content leads to reduced hydration, and consequently to reduced disc height and increased fissures. Degradation of aggrecan, the major proteoglycan of the disc, occurs primarily via action of MMPs and ADAMTSs [10], [11], [22], [23]. In the degenerated discs, 55 kDa and 70 kDa aggrecan fragments due to cleavage by the MMPs and ADAMTSs, respectively, within the IGD region are predominantly found [24]. Consequence of this proteolytic cleavage by either of the proteases is that two fragments are generated - one fragment will possess the G1 region and remain bound to the HA, whereas the other G2-G3 fragment containing GAG that is essential for attracting water is no longer able to interact with the HA and is free to diffuse in the extracellular matrix (ECM) [7], [10]. The aggrecan fragment that is bound to hyaluronan impedes the proper functioning of the disc as it cannot participate in resisting compression due to the loss of its GAG chains. The other proteolytic fragment of aggrecan, which is able to diffuse freely within the ECM, may still help in resisting compressive loads only if it does not undergo further proteolytic processing and loss by diffusing out of the disc via the tissue fissures. Although less obvious

than the changes to proteoglcyans, the collagen II in the NP also undergo denaturation due to the action of proteases such as MMP -1, -8, -13 [25], [26]. As a result, the damaged collagen molecules that persist in the disc tissue cannot contribute fully and appropriately to the biomechanical function of the disc.

The lower proteoglycan content of the NP have major impact on the disc's ability to bear load [27]. The loading of a disc with lower proteoglycan content results in loss of height and fluid more rapidly than compared to normal disc [28], [29]. Such loading of a degenerative discs results in distribution of stress inappropriately to other adjacent structures. In accordance with this, the later stages of IDD include loss AF lamellae structure, disorganization of the AF collagen and elastin networks, increased AF fissures, and formation of neo-osteophytes in the adjacent cartilaginous endplate and vertebrae [14], [30].

It has become imperative to understand the mechanism that contribute to IDD as it is one of the predominant contributor to chronic back pain, a debilitating disorder that erodes the quality of life for those who suffer from it. Basic science studies have begun exploring mechanisms that contribute to IDD and which eventually lead to back pain. From multiple studies, a narrative regarding IDD leading to back pain has emerged- following insult or injury [31]–[36], disc cells upregulate expression of inflammatory cytokines and chemokines, such as TNFα, IL-1β, IL-6, IL-8 as well as IL-17 and CCL [37]–[41], which suppress matrix protein production and promote synthesis of matrix proteases (MMPs and ADAMTSs), and consequently, the breakdown of predominant matrix proteins such as aggrecan and collagen II [40], [42]–[45]. If the cycle of cytokine secretion and protease production continues, the disc undergoes severe degenerated changes resulting in compromised functional ability and herniation. The chemokines and the cytokines increase disc cells expression of the neurotrophins

NGF and BDNF and induce nerve ingrowth, and act directly on dorsal root ganglions (DRG) to induce expression of the pain associated cation channels like ASIC3, a pH sensitive Na^+ channel associated with ischemic and inflammatory pain, and TrpV1, a nociceptive cation channel [46], [47]; activation of TrpVI and ASIC3 likely contributes to back pain [48].

1.3 CAUSES OF INTERVERTEBRAL DISC DEGENERATION

The etiology of IDD is complex and multifactorial. IDD is a commonly associated with age, genetic predisposition, and occupational hazards. Recent studies have shown association between genetic influences and disc degeneration. So far, gene polymorphism of the collagen I, collagen IX, collagen XI, VDR, and aggrecan gene have been associated with disc degeneration [49], [50]. However, it should be noted that the magnitude of the influence of the polymorphism of the aforementioned genes is modest. Furthermore, the genetic studies conducted so far have included only certain demographics and included small sample sizes. Therefore, increasing the sample size and conducting the studies in various other populations is still warranted to derive at a definite genetic and disc degeneration association. Mechanical insults and environmental factors resulting from occupation, sporting injuries, cigarette smoking and atherosclerosis have been touted to promote disc degeneration as well [12], [51]. Like the genetic studies, no strong causal link between heavy work/trauma and disc degeneration has been found. For instance, lowering the time spent heavy lifting or ergonomic interventions have not reduced the incidences of disc degeneration [52]. Additionally, no greater increase in disc degeneration is found in people with extreme smoking history [53]. If not genetic, mechanical injury or environmental factors, what factor greatly influences disc degeneration? Several studies have reported that the occurrence

rate of disc degeneration significantly increases with age. Increase in disc degeneration from 0% at 20 years of age to approximately 90% at age 70 years was found in 600 autopsy specimens [54]. Similarly, investigation of 1000 autopsy specimens found the rate of degenerative findings to increase to about 72% by the age of 70 years in men; women reached the same grade of degeneration as men about 10 years later [53]. Disc degeneration has been shown to initiate as early as second decade of life [55]. By the fifth decade, 97% of all discs show some degree of degeneration, with 50% demonstrating severe grades of degeneration [54]. The intervertebral disc undergoes striking alteration in volume, shape, structure, and composition with age that ultimately compromise motion and mechanical properties of spine. Infact, spine related pain that limits mobility are the most common causes of impairment in the middle aged and in the elderly. Basic scientific studies have begun to shed light on the underlying changes and the mechanisms that promote the gross morphological changes in the disc tissue with age. The section below discusses the results of such studies conducted so far.

1.3.1 AGING IS A MAJOR DRIVER OF IDD.

The age-related morphological changes in disc tissues include reduction in disc height, disappearance of vascular channels originating from the CEP, increased number of fissures and granular debris, and neovascularization of the peripheral AF lamellae inwards [55]. The clear gelatinous NP is transformed into a fibrous tissue with age. The distinction between AF and NP becomes progressively blurred with ageing. Ossification and thinning of the CEP are also found with increasing age [56].

As in case of IDD, the reduction in disc height with ageing is due loss of proteoglycan content, and consequently, its water content. The decrease in proteoglycan content in the disc

tissue is because of both decrease in synthesis capacity as well as increase in fragmentation and loss of the aggrecan with age[9], [31], [57]. Increased presence of denatured collagen II, resulting from proteolytic action of collagenases (MMP -1, -8, -13), is also found in aged discs [26], [57], [58]. Another major alteration in ECM that occurs with age is increase in collagen I synthesis and decrease in collagen II synthesis in the NP of the disc [55], [59]. These changes collectively weaken the ability of the disc tissue to function normally with age.

The remodeling of the ECM with age in disc tissue suggests that the homeostatic balance between the anabolic and catabolic activities is altered. The protagonists of this imbalance are most likely the MMPs and ADAMTSs. Indeed, expression of certain matrix metalloproteinases, including MMP-1,-3 and ADAMTS-5 is elevated in aged discs compared to young discs [60]–[62]. The disc cells are capable of producing the proteases upon induction by pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, and IL-8. These inflammatory cytokines promote expression of ADAMTS-4 and -5, and MMP-1, -2, -3, -4, -13, and -14, and decrease expression of anabolic ECM proteins aggrecan and collagen II in disc cells [37], [40], [43]–[45]. In rat and bovine organ culture models, treatment with TNFα results in suppression of multiple collagen types, aggrecan, fibromodulin, increased expression of MMPs, ADAMTSs, pain-associated molecule nerve growth factor (NGF), and compromised disc biomechanics [63]–[65]. IL-1ra$^{-/-}$ mice, which lack the functional IL-1ra, an endogenous antagonist of IL-1R, display greater loss of proteoglycans and collagens, and exhibit increased expression of MMPs 3 and 7 and ADMTS4 in their IVD [66]. The discs of IL-1ra$^{-/-}$ mice also have higher grade of histological degeneration compared to wildtype mice. Importantly, expression of TNFα and IL-1β and their respective receptors has been shown to increase with severity of aging in human and animal models. Together, these studies suggest that the inflammatory cytokines are major drivers of the

cascade of matrix protein degradation that occurs with ageing in the disc tissue. Part of the inflammatory response in disc observed during aging may be driven by the infiltrating immune cells like macrophages. However, the resident disc cells are also capable of producing IL-6, IL-1β and TNFα in the context of disc degeneration independent of infiltrating leukocytes [67]. Presently, it is still unclear to what extent infiltrating immune cells and resident disc cells contribute to the production of the inflammatory cytokines observed in aged discs.

The increased inflammatory burden in disc tissue with age is postulated to be propagated by oxidative stress. There is ample evidence of increased oxidative stress in disc aging and degeneration. Malondialdehyde (MDA), a secondary product of peroxidation of polyunsaturated fatty acid residues, was found to be significantly higher in the discs of geriatric rats compared to those in adult rats [68]. Accumulation of intermolecular cross-links in long lived proteins such as collagen, a result of oxidative reactions, with age in disc is also well documented. One of the pathways for cross-link formation is via oxidative modification of glycated proteins, resulting in the formation of advanced glycation endproducts (AGEs) such as CML and pentosidine. Accumulation of both pentosidine and CML was increased in aggrecan and collagen with aging in human disc tissue [69]–[72]. CML was found to be accentuated in degenerative region of disc tissue. In another study, redox proteomic analysis revealed that the disc of aged mice contained higher levels of oxidized amino acids than discs of younger mice. Importantly, oxidized matrix proteins were found to be more susceptible to degradation by MMP -1, -2, -9 and -13 proteases [73]. Additionally, numerous *in vitro* studies have shown that treatment of human and rat disc cells with oxidants such as hydrogen peroxide induces expression of matrix proteases and inflammatory cytokines with concomitant suppression in the expression of aggrecan and collagen matrix proteins [74]–[76]. Importantly, abrogation in disc degenerative markers was

seen in rat NP cells, rabbit annulus puncture model of disc degeneration, and in progeroid mouse model of disc degeneration upon treatment with antioxidants [77]–[79]. It is still unclear what endogenous and exogenous factors mediate the increase in oxidative burden and consequently inflammatory stress and matrix degradation in disc tissue with age. Regardless of the event(s) that start the trend towards impaired anabolic and catabolic balance, the functionality of the disc tissue declines as a result.

1.4 THERAPEUTICS TO TREAT IDD

Since disc degeneration involves the loss of balance between the anabolic and catabolic events, therapeutic targets for treating or delaying age-related disc degeneration have focused on either promoting the synthesis of matrix proteins that support the function of the disc or minimizing the aberrant damage response that exacerbate tissue damage. Protein-, gene-, and cell-based strategies have been explored to counter age-related changes to the disc tissue. Growth factors such as TGFβ, BMP-2, GDF-5, IGF-1 have been shown to stimulate synthesis of aggrecan, collagen II, and enhance glycosaminoglycan content in disc cells in vitro [80]. The rationale for using growth factors for treatment and prevention of disc degeneration lies in their capacity to enhance matrix synthesis. Modest improvement in aggrecan, collagen II, and restoration of disc height has been reported upon injection of TGFβ and rhGDF-5 into discs of mice and rabbits induced to undergo degeneration via static compression or via annular puncture, respectively [80]. One of the major limitations of growth factor therapy for disc degeneration is that the half-life for these therapeutic proteins is on the order of few minutes, and this limits its clinical utility for a chronic disorder like disc degeneration. Additionally, several of the growth factor therapy

studies utilize younger aged animals. It therefore is still unclear if growth factor therapy benefits only early stages of degeneration or the improvement in disc will occur if the growth factors are injected into an older aged animal as well. Unlike the growth factor therapy, the goal with gene therapy approaches is to provide long term expression of matrix proteins. The gene therapy studies done so far have involved introduction of MMP and ADAMTS inhibitors or growth factors that promote matrix protein synthesis via direct application of viral vector constructs or via injection of disc cells that have been transduced with these factors in vitro [81]. Though these studies have been promising, several complications including long term assessment of the expression of the transgenes, feasibility in humans, and cost associated with such therapies are yet to be explored. Much like the gene therapy approach, the goal of cell-based therapy is to restore the loss of anabolic synthesis capacity of disc cells and regenerate a biologically active environment. The majority of the investigations carried to date have utilized bone marrow derived mesenchymal stem cells (BM-MSCs) either alone or in combination with carrier agents and have shown modest improvement in proteoglycan content, annulus fibrosus structure, and disc height upon implantation of the BM-MSCs [82], [83]. Despite these encouraging results, it is still unclear whether the injected cells only provide a transient response or whether they exert a sustained response, the mechanism by which they act on disc, the ability to survive in disc which faces loss of capillaries, and hence nutrients, with age, and the risk of ectopic ossification or calcification.

In addition to aforementioned therapeutic strategies, multiple studies have assessed the impact of anti-oxidative and anti-inflammatory strategies to ameliorate the cascade of age-related degradative changes in the disc. Systemic administration of a mitochondria-targeted ROS scavenger, XJB-5-131, to the *Ercc1*$^{-/\Delta}$ mice, which are deficient in the DNA repair enzyme

Ercc1 and recapitulate several features of age-associated disc degenerative changes, resulted in significant improvement in the glycosaminoglycan content as well the proteoglycan synthesis capacity [77]. Similarly, intradiscal injection of anti-oxidant fullerol increased water and proteoglycan content and inhibited ectopic bone formation in discs of rabbits induced to undergo disc degeneration by stab puncture of the annulus fibrosus [78]. It was shown via in vitro studies that fullerol was able to reverse the suppression of aggrecan and glycosaminoglycan in human nucleus pulposus cells treated with hydrogen peroxide or IL-1β. A number of other anti-oxidants such as curcumin, resveratrol, pyrroloquinoline have been shown to rescue loss of proteoglycan synthesis and reduce catabolism of aggrecan via inhibition of catabolic factors including IL-6, IL-1β, IL-8, MMP-1,-3, and -13 [79], [84]–[86]. These studies demonstrate yet again that oxidative stress is one of the key drivers of age-associated disc degeneration and oxidant scavengers may play a promising role in slowing disc aging. In addition to anti-oxidants, several anti-inflammatory agents have been reported to be therapeutic for treating IDD as well. Application of IL-1Ra, an endogenous antagonist of IL-1 receptor, and etanercept, TNFα inhibitor, to tissue explants or rat discs resulted in reduced biochemical markers of disc degeneration, such as IL-1 and IL-6 [87]. Another strategy to lower the inflammatory burden and the ensuing degenerative changes has focused on targeting pathways or molecules that induce expression of the inflammatory factors. One such candidate is the NF-κB pathway whose chronic activation has been implicated in several age-related pathologies. The NF-κB activity has been found to accompany the increase in oxidative stress with ageing in disc and several of the cytokines involved in disc degeneration, including TNF-α, IL-1β, IL-6 and IL-8, are considered typical NF-κB target genes [88]. Indeed, previous studies from our lab have shown that the *Ercc1*$^{-/\Delta}$ mouse with genetic and pharmacological block of NF-κB activity, have higher

proteoglycan content and GAG content compared to untreated controls. [89]. Furthermore, injection of "NF-κB decoy oligonucleotides" resulted in amelioration of degenerative changes in animal model of degeneration [90]. Together these reports suggest a promising alternative to the invasive cell and gene therapy approaches. However, the precise mechanism by which the anti-oxidant and anti-inflammatory agents elicit the anabolic activity while suppressing the catabolic events in disc has yet to be investigated. Moreover, disc aging probably does not occur in isolation and is likely influenced by systemic changes. Therefore, it is essential to investigate whether improvement in disc structure and function is plausible with strategies that aim to delay or prevent disorders prevalent during ageing. Equally important is to conduct studies to identify certain common age-related changes in different organ systems as this will enable development of therapeutics which can be beneficial in improving health of multiple tissues.

1.5 CELLULAR SENESCENCE AND AGING

1.5.1 HISTORY OF CELLULAR SENESCENCE

Cellular senescence is a state of irreversible growth arrest that can be induced by a wide variety of stressors [91]. Cellular senescence was first reported by Hayflick and colleagues after they observed that human fibroblasts replication ceased after a finite number of divisions [92]. They termed this phenomenon as replicative senescence. Despite supply of nutrients and appropriate growth conditions, the replicative senescent cells did not proliferate further. The study included two contradictory speculations. First, it speculated that the dysfunctional telomeres, that result because of successive replications and contribute to malignant transformation of cells, can be

prevented if the cells senesce. In this context, cellular senescence protected organisms from cancer, and hence, was beneficial. The second speculation proposed cellular senescence to promote ageing as it could limit the proliferative capacity of the resident cells and thus could contribute to decline in tissue regenerative ability.

The answer to the paradox of cellular senescence as both a beneficial and detrimental phenomenon is explained by the antagonistic pleiotropy theory [91]. This posits that processes that are beneficial in the younger organisms (example, tumor suppression) can be deleterious in older organisms (example, senescent cells that escape clearance or accumulate). There is now substantial evidence that cellular senescence can promote both tumor suppression and age-related pathologies.

In the ensuing decades since replicative senescence was described by Hayflick *et. al*, it has become apparent that a plethora of stressors can induce cellular senescence. Overexpression of oncogenes in normal cells can induce senescence, a phenomenon termed as oncogene-induced senescence (OIS). Cellular senescence induction also occurs upon epigenetic modifications as well. Lastly, DNA-damaging stresses have been shown to induce senescence as well. Such stresses include UV and IR radiation, drugs generating DNA breaks such as bleomycin and ciplastain, and agents capable of generating oxidative stress such as hyperoxia, hydrogen peroxide, tert-butyl hydroperoxide etc. [91]. These different stressors all engage the DNA damage response (DDR) pathway [93]. In fact, cellular senescence is viewed as the outcome of a protracted DDR. The DDR in stress induced senescent human fibroblasts is found to persist for days, months in some cases, and is essential for sustenance of the growth suppressive mechanisms [94].

The diverse stimuli that induce senescence response seem to converge on two pathways to mediate the DDR and establish and maintain growth arrest. p53-$p21^{Cip1}$-Rb and $p16^{INK4a}$-Rb pathways are central to induction of senescence [95]. p53-$p21^{Cip1}$-Rb and $p16^{INK4a}$-Rb are the two well-known and extensively studied pathways in context of cell cycle control, tumor suppression, and cellular senescence. p53 guards genomic integrity by regulating several cellular processes, including cell cycle arrest, DNA repair, apoptosis and senescence, resulting from various stress signals. Upon genomic damage induced by endogenous and exogenous stressors, the ATM protein undergoes autophosphorylation and activation. The active ATM protein phosphorylates p53 directly to activate it or indirectly via phosphorylating Chk1 and Chk2 proteins [91]. H2A.X, a variant of the H2A histone protein family and a component of the histone octomer in nucleosomes, is another downstream target of ATM and is activated by phosphorylation by ATM. The phosphorylated protein, γH2A.X, serves to recruit and repair proteins to the DNA damage site. Persistent DNA damage response (DDR) is needed to maintain the cellular senescence phenotype [94], [96], [97]. p53 also stimulates expression of the cyclin-dependent kinase inhibitor (CDK1) $p21^{Cip1}$, which prevents cyclin-dependent kinase-2 (CDK2) mediated phosphorylation of retinoblastoma protein (pRb). Consequently, the cell cycle progression from G1 to S is inhibited as the hypo-phosphorylated pRb blocks the transcriptional activity of the E2F factor to promote the expression of the genes necessary for G1 to S progression [91]. Similarly, expression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ is increased by numerous cell stressors including oxidative stress, inappropriate activation of signaling pathways, oncogene activation, and telomere shortening. $p16^{INK4a}$ binds CDK4/CDK6 and suppresses the association of CDK4/6 and cyclin D. This leads to disruption of D-type

cyclins. This consequently leads to hypophosphorylation of pRB and suppression of G1 to S phase progression [95].

It is not clear whether both the p53-p21^{Cip1}-Rb and p16^{INK4a}-Rb pathways are needed to establish senescence in response to diverse stimuli. The consensus is that p53 mediates senescence primarily due to telomere dysfunction and DNA damage and p16 ^{INK4a} mediates senescence primarily due to oncogenes, chromatin disruption, and other stressors [98]. However, currently, there are no studies which unambiguously provide evidence to support this consensus. Upregulation of both the pathways is found in different human cell strains induced to undergo replicative senescence, OIS, and senescence induced by stressors such as hydrogen peroxide, hyperoxia, U.V, and IR [99] [100][98]. It may be that cells within a tissue may contain a mosaic of cells that senesce either due to expression of p53 or p16 or both. The reports published on the roles of these pathways in regulating cellular senescence thus far are still fragmented and incomplete.

