A REVIEW ON CELLULAR SENESCENCE AND PROSPECT OF SENOLYTIC DRUGS AGAINST CHRONIC AGE-RELATED DISEASES

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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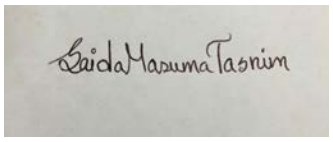

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Approval

The thesis/project titled “A Review on Cellular Senescence and Prospect of Senolytic Drugs Against Chronic Age-Related Diseases” submitted by Saida Masuma Tasnim of Spring, 2017 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelors of Pharmacy.

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Ethics Statement

This study does not involve any kind of animal or human trial.

Abstract

Cellular senescence can be described as a condition in which dividing of the cells stops permanently and undergo distinctive epigenetic modifications, including profound chromatin and secretory changes, resulting in the formation of senescent cells. Senescent cells accumulate in multiple tissues throughout the course of ageing and the formation of excessive senescent cells in various tissues results in numerous chronic disorders, tissue degradation, age-related diseases, and ageing of organs. This indicates that the removal of senescent cells can minimize ageing and thus serves as a potential target for therapeutic intervention. Senolytic drugs are substances that selectively cause senescent cell apoptosis. Several age-associated diseases including cardiac dysfunction, heart failure, atherosclerosis, liver diseases, osteoporosis, pulmonary fibrosis, Parkinson's and Alzheimer's disease have been shown to be mitigated by the use of senolytic agents in preclinical as well as clinical studies.

Keywords: Ageing, Cellular senescence, Chronic disorders, Senolytic drugs, Apoptosis

Dedication

Dedicated to my parents

Acknowledgement

I would like to start by thanking Allah SWT Almighty for His unbounded blessings, mercy and kindness. All praise to Him for blessing me with the enormous patience, strength, courage, knowledge, wisdom and hope needed to finish this project.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

AAA	Abdominal aortic aneurysm
ADCC	Antibody-dependent cell-mediated cytotoxicity
AD	Alzheimer's disease
AlphaSMA	Alpha-smooth muscle actin
AMPKα	AMP-activated protein kinase-alpha
APCs	Antigen presenting cells
AP-1	Activator protein-1
ATF6	Activating transcription factor 6α/β
ATP	Adenosine triphosphate
β-Gal	Beta-galactosidase
CAR	Chimeric antigen receptor
CCFs	Cytoplasmic chromatin fragments
CDKi	Cyclin-dependent kinase inhibitor
CDK4	Cyclin-dependent kinase 4
CDKN2A/B	Cyclin-dependent kinase inhibitor 2A/B
CDKs	Cyclin-dependent kinases
CM	Conditioned medium
CML	Chronic myeloid leukemia
COPD	Chronic obstructive pulmonary disease

CpG	5'—Cytosine—phosphate—Guanine—3'
CREB	cAMP response element-binding protein
CSF	Cerebrospinal fluid
CVDs	Cardiovascular diseases
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DDR	DNA damage response
DIMC	Dimethoxycurcumin
DNAM1	DNAX accessory molecule-1
DPP4	Dipeptidyl peptidase 4
DPS	Developmentally programmed senescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Endothelial growth factor receptor
EndoG	Endonuclease G
ER	Endoplasmic reticulum
GM-CSF	Granulocyte macrophage colony stimulating factor
HDACs	Histone deacetylases
HLA	Human leukocyte antigen

HP-1	Heterochromatin protein 1
HMSCs	Human mesenchymal stem cells
HSCs	Hepatic stellate cells
HSF1	Heat shock factor 1
Hsps	Heat shock proteins
HUVECs	Human umbilical vein endothelial cells
IFNγ	Interferon gamma (IFNγ)
IGF	Insulin-like growth factor
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
IRE1	Inositol requiring enzyme 1α/β
MCP	Monocyte chemoattractant proteins
MIF	Macrophage migration inhibitory factor
MIP	Macrophage inflammatory protein
MMPs	Matrix metalloproteinases
MOs	Macrophages
NF-kB	Nuclear factor-kB
NG2	Neuron-glial antigen 2
NK	Natural killer cells
OA	Osteoarthritis

OPCs	Oligodendrocyte progenitor cells
PAMPs	Pathogen-associated molecular patterns
PDGF-AA	Platelet-derived growth factor AA
P-ECs	Pulmonary vascular endothelial cells
PERK	PKR-like ER kinase
PRRs	Pattern recognition receptors
PVR	Poliovirus receptor
RA	Rheumatoid arthritis
RB	Retinoblastoma
ROS	Reactive oxygen species
RS	Replicative senescence
SASP	Senescence associated secretory phenotype
SAHF	Senescence-associated heterochromatin foci
SF	Synovial fibroblasts
SIPS	Stress induced premature senescence
SNpc	Substantia nigra pars compacta
ST	Synovial tissues
TAA	Thoracic aortic aneurysm
TGF	Transforming growth factor
TIMPs	Tissue inhibitors of metalloproteinases

VSMCs	Vascular smooth muscle cells

Chapter 1

Introduction

Ageing is a stage of becoming older, displaying signs of age, a deterioration in physical as well as mental well-being over time, ultimately resulting in death. It is characterized by a gradual decline in the regeneration of tissues, accumulation of a broad spectrum of cellular and molecular alterations over time, having a detrimental effect on the structure and biological activity of different organs (Ageing and Health, n.d.; Kirkwood, 2005). This reduction in physiological integrity, eventually disables the body to maintain a homeostatic environment and makes it vulnerable to different kinds of diseases, which may ultimately lead to death (WHO | Ageing and Life Course, n.d.). Due to the changes in physical appearance and an elevation in the risk of illnesses and mortality associated with ageing, man has been concerned with trying to decelerate, prevent, or even counteract the signals of ageing since the beginning of time. Many have stepped as far as conducting experiments with dietary regimens, intense exercises, relaxing rituals, and naturally occurring or synthetic wonder-elements to prevent the signs of ageing (EMD Millipore Corp., 2015).

Every living cell exists in a persistent and continuous battle with external and internal agents that can harm them. The life of the living organisms would be significantly limited without its own compensatory mechanisms, as the accumulation of toxic materials would destroy the cellular components and their functions, eventually leading to injury of different organs and intensifying the premature ageing of the whole body (Dodig et al., 2019). Cellular senescence can be described as a condition in which division of the cells stops permanently and undergo distinctive epigenetic modifications, including profound chromatin and secretory changes, resulting in the formation of senescent cells (SCs) (Dodig et al., 2019; Li, Qin, et al., 2019). Senescent cells accumulate in multiple tissues throughout the course of ageing. Cellular

senescence normally exists to prevent the spread of damaged cells and preserve tissue homeostasis (Demaria et al., 2014). However, it also promotes irreversible, detrimental cell damage and decline in tissue homeostasis, especially as the organism ages, contributing to age-related diseases (Li, Qin, et al., 2019). The phenomenon, cellular senescence can arise from oxidative stress, telomeric injury/shortening, DNA damage, mitochondrial dysfunction, epigenetic abnormalities, inflammation, chromatin degradation and oncogene activation (Dodig et al., 2019).

During ageing, a decrease in T-cell regeneration along with an elevation in the development of terminally differentiated T cells are often noticed, which results in immune function deterioration and accumulation of senescent cells (Desai & Landay, 2010).Senescent cells formed in younger organisms are probably wiped by the immune system, but they adhere in elderly tissues, secreting dangerous proinflammatory signals that further destroy different organs in our body (EMD Millipore Corp., 2015). With increasing age, the frequency at which senescent cells experience immune clearance decreases, because of immune dysfunction. Additionally, the senescent cells make immune cells senescent which leads to determined and immoderate accumulation of senescent cells (Barbouti et al., 2020). The formation of excessive senescent cells in various tissues results in tissue degradation, age related disorders, and faster ageing of organs. This indicates that the removal of senescent cells can minimize degradation of organs and tissues accompanied by ageing, serving as an effective strategy in the treatment of numerous chronic diseases (Li, Qin, et al., 2019).

Senolytic drugs are substances that selectively cause senescent cell apoptosis. In animal model experiments, aiming senescent cells using pharmacological or genetic interventions interrupt, mitigate or alleviate various age-related phenotypes, persistent disorders, geriatric syndromes, and lack of biological functional resistance. Disorders that have been successfully

treated by diminishing senescent cells in preclinical studies include fragility, cardiac dysfunction, heart failure, atherosclerosis, myocardial infarction, chronic liver disease, osteoporosis, pulmonary fibrosis, Parkinson's disease, Alzheimars disease and radiation-induced damage (Bhatia-Dey et al., 2016; Chinta et al., 2018; James L. Kirkland et al., 2017; Sawaki et al., 2018). An overview on cellular senescence has been depicted in Figure 1.

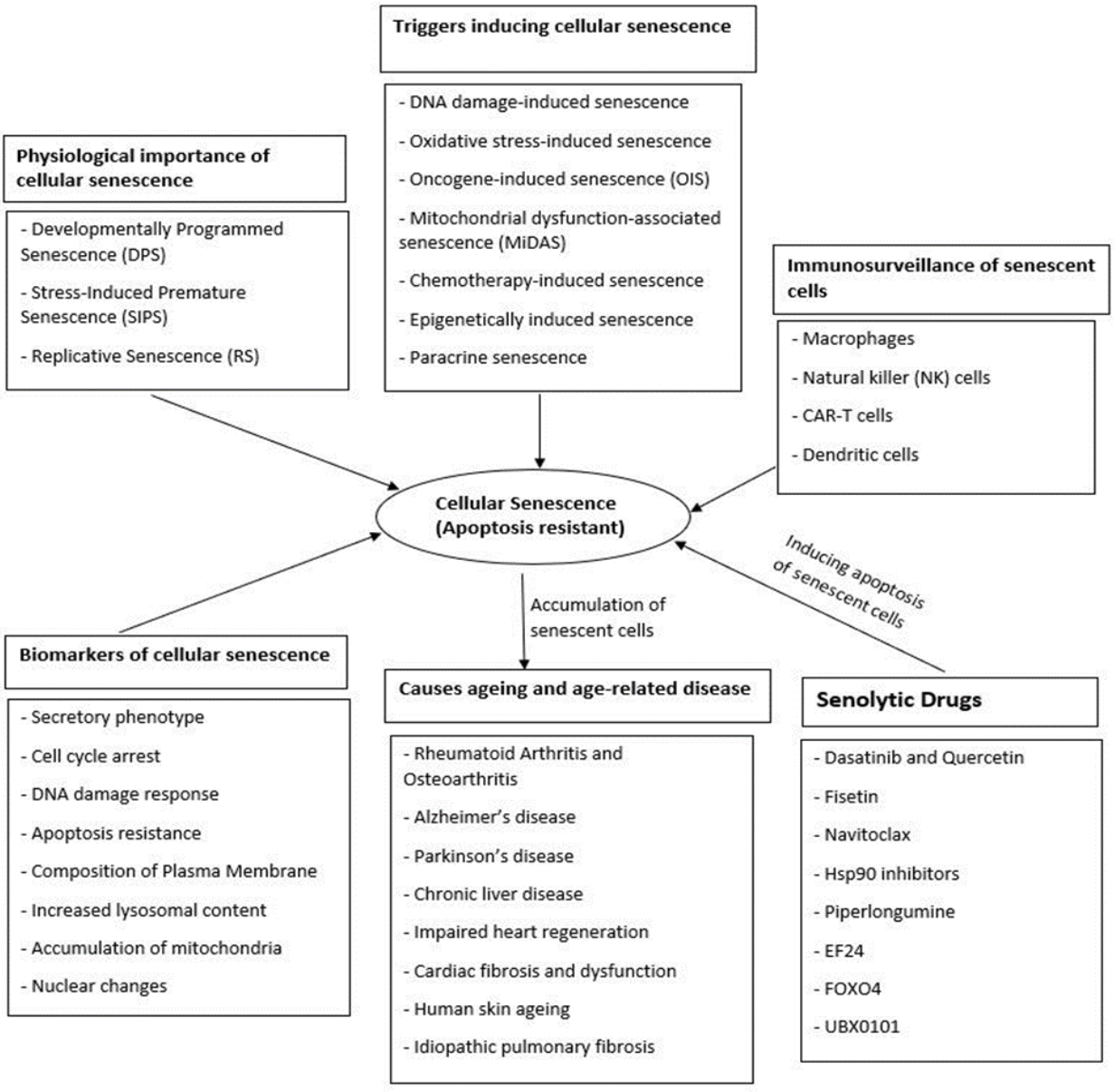

Figure 1: An overview on cellular senescence

1.1 Objectives of the Study

The objectives of this study are-

- to identify the role of cellular senescence in inducing age-related diseases.

