Aging, Age-Related Diseases, and the Zebrafish Model

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Abstract: The entrance of cells into a permanent state of cell cycle arrest with the ability to resist apoptosis is termed “cellular senescence”. The accumulation of senescent cells within the body can lead to tissue aging and the dysfunction of organs. Whether due to external stressors or the passage of time, aging is an inevitable process that afflicts every living being. Current studies that investigate aging rely on the use of cells or rodent models. Although cells present a cost-effective and quick way to analyze aging, they lack the complexity of whole-body systems and therefore require the use of an in vivo model post-in vitro assays. The zebrafish, Danio rerio, presents a cost-effective model with quick development and large numbers of offspring. These fish share 70% similarity of their genes with humans, including genes known to be associated with human diseases, such as those diseases of aging and/or senescence, like Alzheimer’s disease. Major tissues and organs of humans are also found in these fish, and therefore, zebrafish can serve as a useful model when studying diseases, aging, Alzheimer’s disease, and other disorders. In this review, we will discuss some of the major senescence biomarkers and detection methods, as well as discuss how zebrafish models can be used for the study of aging and age-related disorders.

Keywords: aging; senescence; zebrafish; Danio rerio

1. Introduction

Cellular senescence is one of the most rapidly developing fields of science [1]. Because of its relatively new attraction, there are still a lot of unknowns when it comes to senescence and its characterization. The idea of “senescence” itself was first described by Hayflick and Moorhead in 1961 [2]. In their article, they described cells having a “stage III” in which primary cells in culture present a finite lifespan characterized by reduction in mitotic activity. It was not until a later publication by Hayflick [3] in which he observed that there was an increase in cellular debris and degeneration that resembled cell death [1].

Currently, cellular senescence is associated with a cell entering a state of irreversible cell cycle arrest in response to exogenous and endogenous stresses [4]. The word senescence stems from the Latin word senex, which means growing old [5]. It is essentially a tumor-suppressive mechanism cells use to avoid cancer, yet chronic production of inflammatory markers and DNA damage can increase the presence of senescent cells. Senescent cells are known to accumulate with age and have been shown in sites of age-related pathology, thus contributing to organismal aging [6].

Senescence is a biologically necessary process. As mentioned earlier, senescence is a tumor suppression mechanism. The purpose of the induction of permanent cell cycle arrest is to ensure that damage to cells is not implemented into the genome and surrounding cells. However, senescence has other functions that are also aimed at the well-being of an organism. In fact, senescence can be found during embryonic development. In mammalian embryos, senescence has been shown to regulate tissue development by aiding in system remodeling, pruning, and microenvironment modulation [7]. Senescence also plays a role in wound repair by reducing fibrosis and promoting the recruitment of immune cells [8].
The issue with cellular senescence arises after it becomes chronic and unregulated. Put simply, prolonged senescence can lead to the premature occurrence of many age-related diseases. Just like senescence can be beneficial during wound healing, it can also be detrimental. Chronic senescence can lead to a decrease in fibroblast proliferation or to excessive fibrosis [8]. Stem cell exhaustion also occurs, leading to a decrease in cell renewal and an increase in tissue deterioration [9]. Chronic diseases including chronic inflammation, osteoarthritis, neurodegenerative disorders such as Alzheimer’s and other dementias, diabetes, cancer, along with many other age-related diseases can be attributed to the accumulation of senescent cells [10]. Therefore, senescence has often been called a “double-edge sword”.

Because senescence is still not completely understood, there have been numerous studies aimed at identifying senescence-related biomarkers. Many studies have identified different biomarkers that may have a link to aging and cellular senescence [1,11–13]; however, several of these biomarkers are involved in many other physiological processes and diseases. Therefore, as of now, a single biomarker solely related to senescence is yet to be discovered. Despite this fact, there are general hallmarks of senescence that can be used to validate senescence with confidence.

2. What Is Aging?

Aging is an inevitable process that occurs chronologically. Bodily changes due to development, the environment, and diseases all contribute to the process [14]. Aging is essentially the accumulation of these bodily changes, which with time are responsible for, or associated with, an individual’s susceptibility to disease and or death [15]. Individuals with the same chronological age can differ greatly in their biological age, which can be accelerated due to external stressors.

Aging, when observed from the molecular level, is termed cellular senescence. Evidence suggests that senescent cells are accumulated in aging tissues, which contributes to the impairment of physiological processes necessary for the maintenance of health [12]. Cellular senescence is observed in many inflammatory and age-related diseases such as Alzheimer’s, osteoarthritis, and cardiovascular disease [16]. Senescent cells are also thought to play a role in many cerebrovascular diseases like dementia [17]. For example, studies have shown that senescent microglia, astrocytes, and neurons are found in the brain of Alzheimer’s disease patients and animal models [18]. Senescence is a state of permanent cell cycle arrest and apoptosis resistance that can affect any cell type. Internal and external stressors such as DNA damage and telomere shortening can influence the cell’s ability to turn senescent. Although this process can be used physiologically for the inhibition of abnormal cell proliferation, it can lead to a decline in normal organ function [19]. Senescent cells release senescence-associated secretory phenotype (SASP), which is a combination of proteases, immune modulators, growth factors, and inflammatory cytokines [20]. SASP from senescent cells can kill healthy cells or even turn them senescent, thereby exacerbating effects.