1.5.2 CHARACTERISTICS OF SENESCENT CELL

Senescent cells in culture and in vivo are identified by several different markers. However, no single specific marker is exclusively present in all senescent cell types; aggregate of markers is typically used in confirming senescent cells. Senescent cells adopt a “fried egg morphology” and increase in cell size [92]. The senescent cells have increased lysosomal content, and this results in suboptimal functioning of the β-galactosidase enzyme. This phenomenon is termed as senescence associated β-galactosidase (SA-βgal)[101]. Many cell types also acquire resistance to apoptotic signals upon becoming senescent [91]. This resistance has been found to be due

to suppression of apoptotic mediating proteins such as caspase-3 or death-associated protein 3 (DAP3) and persistent upregulation of pro-survival factor Bcl-2 [102]–[104].

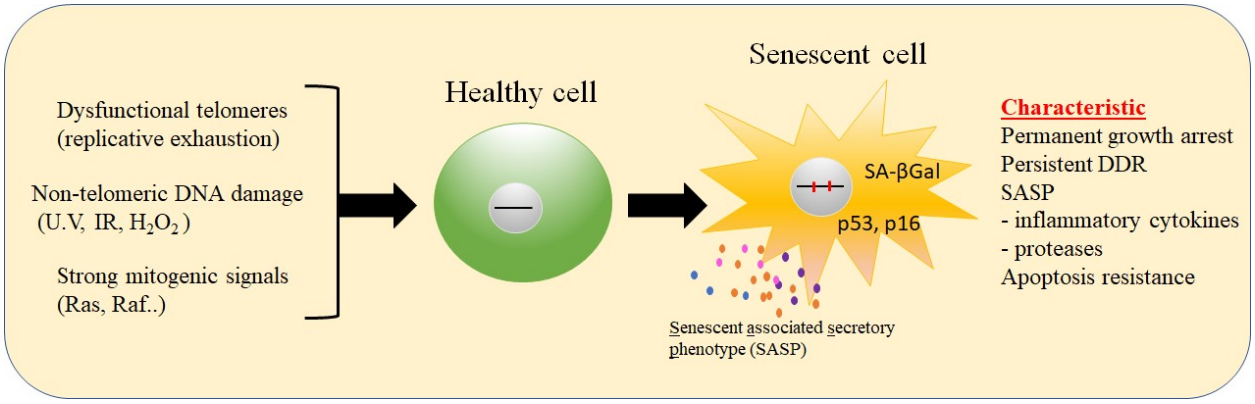

Figure 3. Characteristics of senescent cells
A myriad number of stressors can induce cellular senescence in mitotically competent cells. These stressors include telomere damage generated by replicative exhaustion, non-telomeric genomic damage induced by I.R, U.V, oxidative stress etc., and excessive mitogenic signals produced by oncogenes (which also cause DNA damage). The different stressors all engage DNA damage response (DDR) signaling cascade and ultimately induce cell cycle arrest by activating p53, $p16^{INK4a}$, or both. If the damage induced by the stressors is irreparable and is of sufficient magnitude, the cells undergo permanent growth arrest and chronically secrete elevated levels of inflammatory cytokines and proteases, a phenotype termed as senescent associated secretory phenotype (SASP). The other hallmarks of senescence include senescence associated beta galactosidase (SA-βGal) and apoptotic resistance.

Another characteristic feature of senescent cells is chronic and elevated expression of pro-inflammatory cytokines and chemokines, tissue-damaging proteases, growth factors, and factors that can impact stem and progenitor cell function. This phenomenon is termed as senescence associated secretory phenotype (SASP) [105]. Though a core of the SASP factors are feature of all senescent cell types, there does exists variation in terms of quality and quantity that depends on the cell type and senescence inducer [96], [105], [106]. Furthermore, in cell culture a full SASP develops 5-7 days after cell cycle arrest. Importantly DDR proteins ATM and Chk2, but not p53 and pRb, are found to be needed to initiate and maintain SASP [96]. Functional inactivation of p53 or gain of oncogenic Ras following senescence establishment allows cells to resume proliferation but does not suppress SASP [96].

The SASP factors are reported to be mediated by multiple mechanisms. The most well studied of them is the canonical NF-κB pathway. p65 subunit of NF-κB is found to act as a master regulator of the SASP and its suppression is found to down-regulate expression of several inflammatory factors and matrix proteinases in oncogene-induced, replication-induced, and X-irradiated senescence [107]. Depending on the cell type, the stress-inducible kinase p38MAPK and the transcription factor GATA binding protein 4 (GATA4) are activated by multiple different stressors capable of inducing senescence and are found to act as an upstream activator of NF-κB to initiate SASP [108], [109]. The CCAAT/enhancer-binding protein β (C/EBPβ) is another transcription factor that is found to be tightly associated with senescent chromatin and control secretion of IL-6 and IL-8 SASP factors [110]. Furthermore, inhibition of mTORC1 complex of the mTOR kinase by rapamycin is found to dampen expression of SASP factors in X-irradiation induced senescent human fibroblast cell lines by suppressing the positive feedback loop between IL-1A- NF-κB [111]. Strikingly, the protein synthesis mechanism controlled by the mTOR and protein degradation mediated by autophagy are found to occur in unison in senescence. It is found that suppression in synthesis of certain SASP factor occurs if the mTOR and autophagy are inhibited [112]. The protein degradation controlled by autophagy is speculated to provide the raw materials for facilitating protein translation and consequent protein turnover to establish SASP.

In addition to aforementioned specific proteins and pathways, recent reports have revealed that the senescence induction and SASP are intimately linked to the metabolic state. In replication-induced senescent human fibroblasts, increased glucose consumption as well as increased lactate production were found [113]. However, the increase in glycolysis was not accompanied by increased intracellular ATP levels in the senescent cells. Subsequently it was

found that the replication induced senescent fibroblast cells fail to upregulate certain other glycolytic enzymes such as GAPDH and enolase and it is this failure to co-ordinately regulate all the glycolytic enzymes that contributes to the reduced ATP levels [114]. Other studies have found the mtDNA copy number, mitochondrial protein expression, and respiration are found to increase 2- to 3- fold in human fibroblasts induced to senesce using diverse stimuli; elimination of mitochondria using the uncoupler CCCP, which induces mitophagy by allowing the protein Parkin to localize to mitochondria, resulted in suppression of cell cycle arrest proteins (p21 and p16) and several SASP factors [115]. Increased mitochondrial biogenesis results in increased reactive oxygen species (ROS) and DNA damage. Indeed, reduction in mitochondrial mass lowered mitochondrial derived ROS, 53BP1 foci (marker of DNA double-strand breaks) and DDR signaling, which is required for the initiation and maintenance of SASP [115]. Therefore, the expansion of mitochondria served to maintain the DDR signaling, and thus, the SASP phenotype in this context. Unlike this study, in chemotherapy-induced senescent lymphoma cells, metabolic alteration consisting of increased oxygen consumption, ATP concentration, and fatty acid oxidation were needed to maintain the production of senescent associated proteins, including SASP factors [116]. In addition to protein production, the increased energy generation was found to support increased expression of proteins involved in unfolded protein response (UPR) and autophagy. It was hypothesized that the elevated synthesis of proteins overwhelms the capacity for accurate post-translation processing and vesicular transport and secretion. Indeed, inactivation of NF-κB, which regulates synthesis of numerous SASP factors, was shown to suppress expression of UPR transcripts- ATF4, CHOP- and autophagy markers-p62, LC3 I/II. Thus, in this context, the high and presumably not always proper production and processing of senescence-associated secretory peptides was found to elicit

the demand for high energy generation. Similarly, in Ras-induced senescent fibroblasts, the metabolic alteration consisting of increased fatty acid catabolism and oxygen consumption due to oxidation of fatty acids were found to support the increased secretion of SASP factors [117].

From the studies discussed above, it is evident that the mechanism driving senescence establishment, SASP secretion, and the ensuing metabolic alteration are dependent on the cell type, senescent stimuli, and the magnitude of the stimuli. However, our understanding of the changes that occur in senescent cells is still evolving. Nevertheless, the differences between senescent and non-senescent cells highlighted by the different studies can be utilized in preparing therapeutics targets.

1.5.3 CELLULAR SENESCENCE IS A MAJOR DRIVER OF AGE-RELATED PATHOLOGIES

Cellular senescence can contribute to loss of regenerative potential of the tissue due to the proliferate arrest. Markers of cellular senescence such as SA-βGal, telomere attrition, and $p16^{INK4a}$ have been found to accumulate in multiple tissues with age in humans and rodents [118]. Additionally, senescent cells have been identified at the sites of age-associated pathologies. For instance, increased SA-βGal and chondrocytes with shorter telomere length are found in articular cartilage in patients with osteoarthritis (OA) [119]. Similarly, in the pancreas, $p16^{INK4a}$ positive β-cells levels increase with age in humans and in mice [120]. This restricts the regenerative potential of β-cells and limits the adaptive response to insulin resistance. It is hypothesized that the increase in β-cell senescence with age could contribute to the age-related increase in prevalence of type 2 diabetes mellitus (T2DM). Much like OA and T2DM, loss of proliferation competent cells due to senescence have been attributed to the

development of other age-related pathologies such as glaucoma, cataracts, atherosclerosis, and cardiovascular disease [121]–[124].

In addition to loss of proliferative potential, cellular senescence can alter the tissue microenvironment by virtue of the SASP. Induction of secondary senescence in neighboring cells is one scenario by which SASP can impact tissue health [125]. SASP factors, especially IL-1β and TGFβ, produced by fibroblasts cells undergoing replicative- or oncogene- induced senescence were capable of inducing DDR, senescence arrest, SASP in non-senescent fibroblasts [126]. Additionally, senescent cells can disrupt the architecture of the tissue and synergize with cells with mutations to promote malignancies [127]. For instance, senescent fibroblasts transform non-tumorigenic, immortal mammary epithelial cells into cells capable of producing tumors in mice. In vitro experiments suggested that the transformation was brought by the soluble factors secreted by the senescent cells [128]. Similarly, another study revealed that human fibroblasts induced to senesce using DNA damaging agent bleomycin when injected with cancer cells (MDA-MB-231) in mice, causes enhanced growth of cancer cells and the growth is abolished if an MMP inhibitor is incorporated into the transplants [129]. Taken together, these studies suggest that senescent cells can stimulate hyperproliferation and malignant progression of preneoplastic and neoplastic cells. SASP factors are also implicated in progression of other age-related disorders as well. Senescent bronchial cells are present in lungs of humans with idiopathic pulmonary fibrosis (IPF) and are known to secrete elevated levels of IL-1β and other extracellular matrix proteins [130]. In brain tissue from patients with Alzheimer's disease (AD), elevated levels of astrocytes co-expressing p16 and MMP1 was found, suggesting a link between senescence and age-related pathology such as AD [131].

Other age-related pathologies associated with SASP include cystic fibrosis, intestinal bowel disease, and cataracts [132]–[134].

The association between age-related dysfunction and cellular senescence is more than correlative. Evidence for the causal role of senescence and age-associated disorders was established by a study which showed improvement in skeletal, eye, and adipose tissue upon selective and life-long clearance of $p16^{INK4a}$ positive senescent cells [134]. Additionally, late life clearance of $p16^{INK4a}$ expressing cells, when age-related pathologies are apparent, attenuated the decline in physical activity and subdermal adipose layer thickness. This study achieved selective clearance of $p16^{INK4a}$ expressing cells by using a transgenic mouse that contained GFP and caspase-8-FKP fusion protein under the control of 2.6 kbp $p16^{INK4a}$ mini promoter; upon administration of AP20187 drug, the caspase-8-FKP undergo dimerization and activation and induce apoptosis only in $p16^{INK4a}$ positive cells. Though this study provided solid evidence for targeting senescence for delaying or treating age-related diseases, the transgenic mice carried a progeroid genetic background *(BubR1* hypomorphism), and therefore, the stress for senescence development could be non-physiological compared to natural aging mice. Nevertheless, the results of subsequent studies which selectively cleared senescent cells in a natural aging mouse strongly support the causal role of senescence in age-related dysfunction.

The investigators of these later studies made use of a transgenic mouse called p16-3MR. These mice contain three proteins - synthetic Renilla luciferase (LUC), monomeric red fluorescent protein (mRFP), and truncated herpes simplex virus 1 (HSV-1) thymidine kinase (HSV-TK), under the control of $p16^{INK4a}$ promoter [135]. To generate the p16-3MR mice, the 3MR cDNA was inserted into bacterial artificial chromosome (BAC) containing approximately 50 kbp of the murine INK4a/ARF locus, which encodes two proteins- $p16^{INK4a}$ and $p19^{ARF}$ by

alternative splicing mechanism. The 3MR cDNA was inserted in frame with p16 into exon 2, creating a fusion protein containing first 62 amino acids of p16, but no full-length WT p16. Insertion of the 3MR cDNA also resulted in a stop codon in p19 reading frame in exon 2, thereby preventing full length p19 expression from BAC. The transgenic mouse line that was created contained a single integrated copy of the engineered p16-3MR BAC and contained diploid copies of $p16^{INK4a}$ and $p19^{ARF}$. In these p16-3MR mice, the cells that express HSV-TK are killed by ganciclovir (GCV), a nucleoside analog which has high affinity for HSV-TK but not cellular TK. When normal dividing cells expressing 3MR are administered GCV, the HSV-TK phosphorylates GCV which then competes with endogenous nucleotides for incorporation into nuclear DNA, causing replication dependent double strand breaks, and apoptosis subsequently [136]. However, when GCV is administered to senescent 3MR expressing cells, S-phase independent incorporation of GCV into mtDNA, mtDNA fragmentation, and caspase-dependent cell death ensues [136]. Elimination of chemotherapeutic agent (doxorubicin)-induced $p16^{INK4a}$ senescent cells in p16-3MR using GCV resulted in reduced serum and tissue inflammatory cytokines, cardiac dysfunction, bone marrow suppression, cancer recurrence, and improved physical activity and strength [137]. Similarly, clearance of total body irradiation-induced $p16^{INK4a}$ senescent cells using GCV, suppressed expression of several inflammatory SASP factors, attenuated the disruption of hematopoietic stem cell (HSC) quiescence, which can lead to premature HSC exhaustion, and age prominent phenotype of HSC myeloid skewing [138]. Together, these studies provide convincing evidence for the healthspan benefits that can be achieved by elimination of senescent cells.

1.5.4 THERAPEUTIC STRATEGIES TO ELIMINATE CELLULAR SENESCENCE

Targeting senescent cells is an attractive strategy to prevent or delay age-related disorders and thereby achieve healthy aging. In this regard, approaches including selectively ablating senescent cells and blocking the secretion of SASP factors can be utilized. In particular, senolytic compounds, that specifically kill senescent cells, hold much promise. These drugs are dependent on senescent-specific vulnerabilities compared with normal dividing or differentiated cells. For example, ABT263 (navitoclax) and ABT737, specifically inhibit the anti-apoptotic proteins B-cell lymphoma 2 and B-cell lymphoma–extra-large, which are upregulated in certain types of senescence, and can induce rapid apoptosis in senescent cells [138]–[141]. Indeed, clearance of senescent cells using ABT737 in natural aging mice ameliorated the cartilage degradation and development of post-traumatic osteoarthritis [142]. Two other pharmacological agents that also target the pro-survival pathways upregulated in senescent and which can be employed for targeted killing of senescent cells include dasatinib, a kinase inhibitor that targets a myriad number of kinases, and quercetin, a flavonoid that is derived from plants and which possesses anti-inflammatory properties. Improvement in cardiac and vascular function and later-life physical endurance was seen in mice provided with these compounds [140]. These findings are a significant step towards translating these strategies for clinical applications. However, the senolytic drugs identified to date might have limitations for clinical applications. For example, in a phase II study of ABT263 applied to advanced and recurrent small-cell lung carcinoma patients, transient thrombocytopenia and neutropenia have been reported as side-effects [143]. Thus, identification of additional senolytic drugs is warranted.

Controlling the induction of SASP is also a promising strategy to prevent the negative effects of senescence. This could be achieved by targeting its transcriptional regulatory systems,

such as NF-κB or mTOR signaling, or by blocking the function of several key inflammatory mediators such as IL-1, IL-6 or TNFα [107], [111], [144], [145]. Rapamycin, an inhibitor of mTOR , reduces the secretion of inflammatory cytokines in senescent cells, and has been shown to extend the lifespan of mice [146], [147]. These findings, together with the fact that mTOR inhibitors are already used in clinical settings, suggest that mTOR inhibitors are highly promising drugs for the prevention of deleterious effects of cellular senescence. Likewise, some agents that target SASP factors are already in clinical use and could be candidates for drug repositioning. An IL-1 receptor anatagonist, anakinra, an IL-6 receptor antibody, tocilizumab, and TNFα inhibitors, such as etanercept and infliximab, are currently used to treat rheumatoid arthritis [148], [149]. As IL-6, IL-1, TNFα are core components of SASP of multiple different senescent cell types and appear to reinforce senescence autonomously and non-autonomously, the inhibitors of these factors may be useful for selectively blocking SASP [107], [110], [150].

1.5.5 CELLULAR SENESCENCE IN INTERVERTEBRAL DISC

As discussed earlier, damage due to numerous factors are implicated in driving disc aging [151]. However, of these factors, the cumulative oxidative and inflammatory burden are considered key in disrupting the balance between disc ECM anabolism and catabolism that occurs with age [48], [73]. Oxidative and inflammatory stress are also inducers of cellular senescence phenotype in a myriad number of cell types [99], and thus, could contribute to disc degeneration by inducing senescence in disc cells. Indeed, human NP (hNP) cells exposed to oxidative stress, e.g., hydrogen peroxide, become senescent and display PG homeostatic imbalance [74], [76]. These cells upregulate expression of p53 and p21 proteins and cease to proliferate. Additionally, H_2O_2-treated hNP cells show suppressed expression of aggrecan and collagen II and enhanced

expression of multiple proinflammatory cytokines (IL-6, IL-8, IL1β, TNFα), and matrix proteases (MMP-1, 2, 3, 9, 13 and ADAMTS-4, 5). Consequently, increased fragmentation of aggrecan and collagen is also evident in H_2O_2-treated hNP cells. Similarly, culturing rat NP cells in 20% oxygen upregulated ROS expression, induced DNA damage and cell cycle arrest, and elevated expression of catabolic factors- ADAMTS 4,5 and MMP 2,3, 14 [152]. Treatment of rat disc cells with exceptionally high glucose concentration was also reported to induce senescence via oxidative stress mechanism [153].

There is convincing evidence for the role of inflammatory stress in propagating cellular senescence phenotype in disc cells as well. Exposure of bovine caudal IVDs to TNFα for 21 days resulted in increased aggrecan breakdown, upregulation of IL-6, MMP-3, and SA-βgal [64]. Interestingly, the degradative changes were persistent in IVDs even after a recovery period of 14 days following a 7-day exposure to TNFα, suggesting a non-recoverable catabolic shift mediated by the transition to a cellular senescence phenotype. In addition, loss of proliferative capacity and greater susceptibility to degradative changes is seen in disc of IL-1ra$^{-/-}$ mice which lack functional endogenous antagonist of IL-1R [66]. This could be due to senescence induction as IL-1β has been shown to induce senescence in articular chondrocytes and fibroblasts [105], [154]. Additionally, the collagen proteolytic fragments generated as a consequence of cytokine-mediated stimulation of MMPs and ADAMTS expression induced premature senescence in rat NP cells [155]. Importantly, loss of disc height was observed in rat spine upon injection of rat NP cells induced to senesce with collagen MMP fragments. Together, these studies suggest that disc cells may undergo a phenotypic shift to a cellular senescence phenotype and promote the matrix homeostatic imbalance seen with aging in disc tissue in response to accumulative oxidative and inflammatory stress.