- to provide an insight on the importance of senolytic drugs in the elimination of senescent cells.

- to analyze the therapeutic outcome of senolytic drugs in minimizing diverse age-associated pathologies and in increasing the health span of an individual.

1.2 Rationale of the Study

Ageing, an inevitable phenomenon that occurs over time, has typically drawn interest and enthusiasm throughout human history. It is described by a gradual loss of biological functional integrity and elevated risk of death. The physical decline encompassing ageing is the key risk factor for the formation of several major human diseases for instance diabetes, coronary diseases, cancer and neurodegenerative disorders, which interferes with the healthy lifespan of an individual. Every human being wants to live a long and healthy life without any pain or illnesses. Cellular senescence, which has a beneficial role in the body including tissue homeostasis and remodeling, embryonic patterning, wound healing and tumor suppression, tends to accumulate over time due to telomere shortening, immune dysregulation and persistent exposure to stressful stimuli. This results in the upregulation of factors accelerating the process of ageing and leads to premature ageing syndromes. Therefore, this review was conducted to provide an insight on the importance of eliminating senescent cells which accumulates through ageing by employing senolytic therapies to minimize diverse age-associated pathologies and hence, increase the health span of an individual.

Chapter 2

Literature Review

The current study involved a comprehensive overview on different aspects of cellular senescence and discussed the prospect of senolytic drugs as promising candidates in the removal of senescent cells, thus, delaying premature ageing and age-related chronic diseases. The information for this review paper was collected from the secondary research articles indexed in PubMed, Elsevier, Web of Science, Scopus, etc and the key publications included Springer, Nature, Cells, The Lancet, MDPI, Frontiers, PloS, Science, Aging Cell, Immunity, etc. Prior to compiling the information from the secondary data, an outline was created to look for the information in a systematic manner. Initially, it was important to attain an insight on cellular senescence, its physiological significance, how it is formed and regulated, and its relation with advanced ageing and several diseases. Later, further research was performed on its biomarker identification and evaluation of pre-clinical and clinical study outcomes utilizing senolytic drugs to destroy senescent cells with the purpose of slowing down the ageing process and development of age-related diseases. A large number of articles were scrutinized to present the study with reliable information and all the articles were cited properly.

Chapter 3

Hallmarks of Ageing

Ageing is accompanied by a gradual loss of functional stability, leading to diminished physiological activities and increased susceptibility to mortality. Different human pathologies, including cancer, diabetes, coronary conditions and neurodegenerative diseases develop as a result of this anomaly in bodily functions deteriorating the quality of life (López-Otín et al., 2013). Although ageing is a natural process and people are bound to age over time, however, there are factors which accelerate the process of ageing and so does the risk of developing age-associated illnesses. Some common hallmarks of ageing include rise in free radical production, epigenetic alterations, loss of proteostasis, persistent inflammation and replicative senescence (MacNee et al., 2014).

3.1 Free Radicals

The fundamental chemical mechanism underlying ageing was established in 1954 by the Free Radical Theory of Ageing (FRTA), where the active free radicals naturally formed in the organism interacts with cellular components. The FRTA, and the corresponding observation of the essential, pervasive role of free radicals in disrupting endogenous metabolic processes followed by DNA damage is responsible for accelerating the ageing process over time and death of all living beings (Harman, 2006).

The existence of mitochondria as the powerhouse of the cell due to its remarkable ability to produce energy for cellular activities is undeniable However, during the formation of adenosine triphosphate (ATP) by oxidative phosphorylation in mitochondria of eukaryotic cells, slight proportion of electron leakage in normal respiration lowers oxygen level prematurely, resulting in the formation of reactive oxygen species (ROS) (Liu et al., 2002).

The elevated level of ROS has been associated with DNA damaging events, changes in metabolic control and increase in the rate of ageing. The mitochondrial activity continues to decline as the cells and organisms mature, increasing the electron leakage and the development of ROS (Davalli et al., 2016). The highly unstable ROS interacts with lipids, proteins and nucleic acid and impairs important components of the cell with additional mitochondrial damage. This is the rationale behind free radical theory of ageing, which centered on mitochondria as a growing source of free radicals with ageing (MacNee et al., 2014). Nevertheless, age-associated mitochondrial dysfunction, may lead to ageing free of ROS as a consequence of abnormality in mitochondrial biogenesis, which is controlled by nuclear factors and genes relying on mitochondrial metabolic processes.

The free radical/mitochondrial theory of ageing suggested that ageing is the result of accumulated damage caused by ROS (Harman, 2006). For effective ageing, an optimal amount of ROS is needed to deal with the physiological stress by mediating survival signals (Sena & Chandel, 2012). However, when ROS exceeds its optimal value, they accelerate age-related damages instead of lowering them (Barja, 2014; Hekimi et al., 2011). Apart from normal cell metabolism, some other events that stimulate ROS production inside the body include radiation, environmental contaminants and inflammation. These free radicals can accelerate cellular senescence through telomere shortening; stimulation of redox sensitive transcription factors such as nuclear factor-kB (NF-kB) and activator protein-1 (AP-1) that regulates the transcription of several proinflammatory cytokine encoding genes and cause DNA damage. Persistent inflammation from elevated levels of proinflammatory cytokines and permeation of inflammatory cells into tissues is a characteristic of ageing and most age-related disorders including Alzheimer's disease, chronic obstructive pulmonary disease (COPD), osteoporosis, rheumatoid arthritis, cardiovascular disease and conjunctivitis (MacNee et al., 2014).

3.2 Epigenetic Alterations

Many studies in model organisms have suggested that ageing is accompanied by epigenetic alterations and that any disturbance in regular epigenetic marks such as histone modifications and DNA methylation can influence chromatin remodeling stimulating progeroid syndromes. Changes in the chromatin state regulate gene transcription. It is significant that, with age, a general loss of histones is correlated with chromatin remodeling at local and global levels, a disparity of activating destructive and repressive histone changes and transcriptional modifications in all ageing models (Sen et al., 2016). DNA methyltransferases, histone acetylases, deacetylases, methylases, and demethylases, as well as protein complexes involved in chromatin remodeling, are the numerous enzymatic processes that regulate the epigenetic marks (López-Otín et al., 2013). Sirtuins are type III histone deacetylases (HDACs) that function on histone residues in DNA and are necessary for preserving silent chromatin state throughout histone deacetylation (Harms & Chen, 2007). In mammals, there are seven sirtuin homologues (SIRT1–SIRT7) (Beauharnois et al., 2013). SIRT1 and SIRT2 are localized in the nucleus and dispersed in the cytosol while SIRT3, SIRT4 and SIRT5 are localized in the mitochondria and SIRT6 and SIRT7 are localized in the nucleus but not dispersed in cytosol. These seven isoforms have varying characteristics against acetylated substrates which translate into an expanded array of biological functional processes, including cell cycle control, gene expression, apoptosis, metabolism, and ageing. Moreover, sirtuins are NAD-dependent deacetylase enzymes and have been identified to have anti-ageing properties (MacNee et al., 2014). Additional anti-ageing substances are HDAC2 and a group of DNA repairing molecules such as DNA-dependent kinase protein and Ku86 (Espejel et al., 2002; Harms & Chen, 2007). The loss of balance between pro- and anti-aging substances tends to enhance the molecular alterations accountable for the variations related to ageing (MacNee et al., 2014). Some common epigenetic modifications that have been

observed in elderly tissues include enhanced histone 4 lysine 16 (H4K16) acetylation, H4K20 trimethylation or H3K4 trimethylation, additionally reduced H3K9 methylation or H3K27 trimethylation (López-Otín et al., 2013). Moreover, approximately one-third of CpG (5'—Cytosine—phosphate—Guanine—3') islands present in various tissues have shown age-related changes in DNA methylation pattern, among which 40% of CpG sites were hypermethylated and 60% became hypomethylated upon ageing (Wagner, 2019).

3.3 Loss of Proteostasis

Homeostasis of proteins in a cell is essential for the proper functioning of the cell (Aging: Epigenetic Alterations & Loss of Proteostasis |, n.d.). Age-related disorders and aging have been associated with disruption in protein homeostasis or proteostasis. A variety of complex coordinated processes exists in our body to ensure that the protein synthesized are correctly folded and stable, produced in proper amount, and malfunctioned, unfolded or aggregated proteins are eliminated by the ubiquitin-proteasome activity or autophagy-lysosomal process (MacNee et al., 2014). Protein folding and stabilization are performed by cytosolic and organelle-specific chaperones. Proteins damaged due to interaction with free radicals must be rectified or deleted and restored by functional proteins. The autophagy-lysosomal process and the ubiquitin-proteasome process are the two main proteolytic processes involved in degradation and elimination of defective proteins. However, the efficiency of both the mechanisms involved in the maintenance of cellular proteome integrity declines in response to various exogenous and endogenous stresses that accumulate with ageing (Calderwood et al., 2009; MacNee et al., 2014; Rubinsztein et al., 2011). Older cells contain a broader range of irregular, misfolded proteins and proteins undergoing oxidative damage, including increased cross-linked and aggregated proteins, proteins with reduced enzymatic activity oxidized methionine, glycation and carbonylation. The resulting abnormal protein, which is

secondary to proteotoxic stressors, further generate a damage sequence leading to the formation of more degraded and cytotoxic protein molecules. This loss of proteostasis negatively affects both the structure and function of cellular components. The natural protein metabolism is therefore altered by age and can allow altered proteins to accumulate making the organism susceptible to different age-related proteinopathies such as Alzheimers and Parkinson's diseases (MacNee et al., 2014).

3.4 Inflammation

Inflammation can induce the formation of a broad spectrum of age-related chronic conditions. Innate and adaptive immune systems activated by a number of pathogenic microorganisms and contaminants triggers the secretion of several inflammatory mediators in response to the foreign agents. However, immune stimulation and loss of anti-inflammatory responses are likely to contribute to accelerated ageing (Desai & Landay, 2010). Low-grade systemic inflammation in elderly people is related with elevated amounts of pro-inflammatory cytokines, which includes interleukin (IL)-1b, IL-6, IL-8 and TNF-a, that may lead to many age-associated pathologies (MacNee et al., 2014). This has contributed to the inflamm-ageing theory as a prominent physiological component accountable for elderly diseases (Cevenini et al., 2013; De Martinis et al., 2005).

Progressive decline in the fitness of an organism due to persistent inflammation can be the result of a number of different events, for example, accumulation of proinflammatory tissue injury, inability of a defective immune system to efficiently delete infectious agents, pro-inflammatory effects of senescent cells, upregulation of NF-kB transcription factor involved in proinflammatory cytokine production, or a faulty autophagy system against regulation and timely destruction of cytokines (Salminen et al., 2012). Therefore, reducing the inflammation

of the elderly people can slow down their ageing process and increase life expectancy (Cevenini et al., 2013).

3.5 Replicative Senescence

The replicative senescence hypothesis is the other primary theory of ageing. Due to a mechanism called cellular or replicative senescence most cells cannot proliferate indefinitely. Replicative senescence seems to be an essential characteristic in somatic cells, except for the majority of tumor cells and even some stem cells (J. Campisi, 1997). It is because the telomeres are not completely duplicated during each cell division. Telomeres are chromosome-end regions with repeats of one to five kb (TTAGGG) which protects the DNA from breakdown and recombination and consequently maintain the integrity of chromosomes (MacNee et al., 2014). In the majority of somatic cells, telomeres become shorter with each cell cycle, as the DNA polymerases do not have the ability to entirely reproduce the terminal ends of linear DNA molecules, which is a characteristic of the specialized DNA polymerase recognized as telomerase. Nevertheless, not all mammalian somatic cells exhibit telomerase, leading to a gradual degradation of telomere sections on chromosome ends. The telomere length thus represents the birth length and its rate of depletion. The latter has been a consequence of the history of reproduction but also represents some factors, for instance accumulated oxidative stress and persistent inflammation that act on progenitor cells (Saretzki & Von Zglinicki, 2002). Successive cell divisions allow chromosome telomeres to shorten until cells cannot differentiate any further. The equilibrium between cell mortality and reproduction is therefore impaired and there is a growing impairment in body defense, repair and maintenance. Telomere length is regarded as a biological instead of chronological age indicator, also commonly as a biomarker of the body's ability to absorb damage (Boonekamp et al., 2013). The link between short telomeres and the likelihood of mortality

are significant (MacNee et al., 2014). Recent experiments have started to connect the replicative senescence and free radical hypothesis of ageing. Short telomeres are directly affiliated with ageing and high oxidative degradation. Shortened telomeres stimulate p53 that causes suppression of two gene products. These genes are the proliferator-activated receptor-gamma co-activator -1a and -b genes. Their expression is essential to functioning and survival of mitochondria. This causes mitochondrial dysfunction, giving rise to many age-associated diseases, with increased free radical production (MacNee et al., 2014).

Additional hallmarks of ageing include exhaustion of the stem cells resulting from a number of age-associated injury, decreased tissue regenerative potential; and deregulated sensing of nutrients (MacNee et al., 2014).

Chapter 4

Cellular Senescence

Senescence can be described as a process where cells experience permanent cell cycle arrest. After the cells divide many times, eventually it reaches a limit where it can no longer divide or replicate known as the “Hayflick limit” and those cells undergo senescence. This theory was initially explained by Dr. Leonard Hayflick which was led by his experiment with primary fibroblast cell lines that illustrated a definite proliferative capacity (Bhatia-Dey et al., 2016). Moreover, senescence is a cellular response to stress or genomic instability to block the proliferation of damaged cells. There are a wide number of triggers, external and internal, which can contribute to senescence (Grabowska et al., 2020). Such cells which have reached a finite proliferation capacity are accompanied by loss of nuclear DNA replication, increased synthesis tumor suppressor proteins, irreversible replicative arrest, apoptosis resistance, metabolic shifts with enhanced glycolysis, reduced fatty acid oxidation, elevated reactive oxygen species formation, and possession of a senescence-associated secretory phenotype (Bhatia-Dey et al., 2016; James L. Kirkland & Tchkonia, 2017). Furthermore, senescent cells exhibit NF-kB activation, a key transcription factor, which regulates the transcription of genes coding for different proinflammatory cytokines, thereby, inducing and enhancing inflammation (Shelton et al., 1999). An experiment with human lung tissue showed increased presence of phosphorylated inhibitor of NF-kB and tumor necrosis factor alpha (TNF-α) in $p16^{INK4a}$-positive type II alveolar epithelial cells, a cellular senescence marker, indicating that senescent alveolar cells promote inflammation (Amsellem et al., 2011). Senescent cells build up in tissue with advancing age, triggering inflammation caused by SASP, which suggests further contribution to the formation of age-associated maladies. In young adults, cellular senescence maintains homeostasis by inhibiting the spread of weakened cells, thereby blocking their uncontrolled proliferation and preventing cancer formation. In contrast, with

increasing age there is decrease in senescent cell removal and replenishment of senescent cells which leads to their accumulation, and hence a variety of adverse effects on tissue homeostasis, speeding up the emergence of phenotype and ailments associated to ageing (MacNee et al., 2014).

4.1 Characteristics of Senescent Cells

Senescent cells are distinguished by their unique morphological characteristics. These cells are flat and irregular in shape, relatively large in size, vacuolated, and in certain instances multinucleated with eccentric nuclear appearance (Muñoz-Espín & Serrano, 2014). Cytoskeleton defects and increased number or size of the organelles, particularly, mitochondria, endoplasmic reticulum, nucleus and lysosomes, are responsible for this altered appearance (Amaya-Montoya et al., 2020; Ogrodnik et al., 2019). These changes hypothesized to be caused by the impact of the growth factors that are secreted within the SASP increases organelle mass in the process of preparing for cell division, but resulting in organelle accumulation due to inhibition of the cell cycle. Interestingly, the increase in senescent cells' lysosomal content is the basis for one of the most significant assays for their determination (Amaya-Montoya et al., 2020). β-galactosidase present in lysosomes shows the highest enzyme activity in an acidic pH (4.0-4.5). Senescence associated beta-galactosidase (SA-β-Gal) activity, however, is detected at pH 6, a condition where beta-galactosidase enzyme activity normally should decrease by nearly 99%, but in senescent cells its activity increases. Expansion in lysosomal content and increased expression of GLB1 gene encoding for β-galactosidase in senescent cells may be responsible for the increased activity of this enzyme even at an unfavorable pH. In this case, only cells that have elevated lysosomal content and thus elevated lysosomal b-galactosidase are found to exhibit positive staining and allow the histological recognition of senescent cells. Upon taking irreversible escape from the

cell cycle in response to cellular stress, cells undergo profound phenotypic changes, one of which is SASP. One of the key components of SASP is pro-inflammatory cytokines. The most well studied cytokines in relation to cellular senescence are interleukin 1 and interleukin 6. IL-6 is related to the development of multiple inflammatory disorders such as rheumatoid arthritis and some cancers (Rose-John, 2018). IL-1a and IL-1b, also part of SASP, promotes secretion of IL-6 and IL-8 (Amaya-Montoya et al., 2020; Kumar et al., 1992). Besides that, an array of diverse chemokines is overexpressed and released from senescent cells. The chemokines are categorized based on the inclusion of cysteine (C) and other amino acids (X) in their N-terminal domain. CC, CXC, XC and CX3C are the major four forms (Zlotnik & Yoshie, 2000). In addition, a letter L or R is applied to the name to denote whether the chemokine is acting as the ligand (L) or the receptor (R). CCL-2, CCL-7, CCL-8 and CCL-13, also referred to as monocyte chemoattractant proteins-1 (MCP-1), MCP-2, MCP-3 and MCP-4 respectively, falls under the chemokine classes which acts as ligands. Other chemokines acting as ligands include CCL-3 and CCL-20, also known as macrophage inflammatory protein 1a and 3a (MIP1a and 3a) respectively; CCL-16 or monotactin-1; and CXCL-8 or IL-8 (Coppé, Patil, et al., 2010).

Lymphotactin (XCL-1) is released by older CD4 T cells and CD8 T cells together with various other chemokines and is believed to be involved in T cell chemokine-dependent age-associated disorders such as rheumatoid arthritis and atherosclerosis (J. Chen et al., 2003). Fractalkine (CX3CL-1), the only recognized CX3C chemokine, is over accumulated in senescent biliary epithelial cells and is believed to worsen ductal tissue damage in principle biliary cholangitis (Sasaki et al., 2014). Fractalkine is also an important facilitator of interaction between the glial cells and neurons within the central nervous system. In elderly hippocampal cells, this chemokine expression is reduced and it has been linked to possess both advantageous and disadvantageous functions in ageing brain and neurological disorders

(Bachstetter et al., 2011; Mecca et al., 2018). Other secretory substances of the SASP include growth factors. The most important examples are insulin-like growth factor 1 (IGF-1) and IGF-2 ligands such as epiregulin, amphiregulin (Coppé, Desprez, et al., 2010). IGF-1 has demonstrated a complex role in cultured fibroblasts in relation to cell senescence: acute exposure promotes cell proliferation, while sustained exposure promotes senescence through activation of p53 by suppression of SIRT1 deacetylase (Tran et al., 2014). Many other proteases such as matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and serine proteases are also components of the SASP together with cytokines and growth factors (Lopes-Paciencia et al., 2019). MMPs and TIMPs maintain the stability of the extracellular matrix (ECM) by stringently regulating the degradative and synthetic mechanisms respectively (Bonnans et al., 2014). Senescent cells disturb this equilibrium by releasing a broad array of MMPs, which can contribute to the progression of age-related disorders from dermal collagen injury to enzymatic atheromatous plaque destabilization (Amaya-Montoya et al., 2020; Hassona et al., 2014). MMPs also digest inflammatory and immune mediators such as chemokines, in addition to ECM components. For example, MMP -1, -2, -3, -8 and -13 break down CCL-2, -7, -8, and -13 chemokines leading to its deterioration as well as formation of a number of CCR antagonists (Hromas, 2002). It is still ambiguous whether it can be considered as a regulatory method of inflammatory process, or as an approach to decrease the removal of senescent cells by the immune system (Lopes-Paciencia et al., 2019). As discussed above, a wide variety of stimuli stimulate cell cycle arrest and contribute to cellular senescence. Cyclin-dependent kinases (CDKs) phosphorylate essential mitosis regulators controlling either the progression or suspension of the cell cycle. The CDKN2A/p16INK4A locus codes for the major regulators of the transition from G1 to S phase of the cell cycle; the activation of this gene is therefore considered as a cellular senescence indicator. The p16 protein interacts with the CDK4/6 complex, inhibiting the

kinase activity and prevents several downstream signaling proteins, such as the retinoblastoma (RB) protein, to become phosphorylated. The unphosphorylated retinoblastoma in turn prevents the binding of E2F1 transcription factor to its specific DNA binding site and therefore, prevents transition from G1/S phase by inhibiting target gene transcription (Rayess et al., 2012). Senescent cells are metabolically active and an increase in the AMP: ATP and ADP:ATP ratios have been identified throughout senescence (James et al., 2015).

Numerous events, for instance, exposure to ROS, encounters with infectious agents, mutations and chaperone deficiencies can contribute to ER stress, resulting in protein assembly and agglutination. To cope with such stress, the ER commences an unfolded protein (UPR) that decreases protein synthesis, causes the ER to enlarge, and degrade malfunctioned proteins (Pluquet et al., 2015). The unfolded protein behaviour is controlled by three signal activator proteins-inositol requiring enzyme 1α/β (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6α/β (ATF6) (Hernandez-Segura et al., 2018). Possibly, senescent cells exhibit an elevated UPR because of the increase in demand of SASP for protein synthesis (Cormenier et al., 2018; Druelle et al., 2016). The components of senescence associated secretory phenotype have been listed in Table 1.

Table 1: Components of senescence-associated secretory phenotype

Class	Components
Cytokines	Interleukins- IL-1a, IL-1b, IL-6, IL-7, IL-13,1L-15
Non protein molecules	Prostaglandin E-2 (PGE-2) Reactive oxygen species (ROS) Nitric oxide
Chemokines	Monocyte chemoattractant proteins (MCP) -1, -2, -3, -4 (CCL -2, -7, -8 and -13) Macrophage inflammatory protein (MIP) 1a and 3a (CCL -3, -20) Monoactin- 1 (CCL-16) IL-8 (CXCL-8) Lymphotactin (XCL-1) Fractalkine (CX3CL-1)
Growth Factors; Regulators	Insulin like growth factor- IGF-1, IGF-2 Epidermal growth factor- epiregulin, amphiregulin, heregulin Nerve growth factor (NGF) Hepatocyte growth factor (HGF) Keratinocyte growth factor (KGF) Placenta growth factor (PIGF) Vascular endothelial growth factor (VEGF)

Proteases and regulators	Matrix metalloproteinases (MMPs -1, -3, -10, -12, -13, -14) Tissue inhibitors of metalloproteinases (TIMPs -1, -2) Serine proteases inhibitors (PAI -1, -2) Cathepsin B
Insoluble factors	Fibronectin Laminin Collagens
Other inflammatory molecules	Transforming growth factor (TGF-β1, TGF-β2) Granulocyte macrophage colony stimulating factor (GM-CSF) Interferon gamma (IFNγ) Macrophage migration inhibitory factor (MIF)

Chapter 5

Physiological Importance of Cellular Senescence Program

Cellular senescence exhibits pleiotropic effect that relies on the stimuli, the type of cell and the hormonal environment (James L. Kirkland & Tchkonia, 2017). It is an important response to block uncontrolled cell division and proliferation (Importance of Cell Cycle and Cellular Senescence in the Placenta Discovered, n.d.). The formation of senescent cells occurs during lifespan and performs an advantageous role in a number biological activities, including embryogenesis, wound healing, host immunity, suppression of tumors and prevent tissue fibrosis (Cellular Senescence: What, Why, and How | Wounds Research, n.d.; He & Sharpless, 2017). Cellular senescence can be of acute and chronic type. Acute senescence produces transient SASP effect and is observed during embryonic development and wound healing. In contrast, chronic cellular senescence arises from long term exposure to damaging conditions followed by reduction in immune clearance of senescent cells with age and the resulting persistent secretory profile shows detrimental effects on multiple organs, thereby, leading to age-related disorders (Amaya-Montoya et al., 2020). The concept of cellular senescence has been explained in three aspects: developmentally programmed senescence (DPS), stress induced premature senescence (SIPS), replicative senescence (RS) (von Kobbe, 2018).