Chronic unrepaired DNA damage, a well-known inducer of senescence, emanating from factors other than oxidative or inflammatory stress has been implicated in driving senescence in disc cells as well. In old mice, chronic exposure to different types of genotoxic stress, including ionizing radiation and tobacco smoke, resulted in dramatic up regulation of disc $p16^{INK4a}$ and accelerated disc PG loss [156]. Furthermore, reduced nutrient supply, which is an important contributor to the pathophysiology of disc degeneration, has been shown to promote senescence phenotype in disc cells [157].

Evidence for the role of cellular senescence has been explored in human disc tissue as well. Expression of $p16^{INK4a}$ protein was found to increase with age in human disc tissue and was positively correlated with expression of ADAMTS-5 and MMP-13, suggesting senescent disc cells as the source of the matrix degrading proteases [158]. In another study, increase in the fraction of disc cells stained with SA-βGal positively correlated to the degeneration grades of disc tissue and, more importantly, negatively correlated with Ki67 positive proliferating cells [159], [160]. Collectively, both the animal and human studies suggest that age-induced senescence can greatly reduce the percent of proliferative and functional cells in the disc and can contribute to the functional decline of disc tissue by secretion of pro-inflammatory cytokines and matrix proteases.

2.0 SPECIFIC AIMS

Intervertebral disc degeneration is one of the major contributors to back pain, a largest chronic disorder that impacts the functional ability in the elderly. Individuals over 60 years old have a significantly higher risk of developing pain stemming from intervertebral disc degeneration (IDD) [161]. As such, there is now great impetus to understand how aging drives IDD in order to preserve mobility and fitness in the elderly population. Previous reports have examined the changes that accompany, and likely contribute, to the degeneration of the disc tissue with aging. Among these changes, the accumulative oxidative stress has been shown to be the predominant driver of the ensuing increase in inflammatory burden and matrix proteases with age in the disc tissue [55], [73], [151]. Oxidative stress is a well-known inducer of cellular senescence [99], a cell phenotype that has been reported to be causal in other age-associated pathologies, including atherosclerosis and osteoarthritis [124], [142]. Indeed, earlier studies have shown increased cellular senescence with age in disc of humans and mice [156], [158], [160], and *in vitro* studies have established that senescent disc cells have a catabolic phenotype characterized by increased secretion of proinflammatory factors, matrix proteases, and elevated matrix protein degradation ability [74], [76]. However, these studies are correlative and do not assess the changes that sustains the elevated secretion of proteins in senescent disc cells. To address these gaps, this dissertation work proposed the following Specific Aims (SAs):

SA1. To test the hypothesis that elevated protein secretion in senescent disc cells upregulates the energy generation pathway(s). Rationale: Previous studies have shown that metabolic changes characterized by increased energy generation are critical in sustaining the secretion of senescent related peptides in chemotherapy- and oncogene-induced senescent cells. Approach: We will use an *in vitro* cell culture model of oxidative stress-induced senescent disc cells to assess a) glycolysis and oxidative phosphorylation, b) changes that accompany these two energy generation pathways, and c) whether senescent associated protein synthesis requires upregulation of the energy generation pathways. Significance: Completion of these proposed experiments will elucidate the metabolic changes that enable disc senescent cells to secrete proteins, a large part of which are the catabolic SASP factors.

SA2. To test the hypothesis that senescence disc cells play a causal role in driving age-related disc degeneration. Rationale: Previous studies reported an association, not causation, of increased cellular senescent levels in disc tissue of mice, humans, and sand rats with age. Approach: We will make use of the p16-3MR mice model which enables selective clearance of $p16^{INK4a}$ positive senescent cells upon treatment with the drug ganciclovir (GCV). Using three p16-3MR mice groups- young, old treated with vehicle, and old treated with GCV- we will assess changes in histological features, expression of matrix proteases, and aggrecan content and proteolytic fragmentation in their intervertebral discs. Significance: Completion of this study will determine whether the senescent disc cells cause age-associated disc degeneration.

3.0 OXIDATIVE STRESS-INDUCED SENESCENCE MARKEDLY INCREASES DISC CELL BIOENERGETICS

This work, essentially as presented here, was submitted for publication in Mechanism of Aging and Development: Patil P, Falabella M, Saeed A, *et al*. Mech Ageing Dev. 2018 Oct 4. Results from this work were presented as podium presentation at the 44th International Society for the Study of the Lumbar Spine conference (ISSLS, 2017) in Athens, Greece. To provide the proper context, the paper is presented here in its entirety. The supplementary materials are included in Appendix A.

3.1 INTRODUCTION

Intervertebral disc degeneration (IDD) is one of the most common underlying causes of low back pain and disability in older adults [162]. The intervertebral disc tissue undergoes several structural, biochemical, and biomechanical changes with age which ultimately impede the normal disc function and patient mobility. With increasing age, the disc tissue experiences loss and fragmentation of matrix proteins, specifically aggrecan, the major proteoglycan (PG), with concomitant decrease in water content, leading to fissures and decreased disc height [1]. Multiple reports suggest that the elevated inflammatory cytokines, such as IL- 6, IL-8, TNFα, and IL-1β, are closely associated with the development of IDD. These cytokines

suppress synthesis of matrix proteins and upregulate secretion of matrix proteases that includes ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs) -4/5 and MMP (matrix metalloproteinases)-1, -2, -3, -13, -14 [48]. The age-related imbalance in matrix homeostasis of disc tissue suggests that the disc cells undergo phenotypic alteration. Indeed, the tissue milieu of the aged disc contains elevated quantities of inflammatory proteins and oxidants, two well-known inducers of cellular senescence. Thus, the age-related degeneration of disc tissue could be speculated to be driven by transformation of the disc cells into senescence phenotype.

Cellular senescence is functionally defined as the irreversible loss of cellular proliferative potential and can be induced by a wide array of stressors, which commonly involve DNA damage [163]. Historically, senescence was first characterized as the finite proliferative capacity of primary human cells in culture, triggered by erosion in telomere length [92]. Telomere attrition is found to generate persistent DNA damage response (DDR), which is needed to enforce the permanent growth arrest attained by serial cell passaging [164]. In addition to the passage-dependent telomere shortening, other stressors are known to induce senescence as well. The best studied examples come from conditions in which cells are exposed to subtoxic stress that directly damage the DNA (hydrogen peroxide, UV), termed as stress-induced senescence (SIS) [165]. Senescence can also occur when cells experience strong and continuous mitogenic signals, termed as oncogene-induced senescence (OIS) [166]. Both the SIS and OIS share several features with replicative senescence, including characteristic morphological changes, persistent DDR, irreversible growth arrest, and enhanced senescence-associated beta-galactosidase (SA-βGal) expression.

Growing evidence supports cellular senescence as one of the major drivers of aging and age-associated pathologies [167]. Indeed, senescent cells have been documented to increase with age in many rodent, non-human primate and human tissues [168]–[170]. Moreover, elevated number of senescent cells have been identified at the sites of several degenerative age-associated pathologies including osteoarthritis, glomerulosclerosis, and atherosclerosis [118]. Removal of senescent cells, in natural and accelerated aging mice, ameliorates age-related degenerative changes in several tissues and attenuates functional decline of skeletal and cardiac tissue [134], [140]. The increase in cellular senescence levels has also been documented in degenerative and aging human discs [158], [160]. Recently, we reported a dramatic upregulation of $p16^{Ink4a}$ and accelerated loss of disc PG in mice chronically exposed to genotoxic stress, including tobacco smoke and ionizing radiation [156]. All together these studies suggest a correlative relationship between DNA damage-induced senescent cells and IDD.

While senescent cells growth are arrested, they are far from being inert. One of the most defining features of SIS and OIS cells is the elevated and chronic secretion of myriad inflammatory cytokines and matrix proteases. This robust secretory phenotype of senescent cells is termed as senescent associated secretory phenotype (SASP) [105]. It is postulated that the senescent cells drive tissue aging through the action of SASP factors. *In vitro* and mouse xenograft studies have shown that the SASP factors can promote profound degenerative changes that ultimately compromise the tissue structure and function [106]. Furthermore, it was reported that the level of SASP factors provide predictive power regarding onset of disability and mortality in the elderly [171], [172]. The bioenergetic demands of such robust SASP production is becoming an important area of aging research.

Relatively little is known regarding the metabolic alterations that occur with entry into senescence. In Ras-induced and replication-induced senescent human fibroblast cells, impaired mitochondrial function and increased oxidant formation have been documented [173], [174]. However, in oncogenic $BRAF^{V600E}$-induced senescence, metabolic rewiring to increase mitochondrial respiration was necessary to maintain growth arrest and elevated secretion of IL-6 and IL-8 SASP factors [175]. Similarly, in chemotherapy-induced senescence, metabolic shift to elevated mitochondrial energy generation was needed to sustain the synthesis of NF- κB mediated SASP factors [116]. These findings suggest that the elevated and chronic production of the proteins, including SASP factors, caused by multiple stressors drives increased energy production in senescent cells. However, none of the studies to date have elucidated whether such metabolic changes occur in the context of oxidative stress-induced senescent disc cells.

Herein, we performed bioenergetic analysis of hydrogen peroxide-induced senescent human disc cells. In these SIS cells, we observed increased mitochondrial number, protein expression, and mitochondrial-ATP linked respiration, together with elevated fragmentation of matrix proteins and pro-inflammatory cytokines. Furthermore, protein synthesis in senescent disc cells was found to be supported by the increased mitochondrial respiration. Our findings demonstrated that SIS disc cells acquire enhanced bioenergetics to support secretion of senescent associated proteins.

3.2 METHODS

3.2.1 SAMPLE COLLECTION AND ISOLATION

Human nucleus pulposus disc samples were obtained from patients undergoing surgery (mean age ± SD = 50 ± 11.4 years, mean degeneration grade = 2.41 ± 0.5) (IRB #: PRO12100603). Cells were isolated from digested tissues as described before [176]. Cells were cultured in monolayer in F-12 media (Cat. No. 11765-062, *Life Technologies)* under hypoxic conditions (37 °C, 5% CO_2, and 5% O_2 with a bicarbonate buffer to maintain pH 7.2).

3.2.2 SENESCENCE INDUCTION

Hydrogen peroxide was used to induce cellular senescence as previously described [76]. Briefly, primary human nucleus pulposus (hNP) cells were treated with 500 μM hydrogen peroxide in F-12 (Cat. No. 11765-062, *Life Technologies),* supplemented with 10% Fetal Bovine Serum (FBS; Cat. No. S12450, *Atlanta Biologicals*;) and 1% Penicillin/Streptomycin solution (PS; Cat. No. 15140-16, *Life Technologies*), for 2 h. Culture media was then replaced with fresh F-12 with 10% FBS and 1% PS media without hydrogen peroxide. Cells were then maintained in culture for 10 days to establish senescence.

3.2.3 SENESCENCE ASSOCIATED β-GALACTOSIDASE STAINING

Senescence-associated β-galactosidase (SA β-gal) staining was performed as previously described [101]. Images were taken using brightfield microscopy at 10X magnification.

3.2.4 ELISA

Conditioned media was collected at the end of culture (day 10) and concentrated 40x with 3kDa cutoff centrifugal concentrators (Cat. No. *UFC 900324, Millipore Sigma*). IL-6, IL-8, and CTX-II protein levels were determined using *R&D Total Human IL6 and IL8 DuoSets* (*Cat. No.* DY206 and DY208) and *Biomatik Cross Linked C-Telopeptide of Type II Collagen kit* (Cat. No. EKU03505), respectively, as per manufacturer's instruction. Protein concentration was normalized to cell number assessed by tryphan blue exclusion.

3.2.5 IMMUNOBLOTTING

To assess aggrecan fragmentation, conditioned media was collected at day 10 of culture and concentrated as described above. To assess p53 protein expression levels, protein extracts from cell cultures were obtained using *T-PER Tissue Protein Extraction Reagent* supplemented with proteinase inhibitor cocktail as per the manufacturer's instructions (Cat. No. 78510, *Thermo Fisher*). Concentrated conditioned supernatant and cell lysates from cell cultures were separated using Tris-HEPES 4–20% gradient polyacrylamide denaturing gel (Cat. No. 25204, *Thermo Scientific*). The amount of conditioned media and cell lysates loaded on the gel corresponded to equal cell number. After electrophoresis, the proteins were transferred to a PVDF membrane by electroblotting and processed as described before [76]. Immunoreactive proteins were detected using chemiluminescent detection system (Cat. No. 34096, *Thermo Scientific* and *Bio-Rad ChemiDoc MP*). The following primary and secondary antibodies were used: Aggrecan (Cat.No. ab36861 (anti-G1), *Abcam*), p53 (Cat.No. 2524, *CST*) and β-actin (Cat.No. PA1-183, *Thermo Fisher*), anti-rabbit goat secondary antibody with HRP (Cat. No. PI-31460, *Thermo*

Scientific). Quantification was performed with densitometry analysis using *Bio-Rad* ChemiDoc MP.

3.2.6 BIOENERGETIC FLUX MEASURMENTS BY SEAHORSE XFe96

Bioenergetic measurements were performed using Seahorse Extracellular Flux Analyzer. The protocol for adherent cells was adapted to disc cells [177]. Briefly, hydrogen peroxide treated and untreated disc cells were plated at a density of 80,000 cells per well on a XFe96 plate and cultured overnight in F-12 media (10% FBS and 1% P/S). On the day of the experiment, cell culture media was replaced with unbuffered DMEM (Cat. No. D5030, *Sigma Aldrich*) supplemented with 2 mM Glutamax-1 (Cat. No. A1286001*, Gibco*), 1 mM sodium pyruvate (Cat. No. P5280, *Sigma*), 25 mM glucose (Cat. No. G7021, *Sigma*), 32 mM sodium chloride (Cat. No. S3014, *Sigma Aldrich*), and 15 mg phenol red (Cat. No. P3532, *Sigma Aldrich*) and incubated in non-CO2 incubator at 37 °C. Extracellular flux measurements were performed two-three times at six-minute intervals over five different treatment conditions: basal, oligomycin (2.5 µM) (Cat. No. 75351, *Sigma*), carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP) (2.5 µM) (Cat. No. C2920, *Sigma Aldrich*), 2-deoxglucose (2DG) (100 mM) (Cat. No. D6134, *Sigma Aldrich*), and rotenone (Cat. No R-8875, *Sigma Aldrich*) and antimycin A (Cat. No. A8674, *Sigma*) (2 µM each). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were calculated at each measurement. OCR and ECAR calculations were normalized by cell number per well using the tryphan blue exclusion assay. Mitochondrial ATP-linked respiration was calculated by subtracting the OCR values after oligomycin addition from the Basal OCR values. The proton leak was calculated by subtracting the OCR values after rotenone and antimycin A addition from basal OCR values.

To assess the relationship between protein production and upregulation of bioenergetic parameters, 2.5 μg/ml cycloheximide (CHX), an inhibitor of protein synthesis, was added to hydrogen peroxide treated and untreated cells for 24 hours before measuring extracellular flux parameters.

3.2.7 IMMUNOFLUORESCENT STAINING

Cells were washed twice with cold PBS and fixed in 2% paraformaldehyde in PBS for 15 min at room temperature. Cells were then permeabilized with 0.25% Triton X-100, and blocked with 20% serum (using the species in which the secondary antibodies were made) and 1% BSA in PBS (PBB) for 45 min at room temperature. Cells were incubated with the specific primary antibodies for γH2A.X (Cat. No. 05-636, *Millipore*), mtDNA (Cat. No. 03-61014, *American Research Products, Inc.*), TOM20 (Cat. No.PA5-52843, *ThermoFisher Scientific*) or ATP synthase beta (Cat. No. A-21351, *ThermoFisher Scientific*) overnight at 4°C. Cells were then washed three times with PBB and incubated with secondary antibody. The following secondary antibodies were used: 488 anti-rabbit (Cat. No. A21206, *Life Technologies*), Cy3 AffiniPure Goat anti-mouse (Cat. No. 115-165-003, *Jackson Immuno Research*) or Alexa Fluor 647 AffiniPure Goat anti-rabbit (Cat. No. 111-605-003, *Jackson Immuno Research*) for 1 hour at room temperature. To assess mtDNA colocalization with mitochondria, Cy3 (mtDNA) and Hoechst (BIsbenzimide; Cat. No. B-2883, *Sigma-Aldrich*) were collected simultaneously and Cy5 (TOM20) was collected by sequential scanning frames using a Leica TCS SP8 system. The images were then deconvolved through Huygens Professional software (SVI). Images of cells stained with anti γH2A.X antibodies were acquired with an Eclipse TE2000-U (*Nikon*, Minato, Tokyo, JP).

3.2.8 MITOCHONDRIAL QUANTIFICATION

To assess mitochondrial morphology, we performed immunofluorescent analysis of fixed cells using TOM20 (Cat. No.PA5-52843, *ThermoFisher Scientific*) to label mitochondria. Cells were co-stained with Hoechst (nuclei). Confocal Z-stacks were collected using a 60X (1.43NA) optic on a Nikon A1 equipped with GASP detectors and NIS Elements software (*Nikon Inc*., Melville NY). The confocal datasets were imported into Imaris (Bitplane Zurich, Switzerland) for surface rendering and calculation of mitochondrial number, volume, and sphericity.

3.2.9 mtDNA MEASUREMENTS

Total DNA from primary human nucleus pulposus (hNP) cells was isolated by sodium dodecyl sulfate lysis and proteinase K digestion as previously described [178]. DNA was then resuspended at 37°C in Tris-EDTA buffer containing RNAse A and the total DNA concentration determined by fluorescence using AccuBlue Broad range kit (Biotium, Fremont, CA). mtDNA and nDNA abundance was measured by duplex qPCR using three different TAQMAN primer/probes sets (ND1 and B2M; CytB and GUSB; Cox1 and ACTB) and averaged. Serial dilutions of combined samples were also performed to confirm assay linearity and dilution independence of results. qPCR was conducted on a StepOnePlus thermo cycler (ThermoFisher Scientific) using TaqMan Fast Advanced Master Mix (ThermoFisher Scientific), 4.6 ng/reaction DNA, and 5 μM of primer/probes in 10 μl final reaction volume. Relative mtDNA abundance was calculated by the ΔΔCq method [179]. The qPCR amplification profile was: one cycle (95°C for 20 sec) and 40 cycles (95°C for 1 sec and 60°C for 20 sec). Assays for mtDNA (mtND1, mtCYTB, and mtCOXI) were primer limited (1 primer: 1 probe), whereas assays for nuclear

DNA (B2M, GUSB, ACTB) were not primer limited (3 primer: 1 probe). The primers and probes used for the mtDNA quatification were purchased from Integrated DNA Technologies (IDT) and are listed below:

Table 1. Primers used for qPCR for mtDNA measurements

mtND1 probe	5'-/5HEX/CCATCACCC/ZEN/TCTACATCACCGCCC-3'
mtND1 primer 1	5'-GAGCGATGGTGAGAGCTAAGGT-3'
mtND1 primer 2	5'-CCCTAAAACCCGCCACATCT-3'
B2M probe	5'-/6FAM/ ATGTGTCTG/ZEN/GGTTTCATCCATCCGACA -3'
B2M primer 1	5'- TCTCTCTCCATTCTTCAGTAAGTCAACT-3'
B2M primer 2	5'- CCAGCAGAGAATGGAAAGTCAA-3'
mtCYTB probe	5'-/5HEX/ATCATCCGC/ZEN/TACCTTCACGCCAAT-3'
mtCYTB primer 1	5'-CCACATCACTCGAGACGTAAAT -3'
mtCYTB primer 2	5'-GATGTGTAGGAAGAGGCAGATAAA -3'
GUSB probe	5'-/6FAM/AAGAGTGGT/ZEN/GCTGAGGATTGGCA-3'
GUSB primer 1	5'-TGGTACGAACGGGAGGT -3'
GUSB primer 2	5'-ACGATGGCATAGGAATGGG -3'
mtCOXI probe	5'-/5HEX/TGCCATAAC/ZEN/CCAATACCAAACGCC-3'
mtCOXI primer 1	5'-CTAGCAGGTGTCTCCTCTATCT -3'
mtCOXI primer 2	5'-GAGAAGTAGGACTGCTGTGATTAG-3'
ACTB probe	5'-/6FAM/CTGCCTCCA/ZEN/CCCACTCCCA-3'
ACTB primer 1	5'-GTCCCCCAACTTGAGATGTATG-3'
ACTB primer 2	5'-AAGTCAGTGTACAGGTAAGCC -3'

3.2.10 GENERAL STATISTICAL ANALYSIS

Shapiro-Wilk test was used to test for normality. Student independent T-test was used to analyze data found to be normal. For two variable non-parametric data, the Mann-Whitney test was used. Analysis of variance (ANOVA) with Bonferroni correction for multiple comparison was used in cases of data with multi-variables. Statistics were derived using GraphPad Prism from GraphPad Software (San Diego, CA). All graphs show mean values with error bars (SD or SEM, as defined in the figure legends), unless specified otherwise. $P < 0.05$ was considered to be significant.

3.3 RESULTS

3.3.1 ESTABLISHMENT OF OXIDATIVE STRESS INDUCED SENESCENT DISC CULTURE SYSTEM

To better understand the phenotype of cellular senescence and its role in driving disc tissue aging, it would be most ideal to isolate and characterize the native senescent cells from disc tissue. However, this is technically challenging as no single specific biomarker of senescent cells, and disc senescent cells in particular, has been identified to allow for selection and isolation. Therefore, we made use of a previously engineered *in vitro* model to induce senescence in human disc cells derived from surgical specimens using hydrogen peroxide (H_2O_2) [76]. The rationale for this experimental approach was twofold: 1) H_2O_2 induces oxidative damage, an established feature in aged disc tissue; 2) H_2O_2 provokes DNA damage, a well-known hallmark of cellular senescence [96]. hNP cells were treated with hydrogen peroxide to induce DNA damage and incubated in culture media sans hydrogen peroxide for 10 days to allow cells to establish stress-induced senescence (SIS). This treatment regime ensured that the observed changes were in fact due to senescent phenotype and not to H_2O_2-induced transient oxidative stress experienced by cells. To confirm establishment of the senescent phenotype, we assessed the expression of well-known senescent markers, including SA-βgal, γH2A.X, marker of a DNA damage response, and p53 protein, a regulator of cell cycle arrest (Fig 4A-C). At 10 days post-H_2O_2 treatment, 90% of the hNP cells expressed p53 protein and contained nuclear foci of γH2A.X. In addition, a majority of the cells stained positive for SA-βgal and displayed a flattened and enlarged cell morphology that is commonly associated with senescent phenotype in

these cells. Detection of these markers confirmed successful induction and establishment of SIS disc cells.

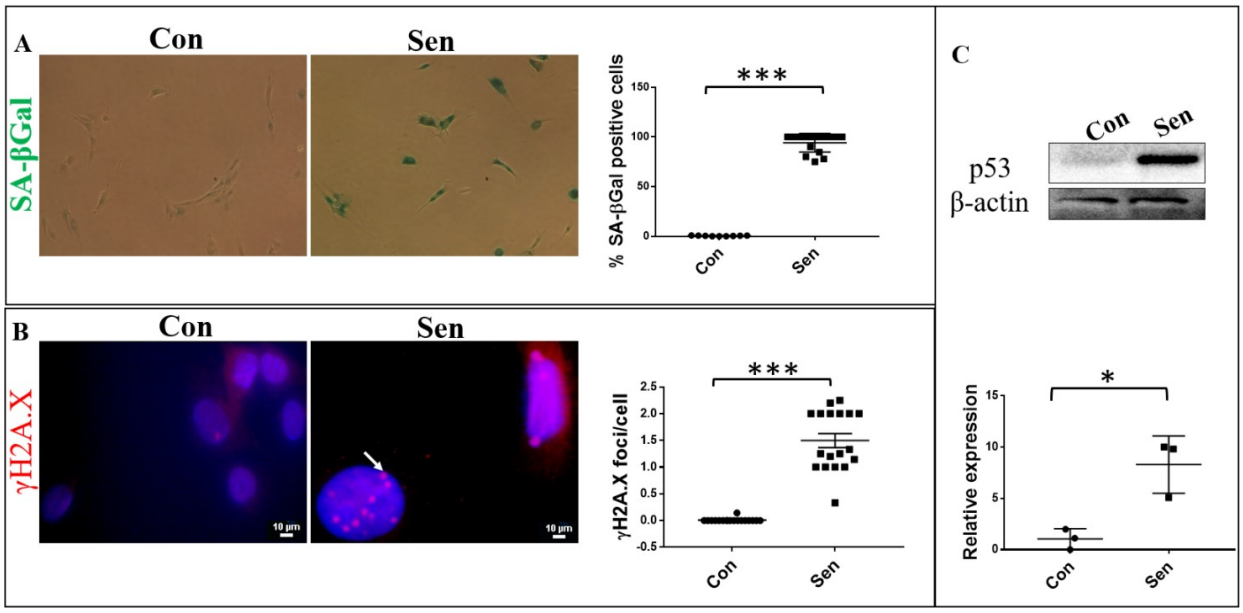

Figure 4. Establishment of stress-induced senescence (SIS) in disc cells
Senescence phenotype establishment in human disc cells treated with hydrogen peroxide (500 μM, 2 hr and examined 10 days later) was verified by SA-βGal staining (A), punctated expression of the DNA damage response marker γH2A.X in nuclei (B), and p53 protein expression (C). The individual points in (A) indicate the percent SA-βGal positive cells in one field of imaging and in (B) indicate the number of γH2A.X foci divided by the number of cells (DAPI) in one field of imaging. Data in (A) and (B) are means ± SEM of 4 independent experiments. The graphs in (C) indicate volume of p53 band divided by volume of β-actin band from 3 independent experiments. Data in (C) is means ± SD; *** $p < 0.0001$; *$p < 0.05$. Scale bar = 10μM.

3.3.2 SENESCENT DISC CELLS ACQUIRE A CATABOLIC PHENOTYPE THAT IS REMINISCENT OF AN OLDER DISC TISSUE PHENOTYPE

To test for markers of age-related IDD, we assessed matrix catabolism in SIS disc cells. The aggrecan fragmentation mediated by ADAMTS and MMP class of proteases was 30x and 5x higher in SIS compared to non-SIS disc cells, respectively (Fig 5A). The collagen II fragmentation in hNP SIS cells was modestly, but not significantly, higher as well (Fig 5B). We next tested levels of proinflammatory cytokines, including IL-6 and IL-8, which are known to

promote the increased collagen and aggrecan fragmentation with age by inducing synthesis of MMP and ADAMTS class of proteases in disc cells. Indeed, we observed greater than 10x increase in lL-6 and IL-8 protein levels in media from hNP SIS cultures compared to that of non-SIS cells (Fig 5C). This suggested that SIS disc cells, by virtue of their elevated secretion of proinflammatory cytokines (IL-6 and IL-8), can promote MMP and ADAMTS catalyzed matrix protein fragmentation seen with aging in disc tissue.

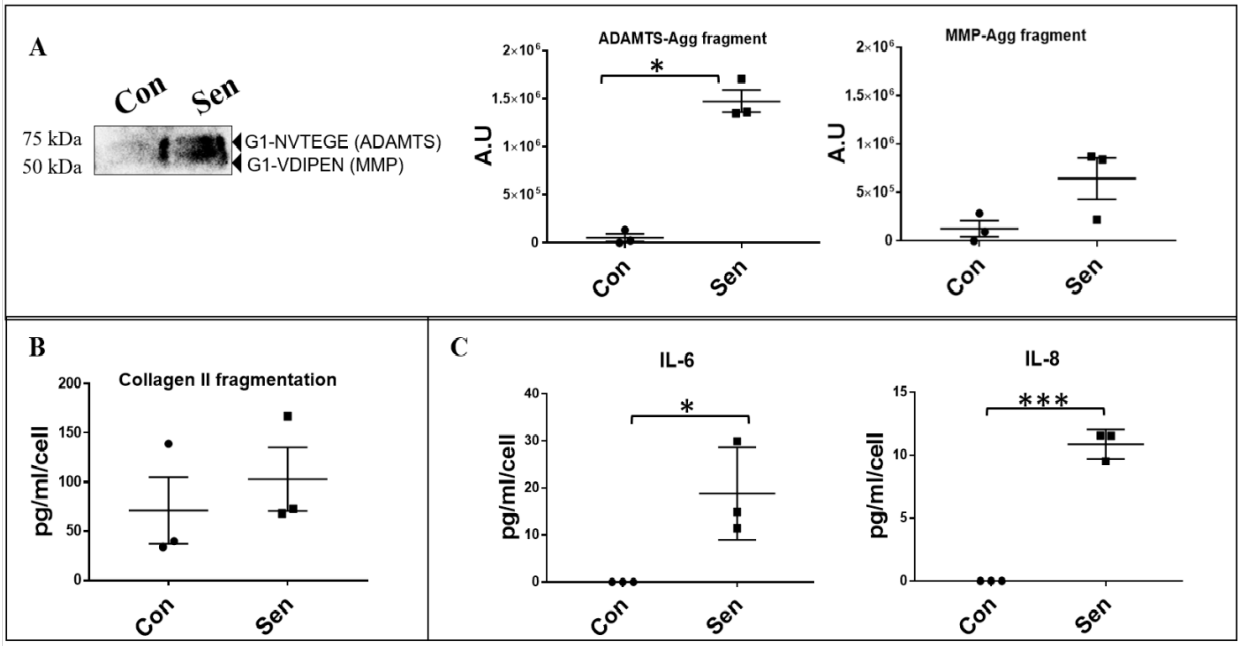

Figure 5. SIS disc cells mirror markers of age-related disc degeneration
SIS disc cells display elevated levels of aggrecan fragments mediated by action of ADAMTS and MMP proteases as assessed by Western blot (A), collagen II fragments (B), and IL-6 and IL-8 proteins by ELISA (C). Data are means ± SD of 3 independent experiments; *** $p < 0.0001$; *$p < 0.05$.

3.3.3 MITOCHONDRIAL RESPIRATION IS INCREASED IN STRESS-INDUCED SENESCENT DISC CELLS

One key feature of senescent cells is their elevated and chronic secretion of numerous proteins, including pro-inflammatory cytokines and matrix proteases. We hypothesized that increased chronic protein secretion is an energetically demanding process, and we therefore examined the

bioenergetic profile of SIS disc cells using Seahorse Flux Analyzer, which measures cellular glycolytic and oxidative phosphorylation (OXPHOS) rates simultaneously. The glycolysis activity is measured via changes in the extracellular acidification rate (ECAR) and OXPHOS activity is assessed via oxygen consumption rate (Fig 16). The SIS disc cells had a significantly higher (182%) basal OCR compared to non-SIS disc cells (Fig 6). The SIS disc cells also had higher ECAR, though not statistically significant, than that of the non-SIS disc cells. Since the increase in basal OCR could be driven by either increase in mitochondrial ATP-linked respiration or proton leak, we examined the sensitivity of the cells to oligomycin, an inhibitor of ATP synthase (complex V) and to rotenone and antimycin A compounds, which inhibit complex I and III, respectively (Mito ATP and Proton leak OCR, described in Fig 16). The mitochondrial respiration associated with ATP production was significantly higher (165%) in SIS cells compared to non-SIS disc cells. However, the mitochondrial respiration associated with proton leak did not change appreciably, suggesting the increase in basal OCR is driven by increased mitochondrial ATP-linked respiration (Fig 6).

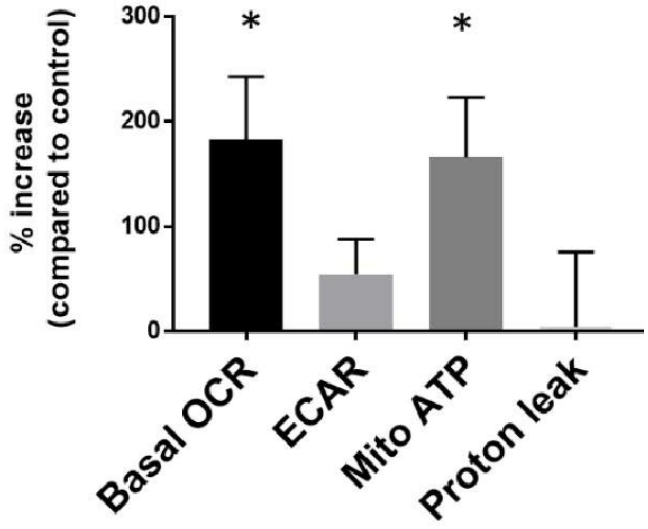

Figure 6. SIS disc cells exhibit alteration in energy metabolism
80x 10^3 SIS and non-SIS disc cells were plated in Seahorse XF96 Extracellular Flux Analyzer plate. The Seahorse Analyzer records rates of OXPHOS and glycolysis via the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR). Basal OCR and ECAR were recorded at the start of the assay. ATP-linked OCR (Mito ATP) is the difference between the basal OCR and OCR after the addition of oligomycin to sample cultures. Proton leak is the difference between oligomycin sensitive OCR and OCR after the addition of rotenone and antimycin A (Fig Sup 1). The data is representative of 5 independent experiments and expressed as means ± SEM; *$p < 0.05$.

3.3.4 INCREASED MITOCHONDRIAL CONTENT ACCOMPANIES THE INCREASED MITOCHONDRIAL RESPIRATION IN SENESCENT DISC CELLS

Increase in mitochondrial ATP-linked respiration is driven typically by increased mitochondrial biogenesis. First, we assessed mitochondrial protein expression by 2-D imaging SIS and non-SIS disc cells labeled with antibodies against ATP synthase beta and TOM20. ATP synthase beta is a mitochondria specific protein which is integral to the electron transport chain complex V. TOM20 is a mitochondrial membrane protein which helps in translocating cytosolically synthesized proteins destined for mitochondria. The expression of both these mitochondrial proteins was higher in SIS compared to non-SIS disc cells (Fig 7A, B). Next, we assessed mitochondrial number and morphology by 3-D imaging SIS and non-SIS disc cells labeled with TOM20.

The data sets from the imaging were imported into Imaris software for surface volume rendering using intensity based segmentation to identify discrete objects (mitochondria). The SIS disc cells had significantly more objects compared to non-SIS disc cells, suggesting a greater number of discrete mitochondrial units (Fig 7C). As the volume per mitochondrial unit was similar, the difference is most likely explained by increased mitochondrial abundance rather than increased fission which would be expected to be associated with increased fragmentation and decreased volume per fragment (Fig 7E). There were no differences in the shape/morphology of the individual mitochondria as reflected by sphericity (roundness) measurements (Fig 7D). The sphericity parameter is defined as the ratio of the surface area of the given object to the surfacearea of a sphere with the same volume as the given object; the closer the sphericity value is to 1, the more spherical the object. Consistently, morphological assessment done using EM imaging of SIS and non-SIS disc cells revealed similarity in terms of mitochondrial cristae and membrane structure between SIS and non-SIS disc cells (Fig 17).

Next, we examined the levels of mtDNA by quantitative PCR in SIS and non-SIS disc cells (Fig 8). To ensure against sequence variation, we used three mtDNA/nDNA multiplex TAQMAN primer/probe sets. Unexpectedly, the qPCR results revealed the SIS disc cells to contain ~37% of the mtDNA levels of non-SIS disc cells (Fig 8). Thus, the increase in mitochondria respiration in SIS disc cells occurs without the expected increase in mtDNA content. Additionally, we performed immunohistochemistry for mtDNA levels in both cell types (Fig 9) and found altered in situ levels and distribution of mtDNA in SIS disc cells. Specifically, while most mtDNA localized with TOM20 in non-SIS disc cells, much of mtDNA did not localize with TOM20 in SIS disc cells. Hence, SIS disc cells not only contain lower quantity of mtDNA, but much of their mtDNA is cytoplasmic, i.e., not residing within the mitochondria.

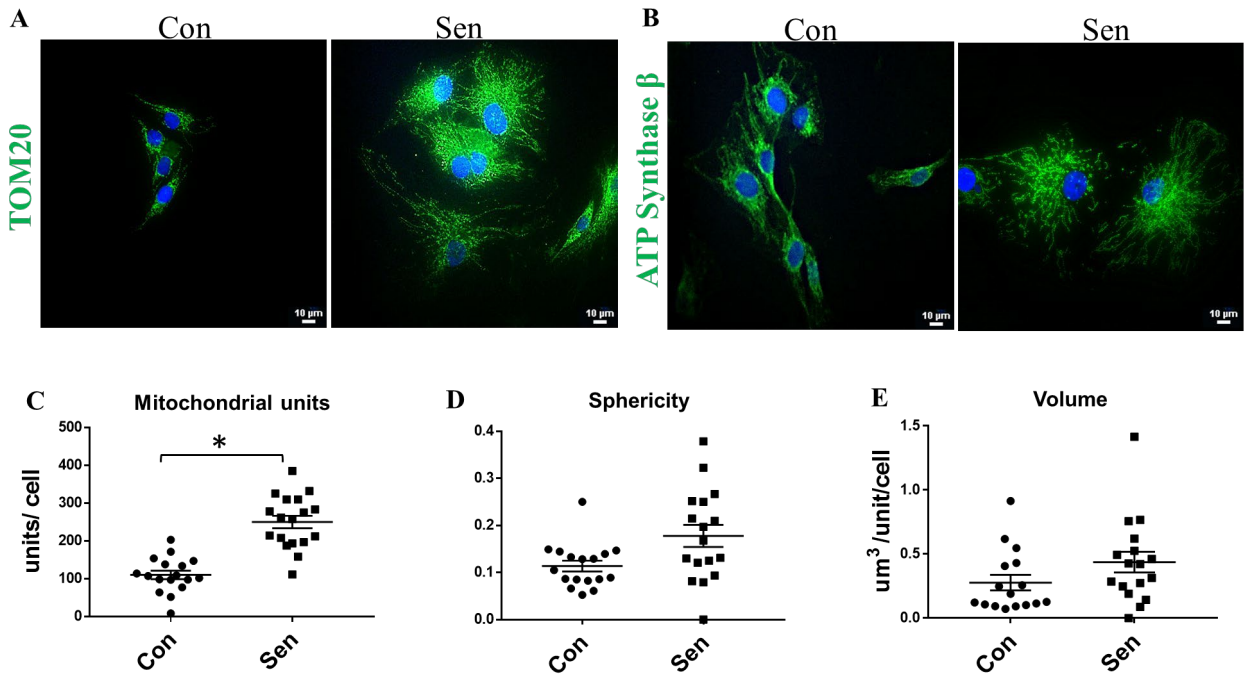

Figure 7. SIS disc cells have increased mitochondrial content compared to non-SIS disc cells
Expression of Tom20 (A) and ATP synthase beta (B) in SIS and non-SIS disc cells as assessed by immunofluorescence imaging. To assess mitochondrial number and morphology, 3-D imaging of SIS and non-SIS disc cells labelled with anti-TOM20 antibody was carried out. The data sets from imaging were imported into Imaris software for surface rendering. The 3-D surface rendering revealed higher number of mitochondria in SIS disc cells (C) but similar sphericity (D) and volume (E). The individual data points in C and E represent the number of surface unit or volume divided by the number of cells (DAPI) in one field of imaging. Data are means ± SEM of 3 independent experiments; *p < 0.05. Scale bar = 10µM.