5.1 Developmentally Programmed Senescence (DPS)

Developmentally programmed senescence is a kind of cell senescence demonstrating tissue remodeling and proposing the root of damage-induced senescence (Muñoz-Espín et al., 2013; Storer et al., 2013). During mammalian embryogenesis, the onset of acute senescence has been identified at several sites, including the mesonephros and the inner ear endolymphatic

sac (Muñoz-Espín et al., 2013). Unlike adult cells, in case of mammalian embryonic patterning of these structures, senescence phenotype is strongly mediated by the expression of p21 protein independent of genotoxic stress, p53 or other cell cycle inhibitors. Furthermore, DPS is accompanied by macrophage infiltration, elimination of senescent cells and tissue remodeling (Muñoz-Espín et al., 2013). Natural killer (NK) cells are the most widespread immune cells during early pregnancy in the maternal/fetal interphase and senescent natural killer cells aids in embryo implantation (Rajagopalan, 2014; Vicente et al., 2016). In senescent NK cells, the fetal trophoblast stimulates the CD158d receptor, initiates the p21 signaling cascade and induces a secretory proteome to facilitate vascular remodeling and angiogenesis (Rajagopalan & Long, 2012). The effect of senescence is partly compensated by apoptosis in case of senescence loss due to lack of p21. However, this phenomenon contributes to noticeable developmental abnormalities (Muñoz-Espín et al., 2013).

5.2 Stress-Induced Premature Senescence (SIPS)

Senescence can occur in cells in response to a class of stressors including, but not confined to, DNA damage and independent of telomere shortening (von Kobbe, 2018). After birth, cells are subjected to several stressors that may trigger a more advanced pathway defined as stress induced premature senescence (SIPS) (von Kobbe, 2018). This route is started following an external stimulus that induces cell cycle arrest and plays a key role in biological functional mechanisms such as wound repair (Amaya-Montoya et al., 2020; Demaria et al., 2014). Stress induced premature senescence (SIPS) occurs after certain sublethal pressures along with H_2O_2, hyperoxia, or tert-butyl hydroperoxide. Replicative senescent cells share similar characteristics with SIPS cells in terms of morphology, senescence-associated beta-galactosidase activation, regulation of cell cycle, gene expression and telomeric shortening

(Toussaint et al., 2000). Senescent cells release platelet-derived growth factor AA (PDGF-AA) in reaction to tissue injury and the CCN1 extracellular matrix-associated signaling protein to speed up the healing process (Demaria et al., 2014; Jun & Lau, 2010). SIPS may be an *in vivo* process for the amassing senescent-like cells. Melanocytes subjected to sublethal ultraviolet B (UVB) doses experience SIPS. Populations with dark and light-skinned melanocytes show variations in the regulation of their cell cycle. Delayed SIPS exists in population with light-skinned melanocytes as there is a decreased interaction of $p16^{INK4a}$ with CDK4 (Cyclin-dependent kinase 4) and decreased phosphorylation of the retinoblastoma protein (Toussaint et al., 2000). Senescent cells can also generate proteins that encourage stemness or decrease reparative ability (Herranz & Gil, 2018). SIPS may be caused by cell exposure to sublethal stresses of different natures, with possible modulations of this mechanism by bioenergetics (Amaya-Montoya et al., 2020).

5.3 Replicative Senescence (RS)

All primary cells leave the cell cycle and enter replicative senescence after the Hayflick limit is reached. This phenomenon is followed by modification in cell morphology, lack of capability for cell differentiation, and arrest of cell proliferation (Schellenberg et al., 2014). Progressive telomere shortening and reduced senescent cell removal interfere with the organ and tissue function throughout ageing (Amaya-Montoya et al., 2020).

The influence of accumulation of senescent cells over time have been demonstrated in several age-related diseases due to its unregulated potential to secrete various bioactive molecules including proinflammatory cytokines, growth factors, chemokines and proteases. Premature replicative senescence was observed in cultured pulmonary vascular endothelial cells (P-ECs) from patients with COPD (Chronic obstructive pulmonary disease), with reduced cell doubling, a higher proportion of β-galactosidase-positive cells, decreased telomerase activity,

shortened telomeres, higher p16 and p21 mRNA levels at an early cell passage relative to control subjects (Amsellem et al., 2011).

Chapter 6

Triggers of Cellular Senescence

Cellular senescence can be driven by telomere attrition or dysfunction, oxidative stress, DNA damage, epigenetic alterations, inflammation, activation of oncogene and intake of certain medications (Dodig et al., 2019). The triggers of cellular senescence along with the pathway they follow to induce senescence have been illustrated in Figure 2.

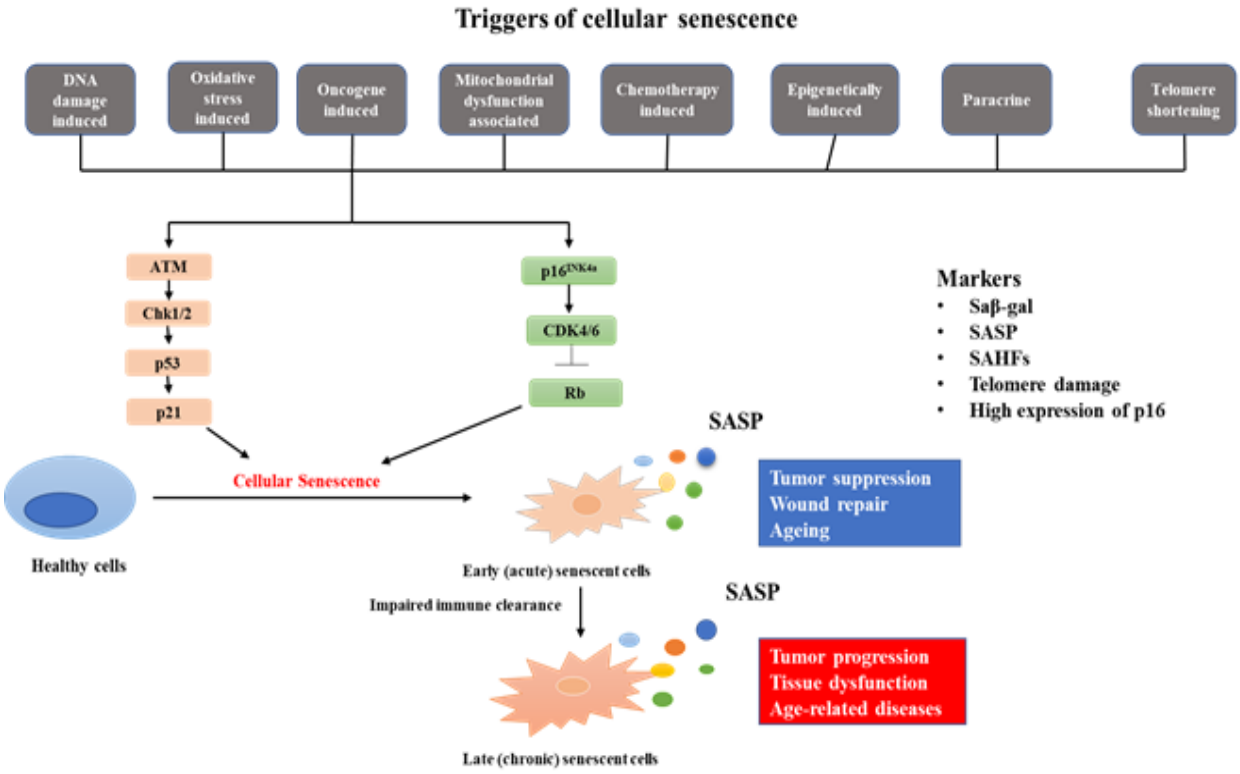

Figure 2: Induction of cellular senescence via p53 and $p16^{INK4a}$ signaling pathway (Adapted from Fujita, 2019; Mijit et al., 2020).

6.1 DNA Damage-Induced Senescence

Depending on the intensity of the damage, the cells with irreparable DNA will experience either senescence or apoptosis (Hernandez-Segura et al., 2018). GATA4 is a transcription

factor, the activation of which depends on the ATM and ATR which are the DNA damage response regulators, but not on p53 or $p16^{INK4a}$. GATA4 in turn activates NF-κB to produce SASP and mediate senescence. In many tissues, including the ageing brain, GATA4 accumulates and could lead to ageing and its related inflammation (C. Kang et al., 2015). Multiple DNA-damaging agents, including ionizing and UV radiation or multiple medications, are used *in vitro* to cause this type of senescence (Muñoz-Espín et al., 2013).

6.2 Oxidative Stress-Induced Senescence

Oxidative damage occurs during elevated ROS level causing cells to reach senescence (Barbouti et al., 2020). The rise in ROS level may occur due to accumulation of oxidizing products from metabolic activities or from exogenous sources (Hernandez-Segura et al., 2017). These oxidizing agents can contribute to irreversible DNA damage as well as affect other cellular components and processes (Hernandez-Segura et al., 2018).

6.3 Oncogene-Induced Senescence

Oncogene-induced senescence is a vigorous and persistent antiproliferative reaction arising from an oncogene-activating mutation or inhibition of a tumor-suppressor gene due to oncogenic signaling (Chandeck & Mooi, 2010). Ras or BRAF oncogene activation, or PTEN and NF1 tumor suppressor inactivation can contribute to oncogene-induced senescence (Calcinotto et al., 2019). Emerging research indicates that phenotypes of senescence vary according to the oncogenic stimulus (H. Zhu et al., 2020).

6.4 Mitochondrial Dysfunction-Associated Senescence (MiDAS)

Mitochondrial dysfunction usually results from disruption in mitochondrial homeostasis, alteration in mitochondrial metabolite formation, changes in mitochondrial membrane

potential and elevated level of ROS generation, gradually directing the cells towards senescence and a separate secretory phenotype (Wiley et al., 2016). Consequently, the deregulated mitochondrial activity has been strongly correlated with ageing phenotypes, especially when the mitochondrial genome integrity is in compromised condition (Barbouti et al., 2020).

6.5 Chemotherapy-Induced Senescence

Senescence can be caused by several anticancer medications, for example- bleomycin or doxorubicin, that induce damage to DNA, while some other drugs like- abemaciclib and palbociclib act as CDK inhibitors (Petrova et al., 2016). The benefit of such genotoxic therapy lies in the fact that it halts proliferation of transformed cells permanently, although in some studies it has been reported that persistent cells may escape senescence and the disease can relapse (Guillon et al., 2019).

6.6 Epigenetically Induced Senescence

DNA methylation, histone alterations, and noncoding RNA species are the three foundations of epigenetic regulation (Gonzalo, 2010). Age-dependent reduction in global DNA methylation has been found to decrease the replicative longevity of cells. This epigenetic alteration, therefore, has linked ageing with replicative senescence (Sidler et al., 2017). Another nuclear phenotype that accompanies cellular senescence includes the formation of facultative heterochromatin, known as senescence-associated heterochromatin foci (SAHF). The highly condensed form of the chromatin formed have been found to be enriched with a variety of epigenetic signatures such as hypoacetylated histones H3K9me3 and H3K27me3, heterochromatin protein 1 (HP1), and histone variant macroH2A. These epigenetic modifications suppress the transcription of genes involved in cell proliferation indicating that

SAHF is related to tumor suppressive proliferative arrest of senescent cells (Nacarelli et al., 2017). Moreover, some studies revealed that DNA methylase inhibitors, for example- 5-aza-20-deoxycytidine or histone deacetylase inhibitors like- suberoylanilide hydroxamic acid and sodium butyrate induce senescence (Petrova et al., 2016).

6.7 Paracrine Senescence

The senescence messaging secretome or SASP produced by the primary senescent cells can influence the bystander cells to also become senescent through paracrine pathways and control senescence response (Hernandez-Segura et al., 2018; Vassilieva et al., 2020).

6.8 Telomere Damage/ Shortening

A precise indicator of the replicative potential of cells is the length of telomeres. The role of telomeres is to shield the ends of chromosomes from depletion and faulty recombination and thus maintain genomic stability (Siderakis & Tarsounas, 2007). Telomere shortening takes place with each cell division as well as in response to various exogenous stress, but can be reversed by the enzyme telomerase which has the ability to preserve the length of telomeres. The length of the telomere at birth is approximately 11 to 15kb, whereas in old age the telomere length is about 4kb which is extremely shorter in comparison to when it began (Dodig et al., 2019). Senescence is thus mostly initiated when the telomere length shortens from 5-20 kb to 4-7 kb (Chandrasekaran et al., 2017). Telomere erosion during normal ageing is regulated by telomerase activity. However, in the absence of telomerase activity, telomere gradually shortens causing the cell to stop proliferating by either apoptosis or senescence depending on the stress level and cell type (Childs et al., 2014).