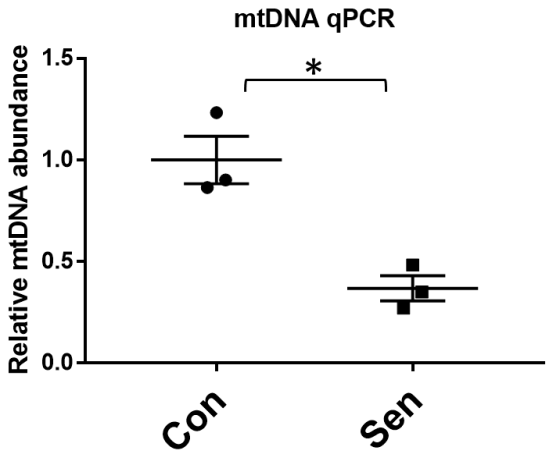

Figure 8. SIS disc cells have lower mtDNA compared to non-SIS disc cells
The relative mtDNA levels in the population was determined by averaging the results of three quantitative PCR assays after normalization to nuclear DNA. SIS disc cells contained significantly lower amounts of mtDNA relative to non-SIS disc cells. Data are means ± SD of 3 independent experiments; *p < 0.05.

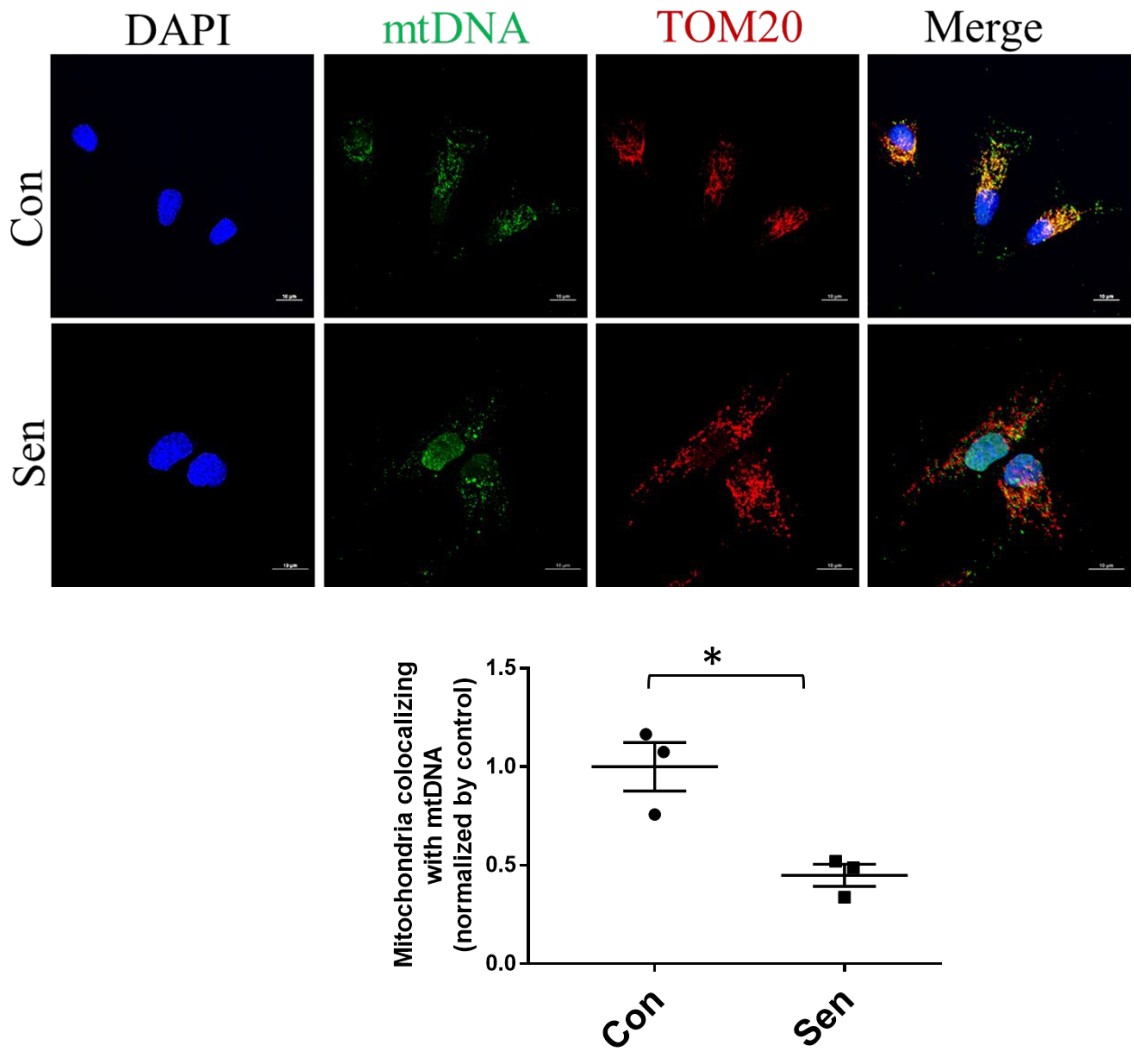

Figure 9. The SIS disc cells mtDNA do not localize with mitochondria
mtDNA (green) and mitochondria (red) were detected in situ by immunohistochemistry in SIS and non-SIS disc cells by fluorescent microscopy (see methods). To quantify mtDNA resident in mitochondria, images were deconvolved and fluorescence volume of mtDNA that co-localized with mitochondria was divided by the total fluorescence volume of mtDNA for each sample. The average of three samples were calculated for each group and normalized to control values (100%). The graph is the quantification of results from three independent experiments. Data is expressed as means ± SD; *$p < 0.05$. Scale = 10 µM

3.3.5 THE ELEVATED MITOCHONDRIAL RESPIRATION IN SENESCENT DISC CELLS IS DRIVEN BY INCREASED PROTEIN SYNTHESIS

To determine whether the increased mitochondrial ATP-linked respiration was in fact needed for the elevated production of the proteins, we assessed the mitochondrial bioenergetics profile of SIS and non-SIS disc cells in presence of the protein synthesis inhibitor cycloheximide (CHX). Consistent with our earlier observation, SIS disc cells had significantly increased basal OCR rate compared to non-SIS disc cells (Fig 10A). However, upon treatment with CHX, the SIS disc cells had 7x lower basal OCR rate compared to untreated SIS disc cells. The basal OCR difference between CHX treated and untreated non-SIS disc cells was very minimal. The reduction in basal OCR rate in SIS disc cells treated with CHX was found to be primarily driven by lower mitochondrial respiration needed for ATP production as the oligomycin sensitive respiration in SIS disc cells treated with CHX was significantly lower compared to untreated SIS disc cells (Fig 10B); no such appreciable difference in proton leak between SIS cells treated with CHX compared to untreated SIS cells was found (Fig 10C). The ATP-linked respiration and proton leak difference in CHX treated and untreated non-SIS disc cells was very modest. These results collectively suggested that the high mitochondrial ATP-linked respiration in SIS disc cells is needed in synthesis of proteins, presumably to maintain SASP where an abundance of inflammatory and catabolic factors is produced and secreted.

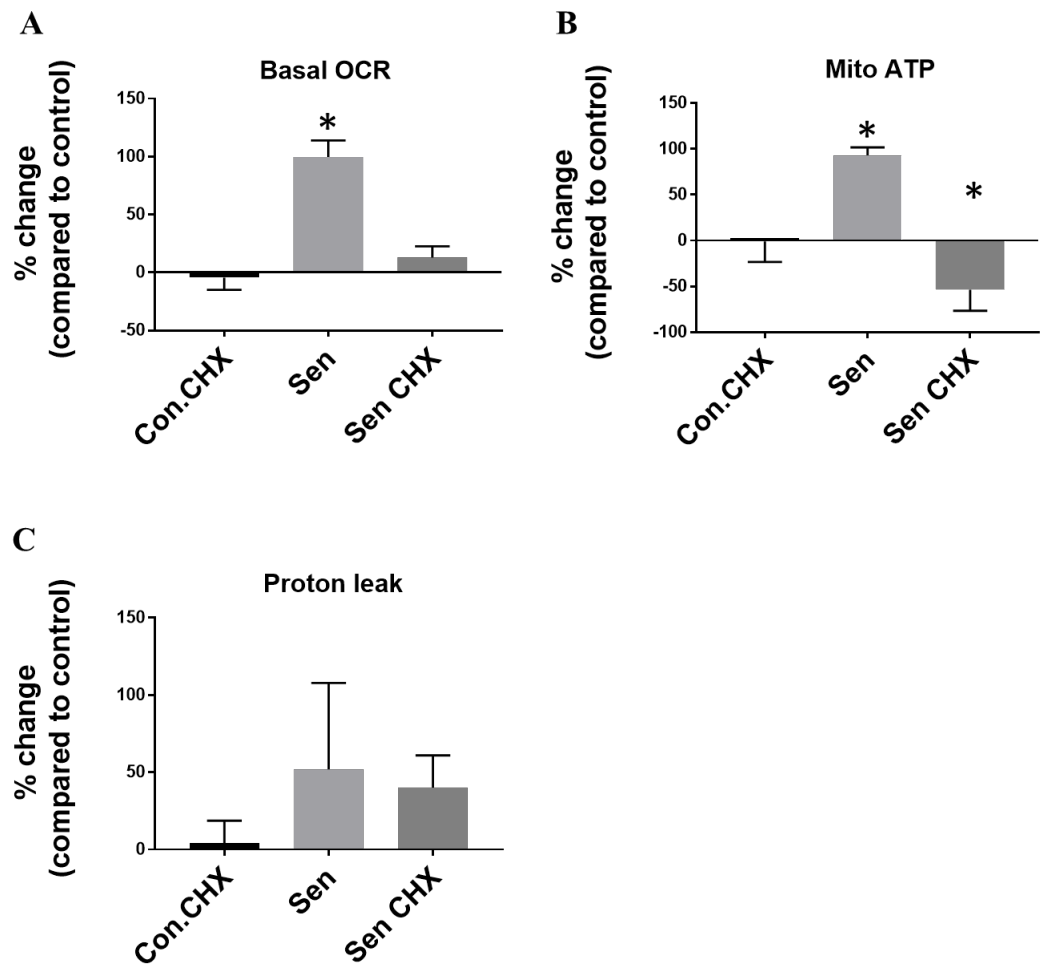

Figure 10. Suppression of protein synthesis dampens mitochondrial-ATP linked OCR in SIS disc cells
80x 10^3 SIS and non-SIS disc cells were plated in a well of Seahorse XF96 Extracellular Flux Analyzer and incubated with or without 2.5 µg/ml of cycloheximide (CHX) for 24 hr before commencing Seahorse recording of the basal OCR and ECAR and OCR after addition of sequential addition of metabolic inhibitors. A) The high basal OCR in SIS disc cells is abrogated after inhibition of protein synthesis using CHX. Assessment of mitochondrial-ATP linked OCR (Mito ATP) (B) and proton leak OCR (C) in presence of CHX suggests that the reduction in basal OCR in SIS cells in A is due to dampening of mitochondrial ATP-linked respiration and not due to proton leak. Data are means ± SEM of 4 independent experiments; *$p < 0.05$.

3.4 DISCUSSION

Senescent cells accumulate in multiple tissues with age in vertebrate organisms and contribute to the decline in tissue homeostasis via secretion of myriad catabolic factors that can

have potent impact on neighboring cells and surrounding tissues. Multiple reports have shown an increase in cellular senescence with age and degeneration in human and rodent discs [156], [158], [160]. The relationship between cellular senescence and age-related disc degeneration maybe more than correlative, as work done by Ngo et al has shown that human senescent disc cells express elevated amounts of matrix proteases as well as proinflammatory cytokines, factors that are known to promote loss of disc tissue health with aging. However, no study to date has explored the underlying metabolic changes of senescent disc cells.

In the present study, we demonstrated elevated production of the key SASP factors IL-6 and IL-8 which mirror the increased aggrecan fragmentation in SIS disc cells. We also discovered that SIS disc cells harbor a substantially greater number of mitochondria and exhibit increased mitochondrial ATP-linked respiration. We speculate that this metabolic alteration, i.e., increased mitochondria ATP-linked respiration in disc cells is necessary to meet the energy demand elicited by the elevated production of proteins in senescent cells. This idea is supported by our experiment showing that inhibition of protein synthesis significantly lowered mitochondrial ATP-linked respiration in SIS but not control disc cells. Our finding is consistent with other reports which show that in chemotherapy and oncogene induced senescent cells, lower macromolecule synthesis (fatty acids) and high mitochondrial ATP production are metabolic adaptations necessary to maintain the persistent and high protein production, including SASP factors [116], [117].

Mitochondria's role in cellular senescence has been widely associated with generation of ROS, which acts as a driver of signaling networks necessary to maintain the senescent phenotype [180]. However, recent work has shed light on the true necessity of mitochondria for senescence in addition to ROS generation. Report by Correia-Melo et al. demonstrated that in irradiation-

induced senescent fibroblasts, suppression of a vast number of genes involved in senescence was seen upon depletion of mitochondria by treatment with CCCP, an uncoupler that targets the ubiquitin ligase Parkin to mitochondria and promotes their degradation [115]. In accordance with this, the protein synthesis in SIS disc cells was seen to drive the elevated mitochondrial ATP-linked respiration. This result was unexpected as disc cells are known to rely on glycolysis for generation of ATP. Therefore, it can be derived that the disc cells upon becoming senescent become adapted to generate energy via OXPHOS to support synthesis of senescent related peptides.

Increased mitochondrial number and protein expression were found to be the changes accompanying the upregulation in mitochondrial ATP-linked respiration in SIS disc cells. However, surprisingly, no concurrent increase in mtDNA levels was seen in SIS disc cells. The conundrum of increased mitochondrial function with lower mtDNA is not unprecedented, however. Heddi et al. observed coordinated increase in nuclear and mitochondrial OXPHOS transcripts in muscle of MERRF (myoclonic epilepsy associated with red ragged fibers) and MELAS (myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) patients with diagnosed mutation in mitochondrial genome [181]. Work by Piechota et al. showed that in HeLa cells, despite a reduction in mtDNA by 90%, the COX2 protein and COX activity were 6-fold higher relative to mtDNA content. Taken together, these results suggest that the depletion in mtDNA can be compensated by increased transcription per genome and efficient protein synthesis [182]. Interestingly, the mtDNA in SIS disc cells did not co-localize with mitochondria and was mostly cytoplasmic. Cytoplasmic DNA is a known trigger for the cytosolic DNA sensor cGAS to produce second messenger cyclic GMP-AMP (cGAMP), which binds and activates the adaptor protein STING, thereby leading to the production of inflammatory cytokines [183]. The

cGAS-STING pathway plays a key role in activation of immune system during microbial infection. Recently, it was shown that cGAS-STING also helps in initiation and maintenance of SASP factors in cells induced to senescence by various means [184], [185]. It therefore could be speculated that the cytoplasmic mtDNA of SIS disc cells plays a role in regulating the SASP factor synthesis. However, this remains to be verified.

Alteration in metabolic state of cells is accompanied by changes in mitochondrial morphology. Specifically, mitochondrial fusion is favored under energetically demanding conditions as it allows for complementation of genes and efficient dissipation of membrane potential [186]. Mitochondrial fission, on the other hand, helps maintain bioenergetic state of the cell by promoting degradation of damaged organelle by autophagy [186]. The mitochondria in SIS disc cells had similar cristae structure, outer membrane definition, sphericity, and volume compared to non-SIS disc cells. In contrast, previous reports show that in human cell lines induced to undergo senescence there is an overall shift to more fusion events, resulting in abnormally elongated mitochondria [187], [188]. However, it is unclear if elongated mitochondria are truly needed to maintain permanent growth arrest in all cell types or whether such morphological changes are only seen in transformed cell lines.

Despite the morphological similarity between SIS and non-SIS disc cell mitochondria, mitochondrial ATP-linked respiration in senescent disc cells is elevated. It cannot be discounted that this upregulation in SIS disc cells, which would consequently lead to higher mitochondrial ATP concentration, could be utilized towards processes other than protein synthesis. In chemotherapy-induced senescent cells, part of the mitochondrial ATP-linked respiration was shown to be devoted to autophagic degradation of the improperly synthesized and processed senescent-associated secretory peptides [116]. It was speculated that the increased secretion of

the catabolic factors by senescent cells overwhelms the cells capacity for proper protein synthesis, post-translation modification, vesicular transport, and secretion. Hence, it is plausible that in SIS disc cells the mitochondrial ATP-linked respiration could be utilized by mechanisms which alleviate the proteotoxic stress. Nevertheless, protein synthesis is positioned at the top of the hierarchy of ATP-consuming processes in cells [189]. Therefore, in senescent disc cells most of the mitochondrial ATP-linked respiration is probably utilized by protein synthesis. In summary, the results of this study collectively suggest that the SIS disc cells attain a catabolic phenotype and display marked upregulation in mitochondrial ATP-linked respiration to support protein synthesis.

4.0 SYSTEMIC CLEARANCE OF P16^{INK4A}-POSITIVE SENESCENT CELLS MITIGATES AGE-ASSOCIATED INTERVERTEBRAL DISC DEGENERATION

This work, essentially as presented here, was submitted for publication in Aging Cell: Patil P, Dong Q, Wang D, *et al*. Aging Cell. 2018 July 9. Results from this work were presented as poster presentation at the Orthopedic Research Society Annual Meeting (ORS, 2018) in New Orleans, USA. To provide the proper context, the paper is presented here in its entirety. The supplementary materials are included in Appendix B.

4.1 INTRODUCTION

Low back pain (LBP) is a major chronic disorder that significantly reduces the quality of life of the elderly. In the United States, direct and indirect costs associated with LBP, including, lost wages and productivity and legal and insurance costs, amount to $100 billion annually [190], [191]. Though the exact cause of LBP is not known, the degenerative changes that accompany the intervertebral disc with age are most frequently associated with LBP [192]. The debilitating back pain stemming from intervertebral disc degeneration (IDD) causes loss of mobility and increases the risk of mortality in the elderly [193]. Furthermore, disc degeneration is associated with clinical conditions such as herniated discs, spinal stenosis and spondylolisthesis, which are major diagnoses which often require surgical treatment. Therefore, there is great need to

understand how aging affects the disc in order to maintain mobility and fitness in the fast-growing population of elderly.

During aging, the intervertebral disc undergoes structural, biochemical and biomechanical changes that ultimately compromise its function. The loss of aggrecan, a major disc matrix proteoglycan (PG), results in reduced capacity to resist compressive forces, a well-established hallmark of IDD. The disc transforms into a fibrous tissue with age because of increased fragmentation and loss of disc PG, with a concomitant decrease in water content, leading to fissures and decreased disc height [8], [24], [151]. Evidence also exists for elevated levels of matrix fragments generated by the proteolytic action of matrix metalloproteinases (MMPs), as well as a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs), in aged discs [60]. Expressions of major pro-inflammatory cytokines such as IL-1ß, IL-6, IL-8, and TNF-α, all known to induce gene expression of ADAMTs and MMPs in IVD cells, are also increased in older adult discs when compared to younger or adolescent discs. Additionally, IL-6 levels are found to be higher in patients with low back pain prompted by disc degeneration [48]. At the cellular level, cellular senescence has been reported to increase with age in discs [194]. Cellular senescence may play a major role in age-related IDD as the senescent phenotype occurs at the right time and has the right characteristics to drive age-related disc degeneration.

Cellular senescence is a response to stress and certain physiological signals by which cells cease to divide and adopt a distinct phenotype [195]. This phenotype includes marked chromatin and secretome changes, and increased expression of tumor suppressor genes such as the $p16^{INK4a}$ cell cycle inhibitor [105]. The senescence response is a potent tumor suppressive mechanism, and has also been implicated in organismal aging [196]. Senescent

cells accumulate with age in most, if not all, tissues of humans, primates and rodents [163], [169], [170]. Furthermore, senescent cells are found at higher levels in diseased tissue compare to unaffected tissue in patients with age-related disorders such as osteoarthritis and type 2 diabetes [197]. Senescent cells are postulated to promote aging via their senescence-associated secretory phenotype (SASP): the increased and chronic secretion of a number of inflammatory cytokines and proteases [105], which can disrupt tissue function and structure. Recent reports showing amelioration of several age-associated pathologies upon senescent cell clearance in naturally aged and transgenic mice strongly support the idea that senescent cells can drive tissue aging [134]. The role of cellular senescence in disc aging and degeneration is less firmly established. Published reports showed an increased number of senescent cells with age and degeneration in human discs [158], [160]. However, these correlative studies do not clarify whether senescent cells play a causal role in age-related disc degeneration.