Chapter 7

Biomarkers of Cellular Senescence

The senescence phenotype is characterized by chronic DNA damage response (DDR) activation, multiple cyclin-dependent kinase inhibitor (CDKi) involvement, increased release of tissue remodeling factors and proinflammatory substances, anti-apoptotic gene expression upregulation, altered metabolic rates and stress of the endoplasmic reticulum (ER) (Hernandez-Segura et al., 2018). Senescent cells exhibit structural aberrations as a result of these signaling pathways, which includes expanded to more flattened morphology, increased senescence-associated beta galactosidase activity, altered plasma membrane (PM) composition, lysosome and mitochondria accumulation, and nuclear modifications (Muñoz-Espín & Serrano, 2014). Several techniques have been established to detect senescent cells which include immunostaining, microscopic imaging, *in situ* hybridization and flow cytometry. However, marker specificity of senescent cells depends on cell type, species, organismal development phase and other factors such as heterogeneity in SASP phenotype (Gorgoulis et al., 2019). The biomarkers of senescent cells have been listed in Table 2.

Thus, there is no universal biomarker that could provide unequivocal determination and quantification of senescent cells (A. S. Wang & Dreesen, 2018). Consequently, a combination of distinctive strategies is frequently implemented for the identification of senescent cells, given the heterogeneous nature of senescent cells and the lack of marker specificity (Hernandez-Segura et al., 2018).

Table 2: Biomarkers of senescent cells

Senescence associated phenotype	Biomarkers
Secretory phenotype	IL-1a, IL-6, IL-8 and CCL2
Cell cycle arrest	$p15^{INK4b}$, $p16^{INK4a}$ $p21^{CIP1}$
Telomere shortening and DNA damage response	p53, gamma-H2AX (γH2AX)
Apoptosis resistance	Bcl-2 protein
Composition of plasma membrane	DEP1 and DPP4
Increased lysosomal content	Beta-galactosidase (SA-β gal)
Accumulation of mitochondria	Endonuclease G (EndoG)
Nuclear changes	LaminB1, SAHF

7.1 Secretory Phenotype

SASP factors including cytokines, chemokines and proteinases are secreted by senescent cells (Hernandez-Segura et al., 2018). The cytokines IL-1a, IL-6, and IL-8, chemokines CCL2, and metalloproteinases MMP-1 and MMP-3 are often used as indicators of senescent cells (Coppé

et al., 2008; Sharpless & Sherr, 2015). It is a common practice to estimate the expression and secretion of a few of these factors, specifically IL-6, using either immunostaining or enzyme-linked immunosorbent assay (ELISA) (Y. Zhu et al., 2015).

7.2 Cell Cycle Arrest

CDKs phosphorylate and activate target proteins that are involved in the progression of cell cycle. During senescence, the key drivers of cell cycle arrest including CDK inhibitors $p16^{INK4a}$ encoded by CDKN2A, $p15^{INK4b}$ encoded by CDKN2B and $p21^{CIP1}$ encoded by CDKN1A are upregulated and hence can be used as markers of senescent cells (Hernandez-Segura et al., 2018).

7.3 Telomere Shortening and DNA Damage Response

Telomere shortening often recognized as DNA breaks by the cells activate DNA damage response signaling pathway through the phosphorylation and stabilization of p53 followed by the expression of cyclin-dependent kinase inhibitor $p21^{CIP1}$ which can be used as senescence markers (de Jesus & Blasco, 2012).Telomere structure can be assessed by flow-cytometric fluorescence *in situ* hybridization and telomere length can be studied using Southern blotting and PCR based approaches (Sharpless & Sherr, 2015).

In the presence of DNA lesions, a robust DNA damage response (DDR) is triggered by the cells. When the damage remains unresolved, the cells may reach a state of senescence (Hernandez-Segura et al., 2018). Persistent DNA damage can be indicated by the presence of phosphorylated H2AX (γH2AX) focal points, normally involved in recruiting and localizing DNA repair proteins (Sharpless & Sherr, 2015). Moreover, measurement of the level of phosphorylated p53 which is a key signaling protein for the activation of DDR can also be used as a biomarker (Muñoz-Espín & Serrano, 2014).

7.4 Apoptosis Resistance

Several prosurvival factors are activated by senescent cells rendering themselves to become apoptosis resistant (Childs et al., 2014). Because of the chronic activation of the transcription factor cAMP response element-binding protein (CREB) responsible for the antiapoptotic Bcl-2 protein expression, senescent cells escape programmed cell death (Ryu et al., 2007). Upregulation of Bcl-2, Bcl-w, or Bcl-xL proteins can be used as senescence marker, although it is not widely employed for senescent cell detection (Childs et al., 2014; Hernandez-Segura et al., 2018; Yosef et al., 2016).

7.5 Increased Lysosomal Content

Senescent cells are characterized by increased lysosomal content and upregulation of several lysosomal proteins (Cho & Hwang, 2012). One of the most well-characterized and frequently used methods in detecting senescence both *in vitro* and *in vivo* is beta-galactosidase (β-Gal) assay which quantifies the increased activity of β-galactosidase enzyme detectable at pH 6 in lysosome of senescent cells by immunohistochemistry (de Jesus & Blasco, 2012; Lee et al., 2006). Senescence-associated β-Gal (SA-β-Gal) assay involves the staining of cells or tissues with a chromogenic substrate of β-Gal, known as X-Gal which cleaves the enzyme resulting in the formation of an insoluble product blue in color. An alternative to using X-Gal is tagging the tissues with fluorescent molecules such as fluorodeoxyglucose (FDG) (de Jesus & Blasco, 2012).

7.6 Accumulation of Mitochondria

An increased number of mitochondria is displayed by senescent cells (Tai et al., 2017). The membrane potential of these mitochondria, however, is diminished, leading to the release of mitochondrial enzymes such as endonuclease G (EndoG) and increased generation of ROS

(Hernandez-Segura et al., 2018). In some experiments, mitotrackers have been used to measure the membrane potential of mitochondria (Passos et al., 2007).

7.7 Nuclear Changes

The reduction of LaminB1, a nuclear lamina structural protein, is a typical marker of senescent cells (Hernandez-Segura et al., 2017). Nuclear modifications, for example- the loss of condensation of constitutive heterochromatin and the emergence of cytoplasmic chromatin fragments (CCFs) enriched with epigenetic marks related to DNA damage, result from the destabilization of nuclear integrity caused by decreased LaminB1 (Adams et al., 2013). Senescence-associated heterochromatin foci (SAHF) have been observed inside the senescent cell nucleus under microscope after DAPI (4',6-diamidino-2-phenylindole) staining as dark condensed regions. These foci are also enriched with chromatin repression marks, such as trimethylation of lysine 9 in histone 3 (H3K9me3), repressive histone H2A variant (macroH2A) and isoforms of silencing heterochromatin protein 1 (HP1) (Sharpless & Sherr, 2015). Different pathways involved in the activation of p53 and p16 are also known to trigger SAHF. This trait, however, is not shared by all senescence types and is a tissue and species dependent senescent marker (Bernadotte et al., 2016).

7.8 Composition of Plasma Membrane

Plasma membrane proteins, such as DEP1 and DPP4 (dipeptidyl peptidase 4), have been proposed as markers of senescent cells (K. M. Kim et al., 2017). Both in vivo and in vitro studies have shown increased expression of dipeptidyl-peptidase 4 (DPP4) glycoprotein in senescent vascular endothelium, and suppression or downregulation of DPP4 were identified to reduce endothelial senescence. Additionally, suppression or inhibition of DPP4 decreased endothelial oxidative stress levels in ageing vasculature and senescent endothelial cells.

Besides, the expression and phosphorylation of AMP-activated protein kinase-alpha (AMPKα) and sirtuin 1 (SIRT1) was normalized by DPP4 inhibition or silencing (Z. Chen et al., 2020).

Mass spectrometry study found that DPP4 was presented selectively on the senescent cell surface. Flow cytometry-mediated isolation of senescent cells was enabled by the differential presence of DPP4. The antibody-dependent cell-mediated cytotoxicity (ADCC) preferentially eliminated these senescent cells, as the presence of DPP4 on their surface made them ideal targets for annihilation by natural killer (NK) cells detecting an anti-DPP4 antibody. However, the selective expression of DPP4 on the surface of senescent cells makes it possible to remove them selectively (K. M. Kim et al., 2017).

Chapter 8

Immunosurveillance of Senescent Cells

Our innate immune system detects danger signs such as cell membrane signals on encounter with cell injury, imminent cell death, viruses, bacterial cell wall extracellular elements, or antigens of fungal or helminth origin. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are identified by pattern recognition receptors (PRRs) located in different cellular compartments (Takeuchi & Akira, 2010). Immune pathways are one of the primary indicators that changes with age, according to an experimental study performed at an individual level on human ageing by repetitive sampling and sustained molecular screening (P. Song et al., 2020). Immune cells become senescent as humans get older, which is referred to as immunosenescence and their response efficiency against pathogens and cellular damage decrease rapidly with age, which is most commonly observed in elderly younger than 100 years. Moreover, the expression of inhibitory natural killer cell receptor NKG2A on the surface of CD8+ T cells has been found to increase sharply within the healthy volunteers' blood with age. The human leukocyte antigen (HLA)-E which is the ligand for NKG2A is released by senescent fibroblasts and the endothelium which suppress the cytotoxic activity of these highly differentiated CD8+ T cells (Pereira et al., 2019). Apparently, a new study utilizing single-cell RNA interpretation for circulating immune cells found that supercentenarians over 110 years of age with stable ageing had an elevated proportion of CD4+ cytotoxic T lymphocytes (25.3% of total T cells) compared to just 2.8% of all T cells in young controls (~50-80 years of age), while supercentenarians as well as control groups both have the same number of T cells. Clonal expansion throughout supercentenarians produces CD4+ cytotoxic T cells with almost the same transcriptome of cytotoxic CD8+ T cells. Supercentenarians, on the other hand, have a significantly lower

number of B-cells than the control population (Hashimoto et al., 2019). Such immune signatures in supercentenarians well illustrate the role of immunosurveillance in providing protection against the triggers of certain pathological conditions including infections and tumor growth (P. Song et al., 2020; Weiskopf et al., 2015).

Inflammation, oxidative stress, DNA degradation, or tissue destruction signals cause normal cells to form senescent cells in multiple organs (Prata et al., 2018). Apoptosis and the immune system can eliminate senescent cells (Ovadya & Krizhanovsky, 2018; Prata et al., 2018). The immune system, including adaptive and innate immune cells, plays a vital role in the elimination of senescent cells at an early stage or under physiological conditions, since most senescent cells are resistant to apoptosis. While senescent cells may undergo apoptosis by senolytic drugs, the immune system must eventually clean up these apoptotic cells. Senescent cell and immune cell interaction influence the role of the immune system. Several *in vivo* experiments have shown that immune cells such as macrophages, natural killer cells, dendritic cells, CAR-T cells, CD4+ T cells, CD8+ T cells and B cells all play a critical role in senescent cell clearance. Senescent cells arising from specific cell type, for example fibroblasts, adipocytes, hepatocytes, astrocytes, cardiomyocytes, melanocytes, chondrocytes, etc. may exhibit different secretory phenotype and the type of immune cell that will be engaged in immunosurveillance of these senescent cells depend on the ligand secreted by that particular senescent cell type. This indicates that different immune cells have distinct ability to precisely recognize and carry out targeted eradication of unique senescent cells (P. Song et al., 2020). The immunosurveillance of senescent cells has been illustrated in Figure 3.

However, with advanced ageing senescent cells attract and defunctionalize immune cells through SASP, resulting in a constant and unnecessary accumulation of senescent cells (Prata et al., 2018). The exact underlying process that causes senescent cells to aggregate within

tissues is ambiguous. It's unclear if this is attributed to an increase in senescent cells that outnumber the defense system's ability to destroy them or immune cell malfunction (P. Song et al., 2020).