To test the hypothesis that senescent cells play a causal role in age-associated disc degeneration, we used a transgenic mouse model in which $p16^{Ink4a}$-positive senescent cells express the herpes simplex virus thymidine kinase, and thus can be selectively eliminated upon treatment with ganciclovir (GCV) [135], [138]. Here we show that year-long clearance of senescent cells mitigates age-associated increases in disc protease and blunts age-dependent disc PG matrix fragmentation and PG loss. These results provide compelling evidence that senescent cells are responsible for generating inflammatory cytokines and catabolic proteases that are known to increase with aging in disc, and thus support a causal relationship between cellular senescence and age-associated disc degeneration.

4.2 METHODS

4.2.1 MICE

Naturally aged 6 month (Young) and 20 month (Old) old C57BL/6 wild-type mice were obtained from the National Institute of Aging and maintained under specific pathogen-free conditions at University of Pittsburgh's animal facility. All animal work was approved and done in accordance with University of Pittsburgh's IACUC. Tissues from the p16-3MR mice were obtained from Dr. Daohong Zhou's group at University of Arkansas for Medical Sciences. Briefly, the p16-3MR mice were either sacrificed at 12 months (Young) or were treated with either PBS (Old-Veh) or GCV (Old-GCV) for additional 12 months (25 mg/kg/day for 5 days/cycle, every other two weeks) before sacrifice [138]. Spines and tails isolated from the p16-3MR mice were immediately frozen using liquid nitrogen and stored at -80^0 C until shipment.

4.2.2 QUANTIFICATION OF MATRIX PROTEOGLYCAN SYNTHESIS

Ex vivo culture of functional spine units (FSUs), consisting of vertebrae-disc-vertebrae, was established as described [77]. FSUs were cultured in F-12/D-MEM containing 10% fetal bovine serum, 1% PS (10000 units/ml penicillin, 10 mg/ml streptomycin), and 25 µg/ml L-ascorbic acid for two days followed by a 12-hour incubation with ^{35}S-sulfate (20 µCi/ml). Proteoglycan synthesis was calculated as fmoles of sulfate incorporated per µg DNA by as described [77]. Average values from three mice, each analyzed in duplicate, were calculated and reported ± one standard error.

4.2.3 HISTOLOGICAL STAIN

Isolated spines were decalcified and embedded in paraffin (*Tissue Tek* processor and *Leica* embedder). Seven-micrometer sections were stained with hematoxylin and eosin (H&E) by standard procedures and photographed under 40-200× magnification (*Nikon Eclipse* Ts100) [156].

4.2.4 IMMUNOBLOTTING

Ten tail discs from each mouse was used to assess aggrecan fragmentation utilizing a previously established method using anti-aggrecan primary antibody (Cat.No. ab36861, *Abcam*) and anti-rabbit-HRP secondary antibody (Cat.No. 31460, *ThermoFisher*) [31]. Expression of p53 protein was examined by extracting protein from spine discs using T-PER Tissue Protein Extraction Reagent with proteinase inhibitor cocktail as per the manufacturer's instructions (Cat. No 78510, *ThermoFisher*) and running a western blot using the p53 (Cat.No. 2524, *CST*) and β-actin (Cat.No. PA1-183, *ThermoFisher*) primary antibody and anti-rabbit-HRP secondary antibody (Cat.No. 31460, *ThermoFisher*).

4.2.5 IMMUNOFLUORESCENCE

Mouse lumbar intervertebral disc tissue were isolated from spines and fixed overnight at 4° C in 2% paraformaldehyde. For immunofluorescent staining, the tissues were cryoprotected with 30% sucrose in PBS overnight at 4° C, then embedded in OCT (Tissue-Tek). Serial axial plane cryosections were cut at thicknesses of 5 μm. The tissue sections were rehydrated in PBS,

permeabilized and blocked with 0.25% Triton X-100, 10% goat serum and 1% BSA in PBS for 30 min at room temperature. Incubation with primary antibodies (anti-Aggrecan, Cat.No. abB1031, *Abcam*; anti-MMP13, Cat.No. ab39012, *Abcam*; anti-IL-6, Cat.No. PAI-26811, *ThermoFisher*) were carried out overnight at 4° C following blocking. The sections were then incubated with secondary antibodies (Cy3-conjugate Goat anti-rabbit IgG, *Jackson laboratory*) for 60 min at room temperature, according to the manufacturer's protocols. Immunostained sections were imaged and analyzed using a Nikon instrument A1 confocal laser microscope and NIS-elements microscopy imaging software.

4.2.6 mRNA ANALYSIS

Total RNA was isolated using TRIzol™ Reagent as per the manufacturer's instructions (Cat. No. 15596026, *ThermoFisher*). Real-time quantitative RT-PCR was run using iTaq™ Universal SYBR® Green One-Step Kit (Cat. No. 1725151, *BIORAD*) and Bio-Rad iCyclyer IQ5 detection system. Target gene expression was calculated by the comparative C_T method ($\Delta\Delta$ C_T) and normalized to the GAPDH mRNA level. PCR primers used in the study are as follows: Cdkn2a (p16) forward: AATCTCCGCGAGGAAAGC; Cdkn2a (p16) reverse: GTCTGCAGCGGACTCCAT; MMP13 forward: TCCCTGCCCCTTCCCTATGGT; MMP13 reverse: CTCGGAGCCTGTCAACTGTGGA; GAPDH forward: GAGGCCGGTGCTGAGTAT; GAPDH reverse: GCGGAGATGATGACCCTTTTGG; Aggrecan forward: GCGAAGCAGTACACATCATAGG; Aggrecan reverse: ATACCCCATCCACACGCCCCG.

4.2.7 STATISTICAL ANALYSIS

Shapiro-Wilk test was used to test for normality. Student independent T-test was used to analyze data found to be normal. For two variable non-parametric data, the Mann-Whitney test was used. Analysis of variance (ANOVA) with Bonferroni correction for multiple comparison was used in cases of data with multi-variables. Statistics were derived using GraphPad Prism from GraphPad Software (San Diego, CA). All graphs show mean values with error bars (SD or SEM, as defined in the figure legends), unless specified otherwise. $P < 0.05$ was considered to be significant.

4.3 RESULTS

4.3.1 INCREASED CELLULAR SENESCENCE IN DISCS OF NATURALLY AGING MICE

Evidence of increased disc cellular senescence with age in humans, rats and in an accelerated aging mouse model was previously reported. However, it was not known whether cellular senescence also occurs in the discs of naturally aged mice. Hence, we assessed senescence markers in the discs of naturally aging mice. We determined the expression levels of p53, p21 and $p16^{Ink4a}$, which are known to regulate cell cycle and senescence in response to stress, in discs of young (6 months) and old (22 months) C57BL/6 mice by Western blotting and real time quantitative RT-PCR. Discs from older mice expressed significantly higher levels of p53 (3x) and p21 (1.5x) protein and $p16^{Ink4a}$ mRNA (1.5x)

compared to discs from younger mice (Fig. 11 A.1-.3). As expected, ADAMTS- and MMP-mediated fragmentation of aggrecan in older mouse disc were 10x higher in discs from older, compared to younger, mice (Fig 11B). In contrast, PG synthesis capacity, assessed by ^{35}S-sulfate incorporation, was lower in discs from older, compared to younger, mice (Fig. 11C). These *in vivo* correlations between disc cellular senescence and perturbed matrix homeostasis are consistent with our recent cell culture study demonstrating a matrix imbalance phenotype of senescent disc cells.

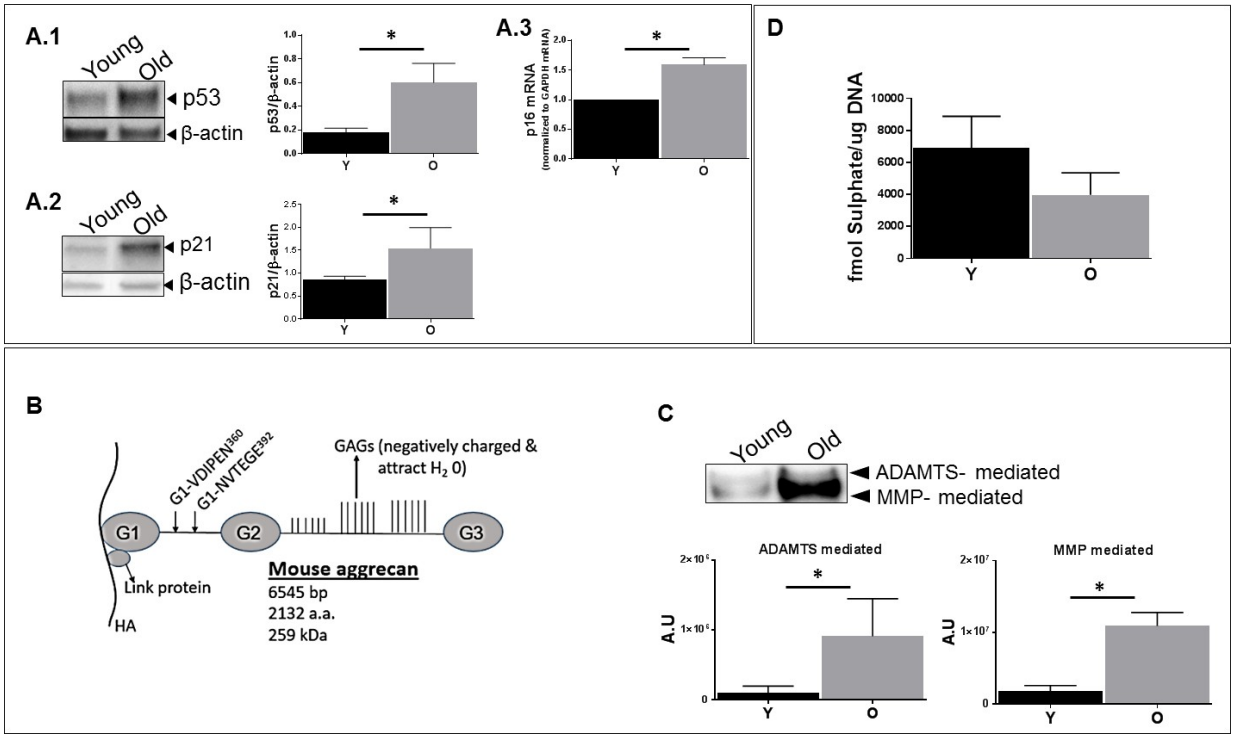

Figure 11. Degenerative changes in intervertebral discs of naturally aging mice
Expression of selected senescent markers, p53 (A.1), p21 (A.2) and p16^{IINK4a} (A.3) in disc tissue from young (6 mo) and old (22 mo) mice determined by Western blotting and qRT-PCR. Graphs on the right are quantification of results. Graphs of A.1 and A.2 indicate volume of respective protein band divided by volume of β-actin band. B) A schematic of the mouse aggrecan core protein covalently linked to the sulfate-rich glycosaminoglycan (GAG) and non-covalently bound to a hyaluronic acid (HA) chain via the link protein. The cleavage sites of ADAMTS (G1-NVTEGE^{392}) and MMP (G1VDIPEN^{360}) proteases between G1 and G2 interglobular domains are indicated. C) Western blot analysis of aggrecan fragments generated by ADAMTS and MMP proteases. Graphs below are quantification of the results. D) Proteoglycan synthesis as measured by ^{35}S-sulfate incorporation using whole disc organ cultures. Data are means ± SD of 4 independent experiments; * $p < 0.05$. Y=young and O= Old.

4.3.2 CLEARANCE OF DISC SENESCENT CELLS USING P16- 3MR TRANSGENIC MICE

To test the concept of a causative role for cellular senescence in driving age-related IDD, we used p16-3MR transgenic mice (Fig. 12A). These mice contain a transgene in which a fusion protein consisting of renilla luciferase (LUC), monomeric red fluorescent protein (mRFP) and herpes simplex virus 1 thymidine kinase (HSV-TK) is driven by the senescent sensitive $p16^{INK4a}$ promoter. $p16^{INK4a}$-positive senescent cells in p16-3MR mice can be selectively killed by ganciclovir (GCV), a nucleoside analog with a high affinity for HSV-TK that is converted into a toxic DNA chain terminator, causing fragmentation of mitochondrial DNA and apoptosis selectively in HSV-TK expressing senescent cells [135], [136], [138]. In our study, expression of RFP in old mice treated with GCV was three times lower compared to old mice treated with PBS, suggesting that GCV reduced $p16^{INK4a}$-positive senescent disc cells in the old GCV-treated group. Notably, disc RFP expression was higher in old mice treated with PBS than young mice, confirming the increased disc senescence observed in aged WT mice (Fig. 12B). It is important to note that ganciclovir treatment eliminates $p16^{INK4a}$-positive cells in all tissues including the disc.

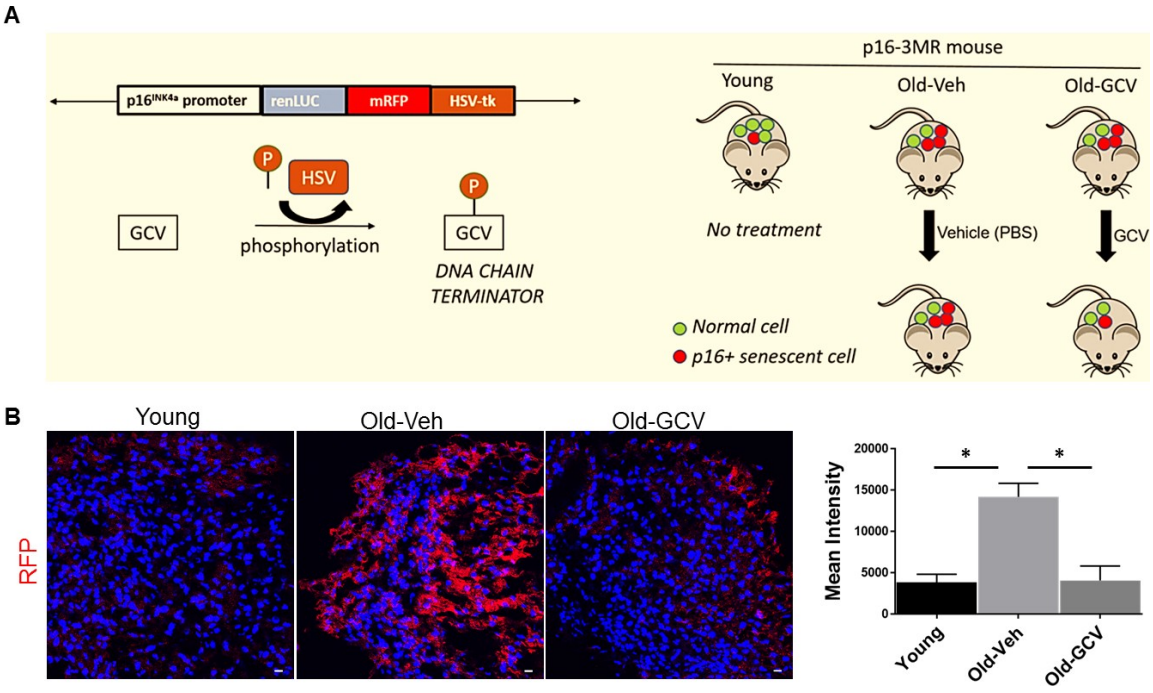

Figure 12. Clearance of senescent cells in p16-3MR mice
A) Schematic of the p16-3MR transgene and strategy used to selectively kill senescent cells. The transgene contains functional domains of renilla luciferase (renLuc), monomeric red fluorescent protein (mRFP), and herpes simplex virus (HSV-1) thymidine kinase (HSV-TK) under control of the senescence sensitive promoter of the $p16^{Ink4a}$ gene. $p16^{INK4a}$- positive cells are selectively killed by ganciclovir (GCV), a nucleoside analog, that upon phosphorylation by HSV-TK, can terminate DNA chain elongation and kill cells. p16-3MR mice were sacrificed at 12 months of age (Young) or were administered PBS (Old-Veh) or GCV (Old-GCV) for an additional 12 months before sacrifice. B) Confirmation of elimination of $p16^{INK4a}$-positive cells in Old-GCV mice was assessed by examining levels of disc RFP protein. Graph on the right is quantification of the results. Data shown are means ± SEM of 4 independent experiments, * $p < 0.05$.

4.3.3 ELIMINATION OF SENESCENT CELLS BY GCV TREATMENT SUPPRESSES DEGRADATIVE HISTOLOGICAL CHANGES AND AGE-RELATED DISC MATRIX PG LOSS

Histologic evaluation revealed fissures in nucleus pulposus, loss of AF-NP boundary, and disorganization of AF lamellae with age, but these declines were blunted in older p16-3MR mice treated with GCV (Fig. 13). These histological changes were supported by the increase in aggrecan content as demonstrated in Fig 15. The GCV treated old mice had 37% of the aggrecan

content of younger mice, whereas vehicle treated mice only had 10% of the aggrecan content of young (Fig. 15B). Aggrecan gene expression upon GCV treatment was significantly improved as well (Fig. 15A).

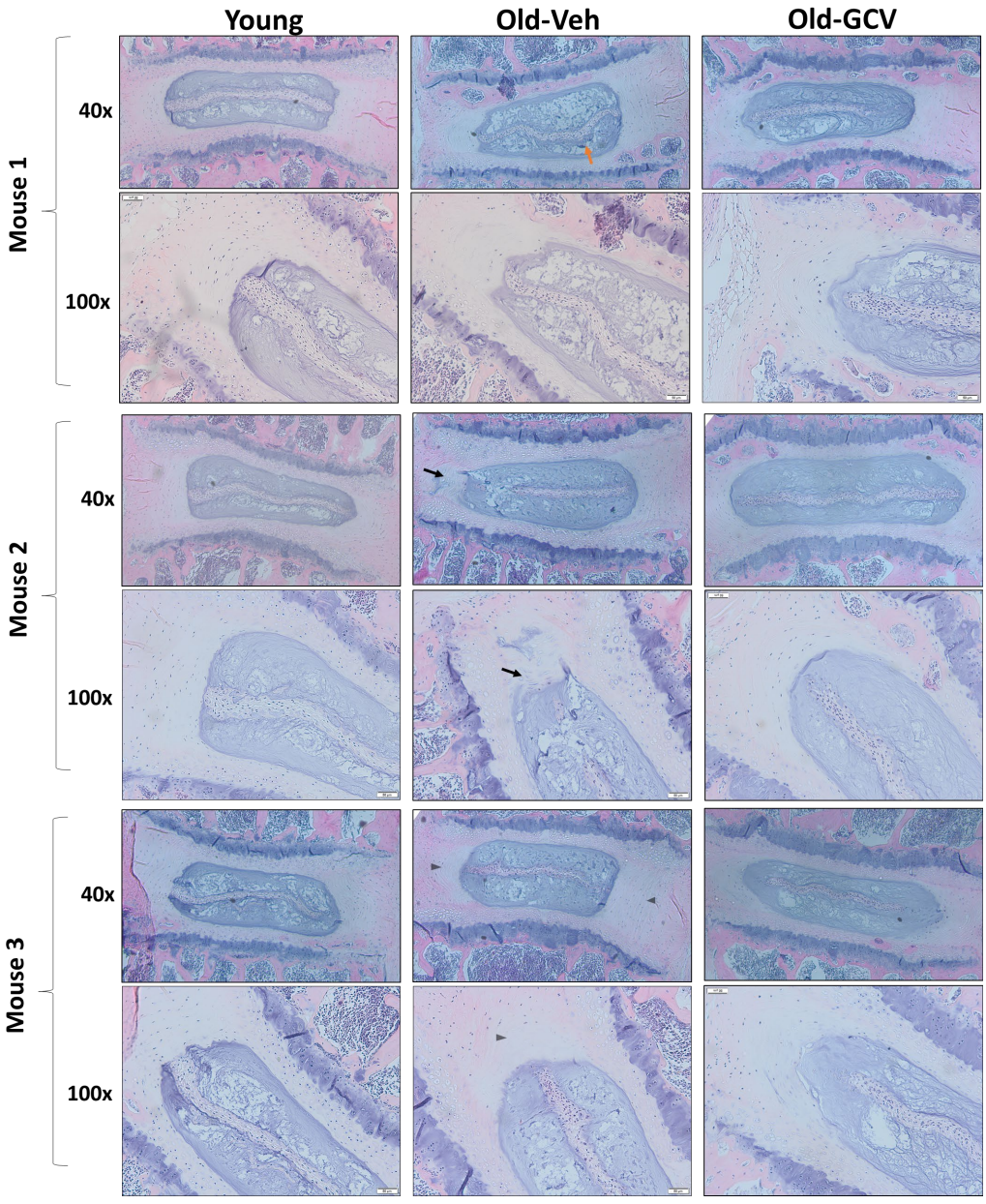

Figure 13. Impact of GCV treatment on gross morphology
H&E staining to assess the gross morphological changes revealed the discs of old p16-3MR mice treated with PBS to contain fissures (orange arrow) in NP, loss of NP and AF boundary (black arrow), and transition to non-concentric serpentine lamellae with each lamella spaced farther apart (black arrow heads) compared to young AF lamellae. These degradative changes seem blunted in the old p16-3MR mice treated with GCV. Discs sections from 5 individual p16-3MR Young, Old-Veh, and Old-GCV were compared.

4.3.4 CLEARANCE OF CELLULAR SENESCENCE BLUNTED DISC MATRIX PG PROTEOLYTIC DESTRUCTION

Disc matrix PG loss is largely due to destruction of aggrecan, the major PG constituent of the disc matrix. Disc aggrecan fragmentation catalyzed by MMP and ADAMTS classes of proteases was suppressed in old GCV-treated mice compared to old vehicle-treated mice. In fact, MMP mediated fragmentation in Old-GCV mice was comparable to that of young and was six times lower than in Old-Veh mice. In contrast, the decrease in ADAMTS mediated fragmentation of aggrecan in Old-GCV mice is less dramatic compared to Old-Veh mice (Fig. 14). Indeed, one of the major MMPs implicated in IDD is MMP13 [48]. MMP13 expression, at both the protein and mRNA level, were blocked by GCV treatment (Fig. 15C&D). Surprisingly, a major disc aggrecanase, ADAMTS4, was not altered by GCV treatment (data not shown). Reduced aggrecan fragmentation was also seen in p16-3MR mice treated with GCV for 6 months to clear senescent cells late in life (Fig 20). However, the effects were not as pronounced, suggesting that prolong senescence clearance is needed to produce beneficial effects.

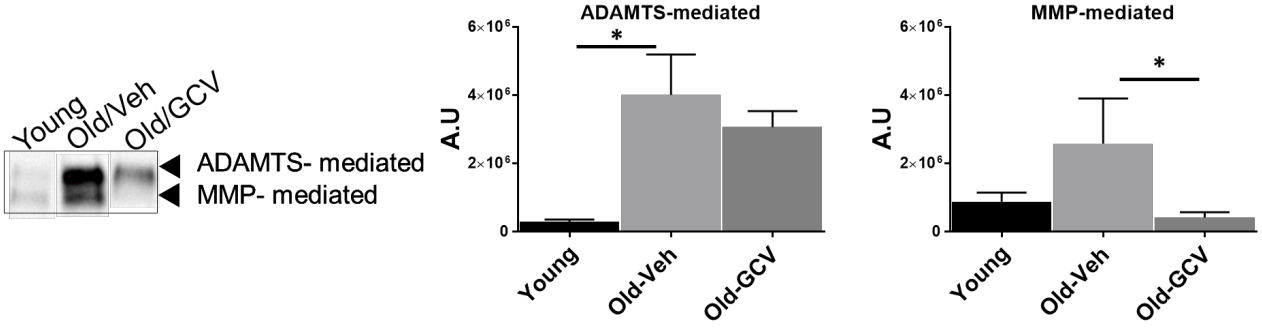

Figure 14. Effects of GCV treatment on disc aggrecanolysis of p16-3MR mice
A) Immunoblot of MMP and ADAMTS mediated cleavage of aggrecan. Graphs on right are quantification of aggrecan fragments shown in panel on left. Data shown is mean ± SD of 4 independent experiments, * $p < 0.05$.

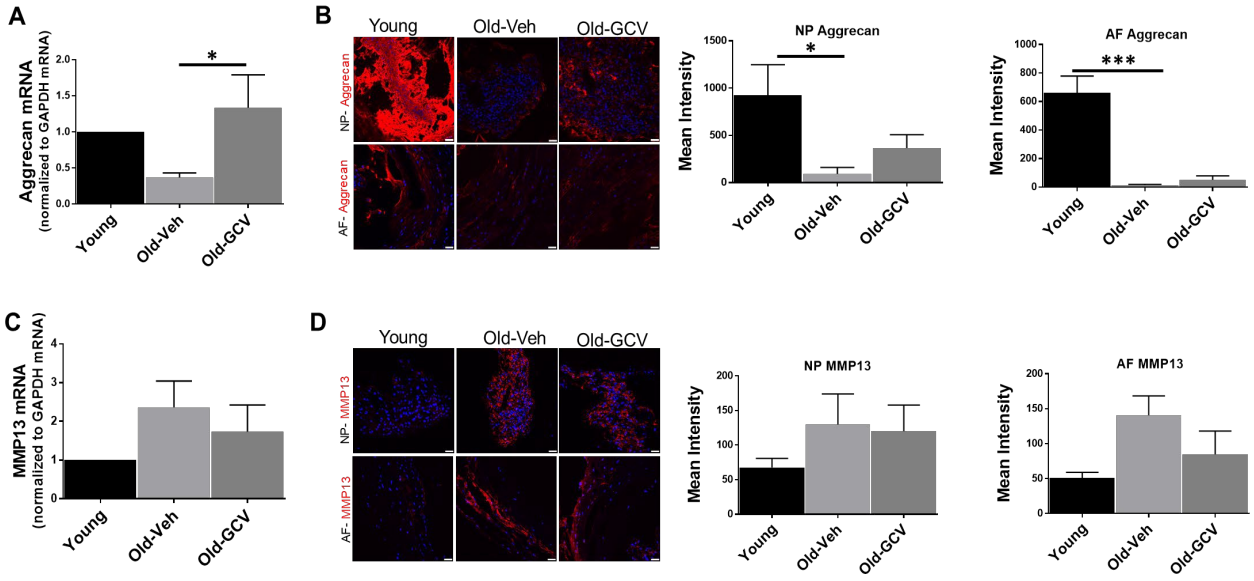

Figure 15. Effects of GCV treatment on aggrecan and MMP13 mRNA and protein levels in intervertebral discs of p16-3MR mice
Aggrecan mRNA levels were quantified by qRT-PCR (A) and protein was quantified by immunofluorescence signals (B) in nucleus pulposus and annulus fibrosus section of disc tissue. MMP-13 expression of whole disc mRNA by qRT-PCR (C) and protein by immunofluorescence (D) in inner nucleus pulposus and outer annulus fibrosus section of disc tissue. Graphs on the right are quantification of the immunofluorescence results. Data shown is mean ± SEM of 4 independent experiments, * $p < 0.05$, *** $p < 0.001$. Scale bar = 10 μm.

4.4 DISCUSSION

Cellular senescence and the SASP negatively impact tissue health and contribute to a myriad of age-associated disorders. Previous studies documented elevated cellular senescence in degenerative discs and a positive correlation between disc cellular senescence level and chronological age in humans [158], [160]. It was not clear, however, whether cellular senescence was a driver or bystander in disc aging and degeneration in these reports.

Since aging is the major contributor to disc degeneration, we hypothesized that cellular senescence is causal in age-related disc degeneration. We found elevated levels of senescence markers, together with repressed PG synthesis and increased aggrecan fragmentation, in

intervertebral discs of aged mice. Notably, the selective removal of senescent cells ameliorated these degenerative changes in conjunction with suppression of matrix protease expression. These findings suggest that the removal of senescent cells attenuated the age-associated disc degenerative changes. This is the first study to evaluate and report a direct adverse impact of cellular senescence on intervertebral disc with age.

The increased fragmentation and loss of proteoglycan, specifically aggrecan, are hallmarks of disc degeneration. Matrix degradative changes invariably lead to altered biomechanics and pathologic outcomes such as disabling chronic back pain and stenosis. Multiple reports show that inflammatory cytokines such as IL-1β, IL-6, and TNFα initiate the cascade that culminates in disc degeneration. These cytokines suppress the synthesis of matrix proteins and upregulate production of matrix proteases that include ADAMTS-4/5, MMP-1, -2, -3,-13, -14 [151]. Indeed, in this study, we saw a concomitant reduction in levels of the major disc protease, MMP13, and aggrecan fragmentation in Old-GCV compared to Old-Veh mice, suggesting cellular senescence phenotype as the source of the inflammatory proteins that trigger the degeneration cascade. This claim is substantiated by the report published by Ngo et. al., which showed decreased PG synthesis capacity, enhanced aggrecan fragmentation, as well as increased production of several cytokines and matrix proteases in oxidative stress-induced senescent human NP cells [76]. Together, these studies support the causal role for senescent cells in driving matrix homeostatic imbalance in aging discs.

Compared to young p16-3MR mice, both MMP- and ADAMTS- mediated proteolysis of aggrecan was elevated in aged p16-3MR mice. However, only MMP-, not ADAMTS-, mediated aggrecanolysis was reduced in discs of GCV-treated old p16-3MR mice. This result is consistent with the effects of GCV on expression of the two predominant matrix proteases in disc

tissue, MMP13 and ADAMTS4. MMP13 expression was reduced, while ADAMTS4 remained unaffected. This failure could be due to the fact that ADAMTS4 is not a SASP factor of $p16^{INK4a}$-positive disc cells and hence are not reduced by GCV administration. Overall, the reduced aggrecan fragmentation seen in GCV treated old p16-3MR mice could be due to elimination of p16INK4a positive disc cells that express MMPs, and increased PG synthesis.

To reduce the levels of other catabolic factors that are not decreased upon elimination of $p16^{INK4a}$-positive senescent cells, it will be important to identify $p16^{INK4a}$-independent stressor(s) that induce senescence in the disc. Studies published to date suggest that oxidative stress, genotoxic stress and chronic activation of NF-κB signaling play an important role in propagating age-related disc degenerative changes [77], [156], [198]. For example, lowering oxidative stress can increase PG content [77]. Likewise, inhibition of classical NF-kB signaling ameliorated several disc aging features, including PG content and cellularity [198]. However, much like this study, these studies relied on models wherein the stressor had systemic effects, so it is unclear whether these effects occur if the stress is induced only locally in the intervertebral disc tissue. Further investigation is needed to resolve the ambiguity about the relative contributions of systemic versus local factors to age-associated disc degeneration.

The observed improvement in disc pathology in p16-3MR mice treated with GCV are consistent with previous results using treatment of aged mice intermittently with senolytic agents. For example, the senolytic combination of dasatinib and quercetin was shown to not only reduce senescence and improve multiple age-related pathologies, but also to improve PG content in the disc of progerioid *Ercc1*$^{-/\Delta}$ mice [140]. Similarly, treatment of *Ercc1*$^{-/\Delta}$ mice with the senolytic HSP90 inhibitor 17-DMAG resulted in not only an extension of healthspan, but also

improved PG content [199]. Furthermore, intra-articular injection of the senolytic drug Navitoclax, a Bcl-2 family inhibitor, was recently shown to slow the progression of age-associated and post-traumatic osteoarthritis in aged mice [142]. However, the effect of these senolytics on disc pathology in natural aged mice is unknown.

The results of this study unambiguously demonstrate the deleterious impact of senescent cells on intervertebral disc degeneration with natural aging. Notably, we found that $p16^{INK4a}$-positive senescent cells promote the production of catabolic factors that are known to contribute to age-associated disc degeneration. As such our findings indicate that therapeutic approaches involving repopulation with functional IVD cells, growth factor supplementation or gene therapy may face challenges unless the inflammatory milieu created by senescent cells is eliminated from the degenerative disc [81], [200], [201]. In addition, these results suggest that a therapeutic strategy utilizing senotherapeutic agents such as D+Q, 17-DMAG or Navitoclax could be developed to ameliorate age-associated disc degeneration [140].

5.0 DISCUSSION

5.1 SUMMARY

Age-related changes in the intervertebral disc tissue predominantly contribute to low back pain, a pathology that imposes substantial societal and economic burden [8], [192]. The persistent oxidative burden and the ensuing inflammatory stress and matrix degradation are characteristic features of aged discs [55], [73]. Evidence in recent years implicate chronic oxidative and inflammatory burden as the stressors in the disc tissue that induce senescent phenotype in disc cells with age [64], [74], [76]. Growing evidence also implicate that the senescent disc cells are key players in propagating the progressive deterioration of disc health with age by robbing the disc of functional cells and exacerbating the degradation of disc tissue via secretion of SASP factors [158], [160]. However, it is not known whether senescent cells play a causal role in driving age-associated IDD despite their presence in aged disc tissue. Likewise, though *in vitro* reports have documented increased secretion of catabolic factors and enhanced fragmentation of matrix aggrecan, none have assessed the underlying metabolic changes that energetically support the enhanced secretion of proteins in the senescent disc cells. Therefore, for this dissertation work, we assessed the metabolic changes in senescent disc cells and determined whether they are causal in driving age-related degradative changes in disc tissue.

In Chapter 3, we described the extent of utilization of the two major energy generating pathways- glycolysis and OXPHOS- and the accompanying changes in oxidative stress-induced senescent (SIS) and non-senescent disc cells. In addition to displaying increased fragmentation of aggrecan and collagen II and secretion of inflammatory factors IL-6 & 8, the SIS disc cells had enhanced mitochondrial content and mitochondrial ATP-linked respiration. This metabolic alteration, i.e., increased mitochondrial respiration associated with ATP production was found to be needed for increased protein synthesis as abrogation of protein synthesis dampened the mitochondrial ATP-linked respiration in SIS disc cells but not in non-senescent disc cell control. This is consistent with other reports which show that in senescence induced by various stressors, lower synthesis of fatty acids and higher mitochondrial ATP production are metabolic adaptations these cells need to sustain their persistent and elevated production of proteins, including SASP factors [116], [117], [175]. The reliance on mitochondrial ATP was unexpected as the disc cells are known to rely on glycolysis for generation of ATP [18]. Therefore, these results suggest that the disc cells can be metabolically flexible and adapt to support the demands elicited by change in phenotype. Interestingly, the increased mitochondrial ATP related respiration in SIS disc cells was accompanied by increased mitochondrial number and protein expression (TOM20 and ATP synthase beta) but not mtDNA copy number. Equally interesting was the fact that the mtDNA did not colocalize with the mitochondria and was mostly cytoplasmic. The presence of cytoplasmic DNA activates the cGas-STING pathway, which was shown to mediate secretion of SASP factors in cells induced to senesce by various means [183]–[185]. Therefore, it is reasonable to conjecture that the cytoplasmic mtDNA in SIS disc cells play a role in regulating SASP factor synthesis as well. However, this remains to be verified. Furthermore, it cannot be discounted that the increased mitochondrial ATP -linked respiration is

to generate ATP for processes other than protein synthesis. Indeed, in chemotherapy-induced senescent cells, part of the increased mitochondrial ATP-associated respiration was needed for autophagic degradation of improperly synthesized proteins [116]. It was reasoned that the elevated secretion of proteins by senescent cells overwhelms their capacity for proper protein synthesis, and thus, autophagy is upregulated to cope with this proteotoxic stress. However, because protein synthesis is the most ATP-consuming process in cells [189], it is likely that most of the respiration associated with mitochondrial ATP production is utilized to produce proteins in SIS disc cells. In summary, these results suggest that the SIS disc cells acquire a catabolic phenotype and metabolic adaptation to support protein synthesis.

We reasoned that removal of senescent disc cells should attenuate the deterioration of disc associated with ageing. This is based on the observations that *in vitro* generated oxidative stress-induced senescent disc cells display the catabolic phenotype similar to that found *in vivo* which is a driver of age-related IDD. In Chapter 4, we described the age-related disc degenerative markers in the natural ageing p16-3MR mice and the changes in these markers upon selective clearance of $p16^{INK4a}$ positive senescent cells. We found elevated levels of $p16^{INK4a}$ positive senescent cells in the discs of old p16-3MR treated with vehicle compared to the young p16-3MR mice. The old p16-3MR mice administered with vehicle had all the histological markers associated with disc ageing, including clefts and fissures in the NP and AF and loss of AF lamellae structure (non-concentric). These histological changes appeared attenuated in the old p16-3MR mice treated with GCV to selectively remove $p16^{INK4a}$ positive senescent cells. In addition, substantial loss in aggrecan content, a major hallmark of disc degeneration, associated with ageing in disc was attenuated upon treatment with GCV. Since aggrecan loss could be due to increased fragmentation and diffusion through clefts/fissures, we

assessed the proteolytic degradation of aggrecan. Whereas the aggrecan fragmentation in p16-3MR old mice treated with vehicle was significantly elevated compared to young mice, the proteolytic cleavage of aggrecan in p16-3MR old-mice treated with GCV was significantly reduced and was comparable to that of the young mice. However, only MMP-, and not ADAMTS- mediated proteolytic cleavage was reduced in discs of GCV-treated old p16-3MR mice. This result was consistent with the impact of GCV on the expression of the two major protease in disc tissue, MMP13 and ADAMTS4. While the expression of MMP13 was reduced, the ADAMTS4 levels remain unaltered. This result was unexpected and could be due to the ability of GCV to eliminate $p16^{INK4a}$ senescent cells that secrete MMP13, and not, senescent cells that secrete ADAMTS4 as part of their SASP. However, this remains to be verified. As mentioned earlier, the disc tissue likely contains a mosaic of distinct senescent cell types. Therefore, as this study highlights, it is important in future studies to identify disc senescent cell types that contain other proteases such as ADAMTS4 and other pro-inflammatory cytokines as part of their SASP repertoire. Overall, the reduced fragmentation and increased content of disc aggrecan and the improvement in histological features could be due to elimination of $p16^{INK4a}$ positive disc cells that express MMPs, and probably other SASP factors, and increased PG synthesis. In summary, this study demonstrates the causal role of senescent cells in age-related disc degeneration.

Collectively, the studies described in Chapter 3 and 4, provide solid evidence for considering senescent disc cells as therapeutic targets to ameliorate age-related disc degeneration. The studies described herein are the first to evaluate the underlying metabolic changes in senescent disc cells and demonstrate their causative role in promoting age-related IDD. As such, the current senolytics which have been investigated in context of osteoarthritis,

atherosclerosis, and stem cell rejuvenation, should be evaluated in context of age-related disc degeneration as well. Senolytics provide a promising alternative to current disc degeneration treatments such as gene-, protein-, and stem cell- based therapies, which are invasive, expensive, and whose affects are short lived.