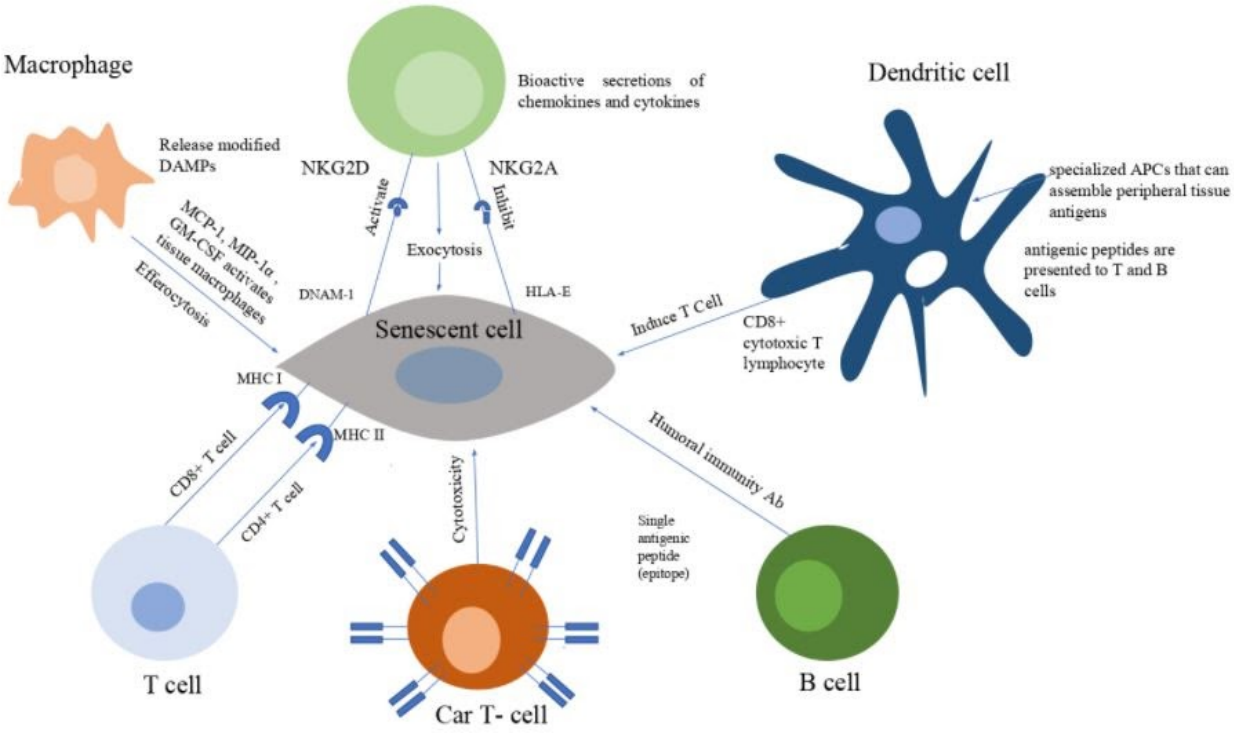

Figure 3: Immunosurveillance of senescent cells (Adapted from P. Song et al., 2020)

8.1 Macrophages

Innate immune-effector macrophages play a vital role in efferocytosis which refers to the elimination of apoptotic cells in specific tissues. This process is important because if the apoptotic cells are not removed in the right time, these cells may undergo secondary necrosis which is characterized by apoptotic cell membrane rupture and release of modified DAMPs inducing a chronic inflammatory response (Roberts et al., 2017; Sachet et al., 2017). Multiple SASP-related factors, including macrophage chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1α), and granulocyte-macrophage colony-stimulating

factor (GM-CSF) attract and stimulate tissue macrophages and circulating monocytes, which serve as scavenger cells and antigen-presenting cells (APCs) for senescent cell clearance. Monocytes/macrophages (MOs) have been observed to migrate in response to conditioned medium (CM) obtained from senescent human adipocyte progenitor cells but do not react to CM acquired from non-senescent fat cell progenitors in an *in vitro* analysis. MOs in immediate contact with SCs have been identified, indicating involvement through membrane surface receptors. The study has also shown that senescent cell clearance was prevented on removal of MOs, which indicates that MOs are necessary for reducing senescent cell burden (Prata et al., 2018).

8.2 Natural Killer (NK) Cells

The natural killer (NK) cells remove compromised cells by inducing apoptosis either via membrane receptors or through enzymatic degradation (Prata et al., 2018). Multiple bioactive secretions including chemokines and cytokines from senescent cells regulate the ability of NK cells to destroy senescent cells (P. Song et al., 2020). Usually, in response to any cell injury or encounter with pathogens, these immune cells are recruited to the site of action (Sivori et al., 2014). For example, hepatic stellate cells (HSCs) are activated in a model of liver fibrosis. These cells begin to express alpha-smooth muscle actin (alphaSMA) and secrete molecules of extracellular matrix that lead to liver fibrosis (Prata et al., 2018). The HSCs might eventually become senescent-like due to increased expression of the cell cycle inhibitors $p16^{INK4a}$ and $p21^{CIP1}$ and impaired replicative capacity with characteristic rise in SA-β gal activity. As a result, NKs become activated to eliminate the senescent cells and have been found to cause recovery of fibrosis associated damage within 20 days (Krizhanovsky et al., 2008). These senescent-like HSCs are identified by the NK receptors-natural killer, group 2, member D (NKG2D) and DNAX accessory molecule-1 (DNAM1),

and cause cell death by the release of cytotoxic perforin instead of Fas ligand induced apoptosis (Sagiv et al., 2013).

DNAM-1 and NKG2D are activating receptors expressed on the natural killer cells, and ligand with poliovirus receptor (PVR) and major histocompatibility complex (MHC) class 1 chain-related protein A (MICA) or MHC class 1 chain-related protein B (MICB) respectively. This interaction of the ligand with its complementary receptors on the NK cells mediate the removal of senescent cells. Chemotherapeutic drugs, for example, melphalan, doxorubicin and bortezomib, have been found to upregulate the expression of ligands for DNAM and NKG2D on myeloma cell lines showing senescent-like phenotype, thereby, increasing NK cell susceptibility and effector function to eliminate those cells. This function of the NK receptors can be further supported by a study where deletion of NKG2D receptors in mice with liver fibrosis led to the accumulation of senescent hepatic stellate cells. On the contrary, interaction of MHC Ib molecule HLA-E ligand with the inhibitory receptor NKG2A on the NK cells blocks the cytotoxic activity of the immune cell resulting in the accumulation of senescent cells. Senescent human dermal fibroblasts demonstrated higher expression of HLA-E through p38 signal transduction pathway during ageing compared to non-senescent cells. This indicates that several age-associated diseases can be delayed or reversed by preventing the interaction between HLA-E and NKG2A or by inhibiting NKG2A protein expression (P. Song et al., 2020).

8.3 CAR-T Cells

T cells play a critical role in the maintenance of a healthy lifespan through the coordination of immune responses on recognition of cellular damage associated patterns and against various extracellular and intracellular pathogens (P. Song et al., 2020). T lymphocytes exhibit highly specific T-cell receptors for a single antigenic peptide (epitope). T lymphocytes must

recognize naturally processed antigen, which is normally presented to them by the MHC molecule on the antigen presenting cells (APCs), to be activated and retain immunological memory from primary antigen encounters (Prata et al., 2018).

CD4+T cells and CD8+ T cells are two types of T lymphocytes with distinct functions. CD4+ helper T cells coordinate the activities of other immune cells, and polarization divides T-helper cells into cells that secrete a variety of cytokines (Fulop et al., 2018). CD4+ cytotoxic T cells have the potential to kill senescent tumor cells by recognizing MHC II components that are usually not present in healthy cells but are expressed in a subset of tumor or senescent cells (T. W. Kang et al., 2011). CD8+ cytotoxic T lymphocytes (T-CD8+ CTLs) facilitate NK cell-like killing and recognize MHC I molecules on the APCs followed by eradication of target cells using cytotoxic molecules (Fulop et al., 2018; Hashimoto et al., 2019).

On the contrary, CD4+ Foxp3+ Treg cells suppress immune responses after successful elimination of invading microorganisms or damaged cells (Fulop et al., 2018). T-CD8+ CTLs can therefore also lead to immune surveillance of SCs. T helper type 1 (Th1) cells are mostly a subset of T-CD4+ cells that produce cytotoxic inflammatory cytokines which may improve the immune regulation of senescent cells (Prata et al., 2018). Th2 cells, in contrast, are a distinct subset of T-CD4+ cells that secrete IL-4 and TGF-, that inhibits SC clearance by downregulating NKG2D in NKs as well as T-CD8+ CTLs (Hu et al., 2016). The selectivity and efficacy of cytotoxic T cells, however, declines as people become older (P. Song et al., 2020).

Chimeric antigen receptor (CAR)-T cells can be utilized as a useful technique for eliminating target senescent cells. As the senescent cells express distinct cell-surface antigens, specific CAR-T cells can be developed to selectively deplete the senescent cells (P. Song et al., 2020).

Senescent cells were observed to express ligands or antigens that activate the NKG2D and DNAM-1 receptors, as well as HLA-E ligands that facilitate the function of NKG2A inhibitory receptors. Based on this concept, senescent cells may be targeted by engineering T cells which express NKG2D-CAR. The NKG2D-CAR would recognize NKG2D ligands on the surface of the senescent cells allowing the T cells to perform its cytotoxic activity (Baumeister et al., 2019).

8.4 Dendritic Cells

Dendritic cells (DCs) are specialized APCs that gather peripheral tissue antigens and then carries them to the secondary lymph organs and tissues, such as lymph nodes, where antigenic peptides are presented to T and B cells by these DCs (Prata et al., 2018). DCs are effective inducers of NKs and CD8+ cytotoxic T lymphocyte activity and can increase the SC clearance facilitated by these types of immune cells (Rahman & Aloman, 2013). Senescent cells may generate unique cell surface antigens which are processed by DCs to express these antigens on its cell surface, followed by antigen recognition by T cells (P. Song et al., 2020). The ability of DCs to monitor the organism and migrate to lymphoid organs activates the T and B cell responses against senescent cell surface antigens. Moreover, cellular vaccine therapies could be developed to artificially activate DCs (Saxena & Bhardwaj, 2018).

Chapter 9

Role of Cellular Senescence on Disease Development

The senescent cell burden has been found contributing to several age-related diseases or conditions, including rheumatoid arthritis, chronic liver diseases, neurodegenerative diseases, cardiovascular diseases, human skin ageing and pulmonary fibrosis. In tissue ageing and different chronic disorders, senescent cells originating from different cell types have shown distinctive characteristics having distinct functions. The initiation and development of both organ ageing and chronic illnesses can be retarded by controlling and balancing cellular senescence (P. Song et al., 2020).

Cellular senescence is referred to as primary senescence and secondary senescence to distinguish between the natural progression of ageing (normal) or disease condition (abnormal) (Childs et al., 2017). Primary senescent cells are based on 'natural' modifications, suggesting the fate of cells as a result of ageing, tissue repair, etc. In contrast, the secondary senescence appears to be the 'abnormal' transition as it emerges from the onset of the diseases and persisted throughout the entire path. Both primary senescence and secondary senescence could induce or aggravate age-related diseases (Chi et al., 2019).

9.1 Rheumatoid Arthritis and Osteoarthritis

Accumulation of senescent cells in various human diseases has been associated with pro-inflammatory effects with deleterious consequences. Premature lymphocyte senescence, along with the aggregation of senescent CD4+ T cells, is a signature feature of rheumatoid arthritis (RA). People with systemic bone loss have more circulating senescent CD4+ and CD28+ T cells than those with typical bone mineral density. Throughout senescent CD4+ T cells, the receptor activator of nuclear factor kappa-B ligand (RANKL), is detected at higher

levels than in CD28+ T cells, and its expression can be induced by IL-15, a key cytokine in the pathogenesis of rheumatoid arthritis. RANKL signaling mediates bone resorption process through the activation of osteoclasts (Fessler et al., 2018).

Immunohistochemistry has been used to evaluate the expression of the senescence marker $p16^{INK4a}$ in rheumatoid arthritis, osteoarthritis (OA), and regular synovial tissues (ST) from variable-aged donors. The fraction of senescent p16(+) cells in normal synovial tissues from elderly people are usually found to be higher than in younger ones. While senescent cell proportions between older synovial tissues with rheumatoid arthritis and osteoarthritis and older synovial tissues without the disorders were comparable, senescence was found to be higher in younger RA ST relative to similar aged control group ST. Moreover, the percentage of SA-β-gal (+) senescent cells in synovial fibroblasts (SF) collected from different aged healthy donors was found to increase proportionally with age. The senescent cell burden was found to be significantly higher in > 40-year-old groups. Stress-induced senescence was also demonstrated by the increased expression of several additional markers including IL6, CXCL8, CCL2 and MMP3 and other protein secretions upon exposure of young and old synovial fibroblasts culture to H_2O_2 or TNFα, which suggests an increase in the risk of developing diseases from the rise in secretion of pro-inflammatory mediators (Del Rey et al., 2019).

9.2 Alzheimer's Disease

Alzheimer's disease (AD) is an age-related neurodegenerative brain condition for which no disease-modifying medications are available till now. This disorder is characterized by local inflammation, lysosomal dysfunction, accumulation of activated microglial cells and astrocytes, and formation of neuritic plaque high in beta amyloid peptide (Aβ) in vulnerable regions of the brain (Scheltens et al., 2016).

In several old age diseases, tissues undergo accumulation of senescent cells with various phenotypes characterized by cell cycle arrest, apoptosis resistance, and proinflammatory molecular secretion (Muñoz-Espín & Serrano, 2014). Neuritic plaques, a pathological characteristic in the brain of Alzheimer's disease (AD), contain extracellular Aβ peptide aggregates and autolysosome-accumulating degenerating neurites. During normal ageing and in affected brain regions of individuals with Alzheimer's disease (AD), senescence markers have been detected to be expressed by astrocytes in human brain tissues (C. Kang et al., 2015). The senescence-like phenotype, characterized by upregulation of p21, $p16^{INK4a}$, and senescence-associated β-galactosidase activity, is exhibited by Aβ plaque-associated oligodendrocyte transcription factor (Olig2) and neuron-glial antigen 2 (NG2) expressing oligodendrocyte progenitor cells (OPCs). Elevated levels of mRNA transcript encoding proteins involved in OPC activity, replicative senescence, and inflammation were identified by molecular interrogation of the Aβ plaque environment. Direct induction of Aβ aggregation in cultured OPCs, furthermore supports the phenomenon of developing cellular senescence in patients with Alzheimer’s diseases (P. Zhang et al., 2019).

9.3 Parkinson’s Disease

A considerable amount of evidence shows that Parkinson's disease (PD) results in inflammation and immune activation (Chinta et al., 2018). There are reciprocal pathological associations between senescence and PD-linked gene mutations. PD-linked mutation in genes coding for alpha-synuclein, leucine-rich repeat kinase 2 (LRRK2) and vacuolar protein sorting ortholog 35 (Vps35) can induce premature senescence while senescence can induce PD-relevant neuropathology and neurological condition (Senescence in Parkinson’s Disease and Related Disorders | Parkinson’s Disease, n.d.). Neuroinflammatory alterations such as microglial cell activation, elevated levels of pro-inflammatory cytokines, including IL-6 and

IL-8 along with immune changes in peripheral blood are well documented in patients with Parkinson's disease (Chinta et al., 2018). Parkinsonian substantia nigra pars compacta (SNpc) tissues showed elevated expression of the senescence marker $p16^{INK4a}$ relative to control tissues, and several SASP components including MMP-3 protease and the pro-inflammatory cytokines IL-6, IL-1a, and IL-8 (Williams-Gray et al., 2018).

9.4 Chronic Liver Disease

Several experiments have shown that senescent cells accumulate in fibrosis induced murine livers. Liver fibrosis is a precursor for developing liver cirrhosis (Krizhanovsky et al., 2008). Cirrhosis of the liver has been linked to telomere shortening. Telomere depletion through repetitive cell division leads to replicative senescence and is thought to be the cause of hepatocyte senescence in chronic liver illness (Paradis et al., 2001; Sekoguchi et al., 2007). It has been proposed that oxidative stress causes both telomeric and non-telomeric DNA damage, resulting in telomere-dependent as well as telomere-independent cellular senescence (A. Aravinthan, 2015). Therefore, both replicative senescence and stress-induced senescence are likely to develop chronic liver disease (A. D. Aravinthan & Alexander, 2016).

9.5 Impaired Heart Regeneration

Cardiac ageing and pathology affect cardiac progenitor cells' (CPCs) potency to function (Castaldi et al., 2017; Cesselli et al., 2011). This means that the aged and diseased myocardium has a decreased capacity to sustain homoeostasis, to heal, and regenerate after injury (Ellison-Hughes & Lewis, 2017). As a result, the ageing environment can limit the efficacy of cell transplantation procedures, preventing cardiogenic proliferation of transplanted cells and activation of endogenous restorative mechanisms. More than half of the cardiac progenitor cells in the elderly human heart are senescent. CPCs in the failing heart

of a person with advanced age showed accumulation of senescent CPCs, as well as decreased self-renewal, differentiation, and regenerative capability *in vivo* (Castaldi et al., 2017). The resulting SASP also makes the surrounding healthy and cycling-competent CPCs senescent (Lewis-McDougall et al., 2019).

9.6 Cardiac Fibrosis and Dysfunction

There is a strong connection between cardiovascular disorders and cellular senescence. Senescent cardiovascular cells lead to chronic cardiovascular diseases (CVDs), including atherosclerosis, thoracic aortic aneurysm (TAA), abdominal aortic aneurysm (AAA), stiffness of the arteries and heart failure (Judith Campisi & Deursen, 2015; H. Z. Chen et al., 2016; P. Song et al., 2020; Watson et al., 2017). Senescence in vascular smooth muscles typically drives atherosclerotic plaque vulnerability, resulting in myocardial infarction and stroke (H. Z. Chen et al., 2016). In humans with end-stage heart failure, increased myocardium levels of p53 have been recorded (H. Song et al., 1999). Mouse models with cardiovascular disorders and human heart tissues were studied for characterizing premature senescence and fibrosis. In fibrotic areas, senescent cells were found to accumulate, indicating that fibroblasts may be the key cell group experiencing senescence upon fibrosis induction (Meyer et al., 2016).

9.7 Human Skin Ageing

Skin ageing results from a mixture of intrinsic and extrinsic variables that eventually compromise the skin's structural integrity and physiological function (Kammeyer & Luiten, 2015). Owing to the interaction of DNA damage response (DDR) proteins with telomeres, telomere dysfunction has been found to drive senescence which increased in multiple mammalian tissues during ageing (Bhatia-Dey et al., 2016). The only epidermal cell type to express the senescence marker $p16^{INK4a}$ during human skin ageing is melanocytes. In mature

melanocytes despite detectable telomere shortening, specific senescence indicators such as reduced HMGB1 (high morbidity group box 1 protein) and abnormal telomeres have indeed been observed (Victorelli et al., 2019). Furthermore, by stimulating CXCR3-mediated mitochondrial ROS, senescent melanocyte SASP causes telomere disruption and restricts the multiplication of surrounding cells through a paracrine mechanism. Ultimately, using 3D human epidermal counterparts, senescent melanocytes inhibit basal keratinocyte proliferation and cause epidermal atrophy *in vitro*. Senescent melanocytes regulate keratinocyte activity and play a role in human skin ageing (Victorelli et al., 2019).

9.8 Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF), a chronic, irreversible fibrotic lung condition, becomes more and more common as people become older. IPF pathogenesis involves increased ageing of the alveolar epithelium, as shown by oxidative damage, telomere depletion, DNA damage, swelling, and matrix remodeling (Justice et al., 2019). Cellular senescence, a characteristic of ageing, may be a central pathway that contributes to IPF (Faner et al., 2012). Senescent cells and SASP can fuel a pro-inflammatory fibrotic reaction that leads to lung damage and severe functional impairment in IPF. Senescence biomarkers including $p16^{INK4A}$, DNA foci damage, telomere malfunction, and SASP factors aggregate in the lungs of IPF patients and are linked to increased disease severity (Justice et al., 2019).

Chapter 10

Senolytic Drugs

The accumulation of senescent cells has been associated with premature ageing accompanied by several chronic diseases. This makes the senescent cells a crucial target for pharmacological intervention. Senotherapeutics are drugs which either eliminate senescent cells or inhibit the expression of deleterious senescence-associated phenotype. Senotherapeutics can be classified into three groups based on their mechanism of action which includes senolytics, senomorphics and immunomodulators. Senolytic drugs work by inducing apoptosis of senescent cells; senomorphics reduces the detrimental effects of SCs by suppressing SASP; and immunomodulators enhance immune-mediated clearance of SCs (E. C. Kim & Kim, 2019). Senolytic agents target molecules associated in senescent cell anti-apoptotic pathways (SCAPs) to induce apoptosis within senescent cells only, leaving non-senescent cells unaffected. SCAPs protect SCs from apoptosis by shielding them from their own pro-apoptotic SASP (Prata et al., 2018). The SCAP associated molecules targeted by several senolytic drugs have been listed in Table 3. A key study in mice model demonstrates that both caloric restriction and possibly a lifespan-extending mutation reduced the age-related senescent cell assembly in mice, which supported the hypothesis of developing agents to attack senescent cells and thereby, slow down the progression of senescence-related diseases (Justice et al., 2019).

A number of synthetic and natural senolytic agents have been found to have undergone pre-clinical studies in mice; and few have already been tested in humans in small scale, while some more are underway of clinical trials (Ellison-Hughes, 2020; J. L. Kirkland & Tchkonia, 2020).

Table 3: SCAP associated target molecules of senolytic drugs

Senolytic drugs	Target molecules
Dasatinib	Ephrins, Bcl-xL, Tyrosine kinase, PI3KCD, p21, PAI1 and PAI2
Quercetin	Bcl-2 Family, p53/p21 and p13k/AKT
Fisetin	P13K/AKT
Navitoclax	Bcl-2 Family: Bcl-2, Bcl-xL, Bcl-w
HSP90 inhibitors	Grp94, Trap 1 and 17-DMAG
Piperlongumine	Bcl-2 Family, Bcl-xL, GSTp1, CRB1 and p53/p21
EF24	Bcl-xL and Mcl-1
FOXO4	p53
UBX0101	MDM2/p53

10.1 Dasatinib and Quercetin

Dasatinib (D), an FDA approved tyrosine kinase inhibitor used for the treatment of chronic myeloid leukemia (CML), has been shown to promote apoptosis of senescent cells via inhibition of ephrin, that modulates the BCL-xL, PI3KCD, p21, PAI1 and PAI2 pro-survival networks (FDA Approves Dasatinib for Pediatric Patients with CML | FDA, n.d.; J. L. Kirkland & Tchkonia, 2020). However, Dasatinib when taken in combination with a nutraceutical-based compound, Quercetin (Q) has been found to render greater efficacy in selectively eliminating SCs and in delaying or alleviating multiple age-associated chronic conditions both in mice models and humans than if it was taken alone. Quercetin is a flavonoid which is ubiquitously found in various fruits, vegetables, tea and red wine (Li, Qin, et al., 2019). This molecule exhibits a wide range of therapeutic properties among which are anti-inflammatory, antimicrobial, antioxidant and anti-carcinogenic effects (Anand David et al., 2016). The most noticeable property of quercetin is its potent antioxidant function, which aids in the prevention of free radicals from forming resonance-stabilized phenoxyl radicals (W. Wang et al., 2016). Since oxidative stress levels increase with the ageing process, quercetin being a strong antioxidant is hypothesized to prevent accelerated ageing by lowering cellular oxidative stress (Singh et al., 2003). In 2007, it was also discovered that this flavonol when administered to *Saccharomyces cerevisiae* improved its tolerance to oxidative stress and increased its lifespan (Belinha et al., 2007). Quercetin had first been identified to function as a senolytic agent in 2015 due to its potential to destroy senescent human endothelial cells (Y. Zhu et al., 2015). It has also been used as a geroprotective agent against rapid and normal ageing in human mesenchymal stem cells (hMSCs), offering a possible clinical intervention to combat age-related disorders (Li, Qin, et al., 2019).

D and Q were found to possess moderate senolytic efficacy independently, but in combination they demonstrated a successful senolytic activity (Y. Zhu et al., 2015). In pre-clinical models, D+Q-mediated clearance of senescent cells has been shown to alleviate lung fibrosis as well as hepatic stenosis; enhance vasomotor activity, ventricular function and neurogenesis; prevent age-related bone loss, anxiety-related behaviour; and increase lifespan (Dookun et al., 2020). D and Q combination has also been found to reduce the adipose tissue senescent cells extracted from patients with diabetes and obesity during surgery by 70% within 2 days of exposure. There was an increase in the amount of cleaved caspase 3 during this period indicating that the elimination of senescent cells was caused by apoptosis. Moreover, there was a significant decrease in the level of SASP factors including IL-6, IL-8, MPC-1, PAI-1 and GM-CSF in the conditioned medium obtained from the cultured adipose tissues treated with D+Q (J. L. Kirkland & Tchkonia, 2020).