5.2 LIMITATIONS

The experimentation performed in this work has a number of noteworthy limitations. First, in the *in vitro* model, the disc cells were cultured in Ham's F-12 media supplemented with 10% FBS, which is a standard practice in the field. However, this is does not completely mimic physiological condition as the glucose concentration (10 mM) [202] is much higher and osmolarity (~300 mOsm/ kg H_2O) [203] much lower in the Ham's F-12 media/10% FBS culture media compared to what disc cells experience in vivo (<<5mM glucose and 500 mOsm/ kg H_2O) [204], [205]. Additionally, the mechanical stress that the disc cells experience in vivo was also absent, possibly resulting in cellular responses that would not be present *in vivo*. This limitation could be overcome by applying the appropriate mechanical load, e.g. using the Flexcell system, and culture media that approximates the physiologic osmolarity and nutrient concentration. While this dissertation work was well under way, media ideal for culture of disc cells, called PrimeGrowth™, was made commercially available [206]. Therefore, incorporation of mechanical load and culture in PrimeGrowth™ media in the engineered *in vitro* culture model would be optimal. Second, the oxidative stress-induced senescent disc cells with the described phenotype may not recapitulate the features of the senescent disc cells that exists in vivo. This limitation could certainly be resolved by isolating the native $p16^{INK4a}$ positive cells from the

discs of p16-3MR mice and comparing their phenotypes to senescent disc cells generated *in vitro*. Lastly, the ability of proteins to upregulate mitochondrial respiration in senescent disc cells *in vitro* was assessed by performing Seahorse assay in presence of cycloheximide, which is a non-specific protein synthesis inhibitor. It would be interesting to assess whether the SASP factor synthesis in senescent disc cells is dependent on the upregulated mitochondrial respiration. This limitation could be addressed by blocking the mitochondrial respiration by antimycin A/rotenone and then assessing the expression of SASP factors.

The *in vivo* study involving the p16-3MR mice has several limitations as well. As in the case of every biological study, the outcomes in the *in vivo* study represent only a small portion of the broad gamut of potential biological markers. Proof of absence of cellular senescence by assessment of additional senescence markers which are known to be expressed in $p16^{INK4a}$ senescent cells, such as loss of HMGB1 [105], DNA damage markers, and cytoplasmic localization of lamin B1 [207], in discs of p16-3MR mice would further strengthen the claim of amelioration of disc degenerative changes with elimination of senescent cells. Additionally, evaluation of other SASP factors such as IL-1β, TNFα, IL-8 was also warranted as these factors are known to play key role in propagating disc degeneration and back pain. Additionally, scoring of the degenerative changes in the NP, AF, and EP in the H&E images would have been useful in understanding the impact of senescence on the whole disc, and not just aggrecan. Other limitations are more difficult to overcome without significant development. The clearance of $p16^{INK4a}$ positive cells in the old p16-3MR mice is systemic, and therefore, the ensuing effects cannot just be attributed to elimination of $p16^{INK4a}$ positive cells in the disc. Overcoming this limitation would require creation of mice model that would enable clearance of disc-specific $p16^{INK4a}$ positive cells. To target genes specifically in the disc, transgenic mice which contain

expression of candidate genes under the control of the promoter aggrecan or type II collagen have been created [208]. However, though the aggrecan and collagen II are abundantly expressed in disc, they are also expressed in other cartilage tissue. Therefore, limitations exist with the currently available transgenic models as they are not completely disc-specific.

5.3 FUTURE DIRECTIONS

Immediate extensions of this work include investigation of the mechanism that regulates the secretion of SASP factors. The classical NF-κB signaling pathway is upregulated with ageing in disc tissue and is known to control expression of multiple different catabolic factors that are involved in promoting matrix degradation of disc tissue [209]. Importantly, genetic and pharmacological inhibition of canonical NF-κB signaling is found to ameliorate age-related disc degenerative changes [198]. Equally important is the fact that the oxidative stress-induced senescent disc cells contain cytoplasmic mtDNA which could activate cGAS-STING pathway, which in turn could activate the classical NF-κB signaling pathway [183]. Therefore, it would be logical to assess whether p65 subunit of NF-κB controls the expression of senescent disc cell SASP. It also would be interesting to assess the capability of the *in vitro* generated oxidative stress-induced senescent disc cells to impact the disc tissue in vivo. This could be achieved by injecting the senescent disc cells into the discs of rat and evaluating the outcomes such as disc height, proteoglycan content and fragmentation, and biomechanical function few weeks post the administration of the senescent cells. Rats injected with vehicle (PBS) and proliferating disc cells would be used as control. Another potential path from the in vitro work would be to evaluate the capability of these in vitro engineered senescent disc cells to induce senescence in proliferating

disc cells. One of the ways cellular senescence promotes loss of tissue health and function with age is by induction of senescence in neighboring cells. Hence, it would be instructive in determining if the SASP secreted by senescent disc cells is capable of inducing senescence in proliferating disc cells.

Over the past few years research has uncovered multiple different compounds which specifically target and kill senescent cells and show improvement in healthspan when administered to mice *in vivo*. Treatment with a combination of dasatinib and quercitin resulted in improvement in healthspan and reduction in PG loss in the disc of progeroid *Ercc1*$^{-/\Delta}$ mice [140]. Likewise, treatment with another senolytic HSP90 inhibitor 17-DMAG also resulted in improved PG content along with improvement in healthspan [199]. However, few of these agents have been tested to improve disc pathology in natural aging mice. Therefore, future work should focus on evaluating the senolytic compounds with proven efficacy to attenuate age-related changes in disc tissue. Furthermore, compounds which have shown to extend lifespan in mice by the National Institute on Aging Interventions Testing Program (NIA-ITP), such as rapamycin, acarbose, 17α-estradiol and nordihydroguaiaretic acid (NDGA) [146], [210], should be evaluated for their ability to improve disc pathology by eliminating or suppressing senescent cells and their SASP. The *in vitro* studies done so far do suggest that compounds such as rapamycin might serve to suppress SASP in disc senescent cells [211]. Metformin is another drug which will be tested in a clinical trial for its ability to delay onset of multiple age-related pathologies by the NIA-ITP (National Clinical Trial number: NCT02432287) [212]. Metformin is a FDA approved drug for the treatment of diabetes. It inhibits complex I of ETC and can therefore suppress production of mitochondrially derived reactive oxygen species (ROS), a major contributor to disc degeneration [213]. Tert-butyl hydroxide-induced senescence in NP cells was suppressed

when treated with metformin [75]. More importantly, metformin treatment was able to ameliorate IDD in a puncture-induced rat model [75]. Examining the impact of metformin *in vivo* on the age-related inflammatory and catabolic events promoted by senescence and other factors holds much promise. The other compounds to consider as well include curcumin [84], [214], an active ingredient of turmeric, and resveratrol [215], a sirtuin agonist, neither of which extend the lifespan of mice but do show amelioration of disc degenerative changes in mice. The *in vitro* oxidative stress-induced senescence model developed in this dissertation work can be used as a screen to test the efficacy of the potential senolytic agents before commencing in vivo work.

Considering the fact that the population in Unites States and elsewhere is ageing rapidly, research work that develop therapeutics to compress the functional decline in old age is imperative as a greater percent of people over the age of 65 years have more than one chronic disease pathology [216]. Cellular senescence has been implicated in driving multiple age-associated pathologies, and therefore therapeutic approaches that specifically kill senescent cells hold potential to extend the healthspan and lifespan. Since IDD is an age-related pathology, it is important to address whether cellular senescence adversely impact disc tissue health with ageing. This dissertation work was therefore carried out to addresses this crucial gap.

APPENDIX A

SUPPLEMENTARY MATERIALS FOR CHAPTER 3.0

This appendix includes the supplementary materials for the manuscript described in Chapter 3.

A.1 METHODS

A.1.1 TRANSMISSION ELECTRON MICROSCOPY (TEM) IMAGING

SIS and non-SIS disc cells were fixed in cold 2.5% glutaraldehyde in 0.01 M PBS for 1 hour at room temperature. The specimens were rinsed in PBS, treated with 1% osmium tetroxide/1% potassium ferricyanide, rinsed in PBS, dehydrated through a graded series of ethanol and propylene oxide and then embedded in Poly/Bed® 812 (Luft formulations). Semi-thin (300 nm) sections were cut on a Leica Reichart Ultracut, stained with 0.5% Toluidine Blue in 1% sodium borate and examined under the light microscope. Ultrathin sections (65 nm) were stained with uranyl acetate and Reynold's lead citrate and examined on JEOL 1011 transmission electron microscope with a side mount AMT 2k digital camera (Advanced Microscopy Techniques, Danvers, MA).

A.1.2 PROTEIN SYNTHESIS

Disc cells were cultured for 24 hours in F-12 media containing 10% FBS and 1% PS in a 6-well plate in the presence of 10 µCi/ml ^{3}H-*L*-proline at 37°C. The cells were either supplemented with 2.5, 5, 10 µg/ml cycloheximide (CHX) or left untreated (0 µg/ml CHX) at the start of the assay. The conditioned media (CM) was collected and the cell samples suspended in homogenizing buffer (20 mM Tris-HCl, 200 mM NaCl, 100 mM glycine, 0.1% Triton×100, 50 µM DTT, 0.1 mg/ml soybean trypsin inhibitor) and subjected to three freeze/thaw cycles at the end of the 24hr incubation period. The samples were then stirred at 4°C for overnight, the CM and cell layer combined, and protein synthesis determined by 3H-proline incorporation using a previously established method [217].

A.1.3 CELL VIABILITY

The quantity of disc cells in presence of 0, 2.5, 5, 10 µg/ml cycloheximide (CHX) was determined using Picogreen assay kit (Cat. No. P7589, *ThermoFisher Scientific*) as before [217].

A.2 SUPPLEMENTARY FIGURES

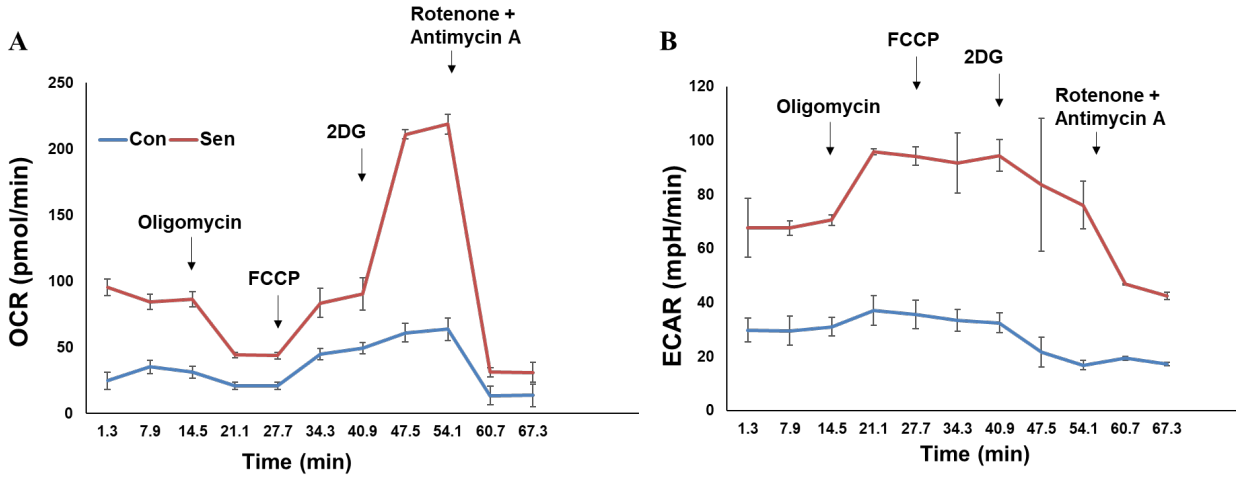

Figure 16. (in support of Fig 6). Representative profiles of SIS and non-SIS glycolytic and oxidative activity by Seahorse assay

80x 10^3 SIS and non-SIS disc cells were plated in Seahorse XF96 Extracellular Flux Analyzer plate. A) The Seahorse Analyzer records rates of oxidative phosphorylation via the oxygen consumption rate (OCR) in each well. Basal OCR is calculated from the mean time points of 1-3. ATP-linked OCR is calculated by taking the basal OCR and subtracting the mean of time points 4 and 5. Proton leak is calculated from taking the taking the mean of oligomycin sensitive OCR and subtracting it from the mean time points of 10 and 11. B) Glycolytic activity is assessed by recording the extracellular acidification rate (ECAR). Basal ECAR is calculated from the mean of time points 1-3. Metabolic inhibitors, oligomycin, carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone (FCCP), 2-deoxyglucose (2DG), and rotenone and antimycin A were added sequentially to distinguish cellular reliance upon glycolysis and oxidative phosphorylation. The plots are illustrative of seahorse trace of a single experiment. Each dot represents a time at which OCR or ECAR was measured. Error bars represent standard deviation ofthe mean.

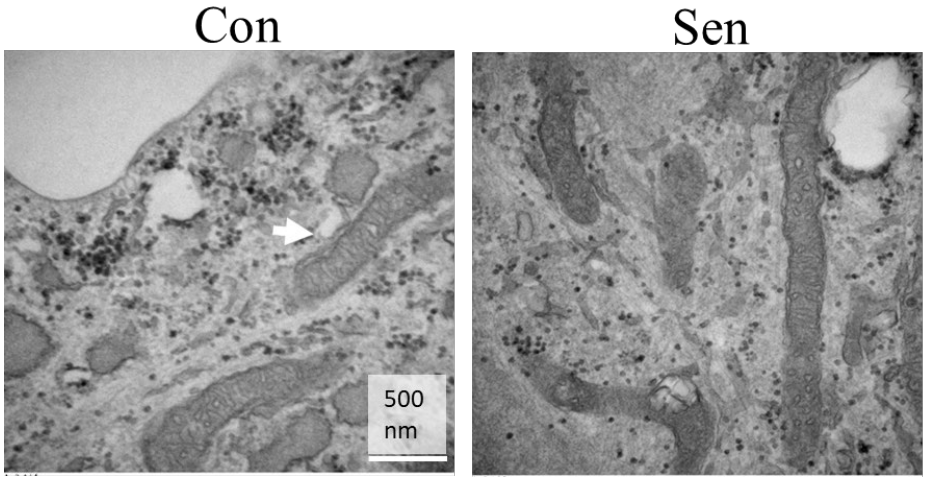

Figure 17. Electron microscopic imaging of mitochondria in SIS and non-SIS cells
The mitochondria in SIS and non-SIS disc cells as assessed by TEM imaging revealed similarity in terms of membrane and cristae definition. Arrow (white) = mitochondria.

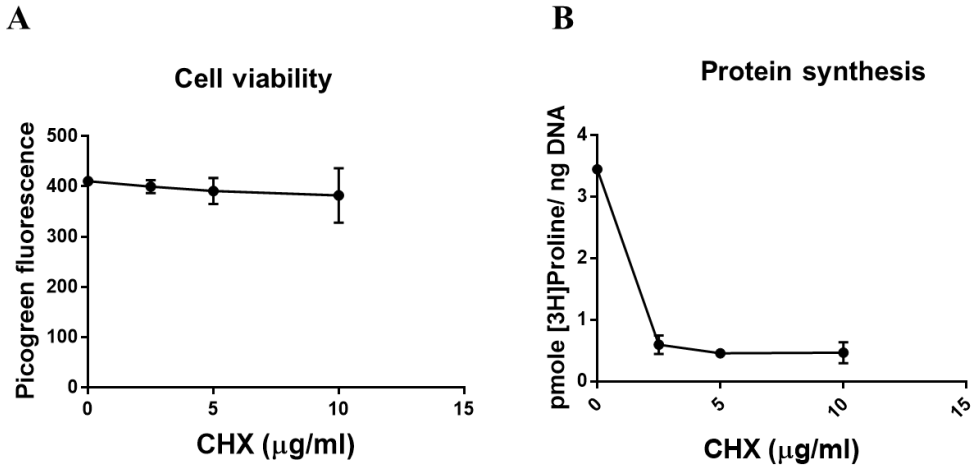

Figure 18. Determination of cytotoxicity and protein synthesis in disc cells treated with CHX
A) Viability of disc cells treated with either 0, 2.5, 5, 10 μg/ml CHX for 24h was assessed by picogreen fluorescence assay. B) Protein synthesis as measured by incorporation of [3H] Proline in disc cells after 24h incubation with different CHX concentrations (indicated). Data are means ± SD of 2 independent experiments.

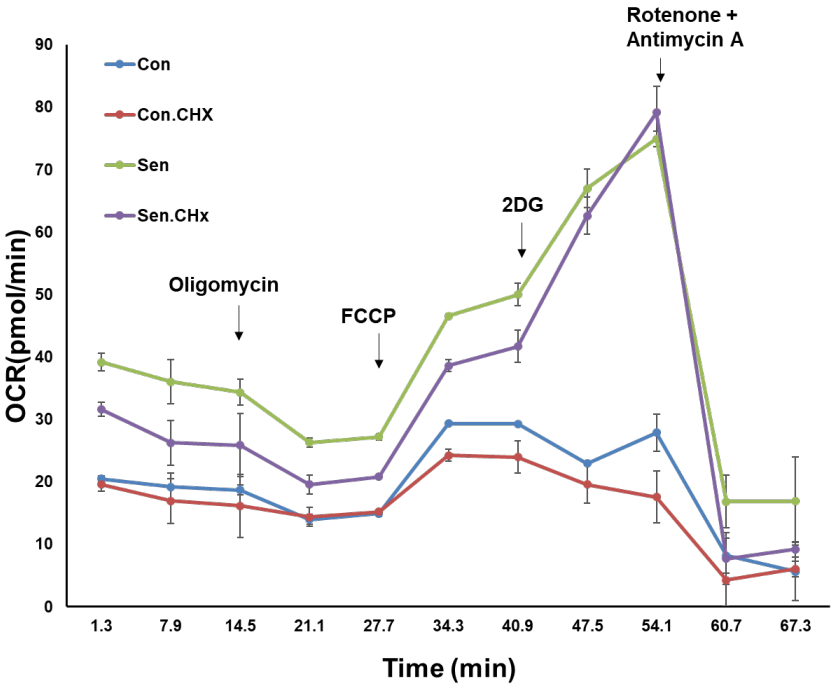

Figure 19. (in support of Fig 10). Representative Seahorse profiles of oxidative activity of SIS and non-SIS disc cells treated with and without protein synthesis inhibitor

Seahorse trace illustrating basal OCR and OCR after addition of sequential metabolic inhibitors (indicated) of SIS and non-SIS disc cells treated with cycloheximide or left untreated. Graph is illustrative of a single seahorse experiment trace. Error bars represent standard deviation of the mean.

APPENDIX B

SUPPLEMENTARY MATERIALS FOR CHAPTER 4.0

This appendix includes the supplementary materials for the manuscript described in Chapter 4.

B.1 RESULTS

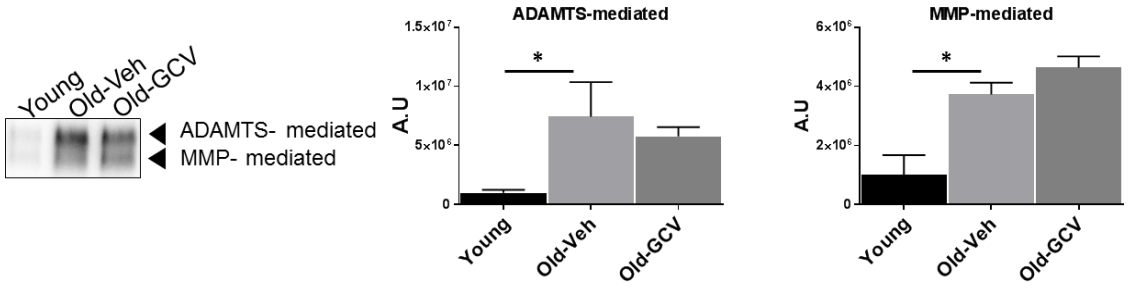

Figure 20. Late life clearance of p16-positive senescent cells in p16-3MR mice
p16-3MR mice were sacrificed at 12 months of age or treated with either PBS or 25mg/kg of GCV for 5 days every two weeks beginning 20 months of age for 6 months. The PBS and GCV treated p16-3MR mice were then sacrificed at the end of treatment and the spines and tails isolated for further analysis. A) Immunoblot of MMP and ADAMTS mediated aggrecan fragments. Graphs on right are quantification of results in left panel. Data shown is mean ± SEM of 3 independent experiments, *$p < 0.05$.

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