The first pilot scale clinical trial with D and Q combination was performed in patients with IPF where the patients were ≥50 years of age. The dose of Dasatinib used was 100 mg/day which was selected based on the FDA approved dose for inducing apoptosis of human cancer cells. Quercetin was given with Dasatinib at a dose of 1250 mg/day. An intermittent dosing regimen of 3 oral doses for 3 consecutive days over a 3- week period was administered to the participants during the study with a total of 9 dosing days. As a result of the treatment, physical mobility of the patients was found to improve. However, the study was performed without a placebo control group. Therefore, the results of this study need to be validated through the employment of large sample size and randomized blinded clinical trial (Justice et al., 2019). Another clinical trial with this drug combination (100 mg D + 1000 mg Q) was performed in patients with diabetic kidney disease who aged from 50-80 years where the regimen was 3 oral doses for 3 consecutive days. In this study, the senescent cell markers like $p16^{INK4a}$, $p21^{CIP1}$, SA- β-gal activity and level of some proinflammatory cytokines were found

to be reduced. This decrease in senescence biomarkers were observed in blood, adipose tissues and skin biopsies collected at 11^{th} day after the 3-day D+Q administration schedule. Therefore, the result suggests that D+Q provides a sustained effect in reducing the senescent cell burden in humans even though they have an elimination half-life of <11 hours (Hickson et al., 2019).

10.2 Fisetin

Fisetin is a nutritional supplement that is readily available. Fisetin was discovered to selectively induce apoptosis of senescent cells, but it had no such effect on the actively dividing human umbilical vein endothelial cells (HUVECs) (Y. Zhu et al., 2017). It falls under the category of flavonoid and can be found in apples, grapes, strawberries, persimmons, cucumbers, and onions (Li, Qin, et al., 2019). Fisetin has a wide range of biological functions, including anti-inflammatory, antioxidant, anti-tumor, anti-angiogenic, hypolipidemic, and neuroprotective properties (Khan et al., 2013; Pal et al., 2016; Sundarraj et al., 2018). The potential of flavonoids to scavenge free radicals confers its antioxidant function (Khan et al., 2013; Markovi et al., 2009). Fisetin has been shown to have a significant anti-tumor activity in a variety of cancer cell lines by suppressing cancer cell proliferation via cancer cell apoptosis, according to the new findings (Li, Qin, et al., 2019). It's intriguing that fisetin's anti-proliferative as well as proapoptotic impact were limited to cancer cells, whereas normal cells were unaffected, indicating strong selectivity of fisetin between normal and cancer cells (Lall et al., 2016; Y. Zhu et al., 2017). However, it is not senolytic towards senescent IMR90 cells (a human lung fibroblast cell line) or primary human preadipocytes, indicating its cell type specific senolytic activity (Y. Zhu et al., 2017). Fisetin's senolytic function was later confirmed in an *in vivo* study involving the testing of a panel of flavonoid polyphenols on senescent fibroblasts obtained from human and mouse

(Yousefzadeh et al., 2018). Among the flavonoids studied, fisetin was the most potent senolytic agent, suppressing senescence markers in multiple tissues (Li, Qin, et al., 2019).

10.3 Navitoclax

Navitoclax is a Bcl-2 family protein inhibitor that is senolytic in some even if not all mice and humans cell types (Fuhrmann-Stroissnigg et al., 2018; Y. Zhu et al., 2016). The Bcl-2 proteins performs its anti-apoptotic activity by blocking the proapoptotic proteins Bax/Bak, thereby preventing the mitochondrial outer membrane from getting pierced by Bax/Bak. Navitoclax has been shown to decrease senescent cell burden by hindering Bcl-2 activity resulting in the improvement of radiation induced hematopoietic stem cell dysfunction and osteoarthritis (Chang et al., 2016; Y. Zhu et al., 2016). Moreover, inhibition of another anti-apoptotic protein Bcl-xL by navitoclax demonstrated elimination of senescent IMR 90 cells and HUVEC but not senescent pre-adipocytes *in vitro* (Dookun et al., 2020). In addition, navitoclax therapy improved the function of hematopoietic and muscle progenitor cells *in ex vivo* assays and prevented ionizing radiation-induced pulmonary fibrosis *in vivo* in old mice (Chang et al., 2016; Dookun et al., 2020).

10.4 Hsp90 Inhibitors

The highly conserved heat shock proteins (Hsps) function to regulate the stability of several misfolded and unfolded proteins and are expressed in response to stressful stimuli such as heat shock, pH change, hypoxia, heavy metal exposure or proteotoxicity. One of the key transcription factors controlling the transcription of Hsps is heat shock factor 1 (HSF1). Hsps can be classified into Hsp27, Hsp40, Hsp60, Hsp70 and Hsp90 based on its molecular weight. These chaperones are mainly involved in the synthesis of properly folded proteins and degradation of irregular proteins (Fuhrmann-Stroissnigg et al., 2018). Several signaling

proteins which are stabilized by Hsp90 contribute to tumor growth and hence, are potential targets of anti-cancer therapeutics. However, due to its crucial role in many cellular processes, it is risky to inhibit its activity. Nevertheless, inhibition of Hsp90 function in an organelle specific manner can minimize the risk of interfering with its normal cellular activity (Fuhrmann-Stroissnigg et al., 2018). In a study with mouse models having human progeroid syndrome, Hsp90 inhibitors were identified to show senolytic activity accompanied by an increase in the healthspan, decrease in the $p16^{INK4a}$ level and delay in the development of age-related characteristics in the model (Fuhrmann-Stroissnigg et al., 2017).

10.5 Piperlongumine

Piperlongumine is a bioactive substance found in the *Piper genus* (Bezerra et al., 2013). It is an amide alkaloid of Piper longum L and is also known as piplartine (Piska et al., 2018). This alkaloid exhibits an array of pharmacological properties against bacteria, platelet agglutination, tumor formation, angiogenesis, metastasis, depression, atherosclerosis and diabetes (Bezerra et al., 2013). Apart from these biological effects, piperlongumine has also been found to eliminate senescent human WI-38 fibroblasts (Li, Qin, et al., 2019). Moreover, when taken together with a Bcl-2/Bcl-xL inhibitor ABT-263, it degraded SCs synergistically. An antioxidant protein known as oxidation resistance 1 (OXR1) is expressed in response to oxidative stress and has been found to be upregulated in senescent cells. Piperlongumine targets OXR1 to induce the degradation of senescent cells through ubiquitin-proteasome mechanism (X. Zhang et al., 2018). This finding suggests that OXR1 is a novel piperlongumine senolytic target that can also be further investigated to develop new senolytic agents (Li, Qin, et al., 2019).

10.6 EF24

Curcumin is a hydrophobic polyphenol extracted from the rhizome of *Curcuma longa*. Curcumin has been shown to exhibit anti-cancer, anti-inflammatory, anti-oxidative, and antimicrobial properties (Li, Qin, et al., 2019). Several clinical studies have examined curcumin's medicinal ability and demonstrated the advantageous aspects of curcumin in slowing ageing and avoiding or curing age-related diseases (Grill et al., 2018; Takano et al., 2018; Yang et al., 2017). Curcumin has also been shown to extend lifespan and improve the healthspan of *Drosophila melanogaster* (fruit fly) and *Caenorhabditis elegans* (Chandrashekara et al., 2014; Liao et al., 2011). However, its low potency and poor absorption properties are one possible issue that hinders the medicinal use of curcumin (Shoba et al., 1998). Moreover, a recent study has confirmed that curcumin itself displays poor senolytic activity (Yousefzadeh et al., 2018). Curcumin analogs such as EF24, HO-3867, 2-HBA, and dimethoxycurcumin have been designed to improve the biological activity and bioavailability of curcumin. They have been found to be more successful than curcumin in minimizing age-dependent deterioration. EF24 has recently been identified as the most potent senolytic agent among the other analogs of curcumin (Li, Qin, et al., 2019). Not only in SCs induced by ionizing radiation, but also in SCs induced by widespread replication or ectopic Ras oncogene transfection, EF24 reduced senescent cell viability. EF24 shows a broad range of senolytic activity against a variety of SCs, including human IMR-90 fibroblasts, HUVECs, and human renal epithelial cells (Li, He, et al., 2019). EF24 has been shown to induce apoptosis in a variety of tumor cells by the synthesis of reactive oxygen species and causing endoplasmic reticulum stress facilitated by oxidative stress. In addition, EF24 can reduce the expression of Bcl-xl and Mcl-1 in SCs but not in normal cells, which could be partially influenced by the proteasomal degradation pathway. These findings shed light on how curcumin analogs function as anti-ageing agents and suggest that curcumin

analog EF24 may be used as a potential senolytic agent for the treatment of age-related diseases (Li, Qin, et al., 2019).

10.7 FOXO4

The FOXO4 D-retro inverso (DRI) peptide works by increasing the amount of active p53 in senescent cells by targeting the FOXO4-p53 axis. FOXO4 DRI improves mouse quality of life in naturally aged and genetically aged senescence models of rodents (fur and activity). In a mouse model of folic acid-induced kidney injury, there was a significant reduction in senescent tubular cells, but no reduction in renal fibrosis, cellular infiltration, and tubular damage (Jin et al., 2019). A FOXO4-interacting peptide that blocks the association of FOXO4 with p53 decreases senescence and enhances fitness, hair growth, and renal function in progeroid as well as old wild-type mice by inducing apoptosis (Fuhrmann-Stroissnigg et al., 2018). In contrast to non-senescent cells, FOXO4 DRI treatment of senescent human fibroblasts resulted in a selective decrease in senescent cells (Knoppert et al., 2019).

10.8 UBX0101

UBX0101 is a small-molecule inhibitor that targets the MDM2/p53 interaction. Primary human chondrocytes extracted from osteoarthritis patients when treated with UBX0101 experienced a senolytic effect and reduced SASP expression. In a post-traumatic osteoarthritis mouse model, pain relief, decreased articular cartilage erosion, and lower expression of senescent markers in the cartilage were also observed. The improvement in physical function was observed for 84 days (Knoppert et al., 2019).

Chapter 11

Challenges

Although preclinical results clearly endorse the removal of senescent cells that can boost or even reverse the pathological development of multiple diseases, there are issues that need to be taken care of to ensure that interventional techniques are effectively translated. In the process of ageing, senescent cells are gradually abundant within tissues. Despite such a reality, in some physiological aspects such as tumor suppression, tissue repair and regeneration, wound healing, vascular remodeling and embryonic growth, cell senescence is important in a damage-independent way (Adams, 2009; Demaria et al., 2014; Hernandez-Segura et al., 2018; Mosteiro et al., 2016; Muñoz-Espín et al., 2013; Ruscetti et al., 2020).

Thus, during these cycles, the non-selective modulation of cellular senescence will impair physiological integrity and harm patient wellbeing. While experimentally, the influence of senolytics on individual physiological processes remains mostly unexplored, existing senolytics typically cause senescent cell apoptosis upon temporary exposure, with their administration limited to a brief period of time to prevent corresponding side effects (S. Song et al., 2020). Moreover, diagnosis of senescence cells in humans is a limitation since currently these cells can only be identified from tissues extracted surgically and in human cell lines.

Chapter 12

Conclusion

Senescent cells have beneficial effects in several physiological processes, however, a disruption in the maintenance of senescent cell homeostasis drives the production of many factors that accelerates the process of ageing and chronic diseases (P. Song et al., 2020). Senolytics appear as promising drug candidates in prolonging lifespan and improving the quality of life of an individual through the selective apoptosis of senescent cells. Studies suggests that senolytic agents can cause recovery of several organ dysfunction, attenuate inflammation of the tissue and reduce metabolic dysfunction across cell types and tissues (J. L. Kirkland & Tchkonia, 2020). There are currently clinical trials underway for several synthetic and nutraceutical based senolytic drugs to assess if the outcomes found in animals can be replicated in humans. Continued senescence research can reveal substantial insights related to the clinical use of senolytics, specifically with regard to changes in senescent cells, SASP, and their cellular fate over time (Martel et al., 2020).

Future Directions

A number of issues still need to be addressed in order to establish the use of senolytic drugs as an anti-ageing agent. There is a great need to identify specific and reliable biomarkers of senescent cells. Moreover, in order to decide the optimum senolytic regimen with regard to the molecular combinations, doses, and treatment schedules, further research with senolytic drugs is needed (Martel et al., 2020). Simultaneously, the short-term and long-term adverse impacts of certain senolytic drugs and cell specific response to senolytic drugs needs to be further evaluated via clinical trials (Ellison-Hughes, 2020).

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