External stressors such as smoking, sun exposure, pollution, etc., can prematurely result in the production of senescent cells. Selective killing of senescent cells can help in eliminating further damage to neighboring cells and organs. For instance, studies have shown that removing senescent cells in the brain ameliorates β-amyloid peptide and tau-protein-induced neuropathologies, and improves memory in Alzheimer’s rodent models, suggesting a possible association between senescence and Alzheimer’s disease [18]. Targeting vasculature early using “senescence killing” drugs (senolytics) has also been found to improve the integrity of the blood–brain barrier in Alzheimer’s disease rodent models, indicating a valid therapeutic target [21]. However, this becomes more of a problem when dealing with senescent cells that are terminally differentiated postnatally or differentiated along a terminal cell lineage, like fibroblasts and neurons [22]. Therefore, it is important to understand and identify the genetic factors that are associated with the
emergence of senescence both due to intrinsic and extrinsic factors to aid in the development of preventative therapies.

2.1. Aging Biomarkers

The identification of biomarkers unique to senescence is difficult [12]. Many of the current biomarkers used to identify senescent cells are not specific to senescence and can be seen in different diseases that present inflammatory markers, tumor suppressor markers, cell cycle arrest-based markers, along with others [11]. A few of the many biomarkers that have been identified to be partly or entirely involved in the process of senescence are discussed below. It is important to note that some of these markers discussed in one section have many cellular roles in relation to senescence, and therefore are applicable to other sections as well. They are discussed only once for the flow of information.

2.1.1. Lysosomal Activity

Among the most characterized biomarkers for the identification of senescence are those pertaining to lysosomal activity, such as senescence-associated β-galactosidase (SA-β-Gal). β-galactosidase is an enzyme that hydrolyzes β-galactosides into monosaccharides in senescent cells at a pH of 6.0. β-galactosidase activity increases in senescent cells due to an increase in lysosomal content, including size and number [23]. This results in the overexpression of lysosomal β-galactosidase found in senescent cells. This enzymatic activity is sometimes shown to be present in quiescent cells and thus presents major limitations with the exclusive detection of senescence [24]. Regardless, senescence-associated β-galactosidase activity is the most widely used biomarker for senescence [23].

Another biomarker linked to lysosomal activity is lipofuscin. Lipofuscins are pigmented granules that are lysosomal waste products that cannot be degraded or removed. They are essentially composed of transition metals, covalently cross-linked proteins, oxidized lipids, and oligosaccharides [24]. In post-mitotic cells, lipofuscin accumulates and forms what is known as the “aging pigment”. Lipofuscin has also been observed during replicative senescence [24]. The more lipofuscin is present in the cell, the “older” the cell is said to be [25]. As a result, lipofuscin can be used as a biomarker for senescent cells.

2.1.2. Reactive Oxygen Species

The presence of reactive oxygen species (ROS) is another biomarker that can be linked to senescence, but is not uniquely present in aging. An overproduction of ROS or the lack of defenses in the form of antioxidants can lead to and escalate cellular senescence [7]. It is believed that mitochondrial ROS are a key initiator of cellular senescence, which has been shown in rodent models to activate SASP [26]. With the increase in oxidative damage within the mitochondria, there becomes an imbalance in fusion–fission events, which could explain the abnormalities in mitochondrial morphology noted earlier—giant mitochondrion being one of them [27].

Cells, and thereby, by extension, tissues and organs, are exposed to ROS from external stressors as well as within the cells themselves. The majority of ROS—close to 90%—that are produced intracellularly are due to the mitochondrial electron transport chain. Electrons can prematurely leak from the chain and form the superoxide ion radical which can be converted to hydrogen peroxide and further to peroxide [28]. Another intracellular source of ROS is through the work of a variety of enzymes. Members of the NADPH oxidase (NOX) family along with other enzymes present in peroxisomes with oxidase activity are natural producers of ROS [28]. Initially, it was believed that the role of these oxidases was to aid in the role of immune defenses by producing ROS to destroy pathogens. However, it is now known that these enzymes participate in many physiological processes such as cell signaling, gene expression, and post-translational protein processing [29]. However, misregulation of these enzymes has deleterious effects that can play a role in cellular senescence.
External stressors play a large role in the creation of ROS and premature cellular senescence. Common sources that can lead to ROS include UV radiation from the sun and tanning beds, as well as other sources of ionizing radiation such as X rays. A lot of environmental pollutants and toxins also play a role in the creation of ROS, such as air pollutants that come from factories and affect the airways as well as skin cells, which are constantly exposed to the environment around them. Smoking and secondhand smoke also lead to the production of ROS, since smoking is linked to lipid peroxidation [28]. Administration of some chemotherapeutics can also lead to the formation of ROS and directly induce cellular senescence.

2.1.3. DNA Damage Markers

Telomeres are regions at the end of a chromosome composed of repetitive DNA sequences whose purpose is to protect the chromosomes from becoming tangled. Telomeres are known to become shorter with every cell division [30]. Research has found that telomere shortening leads to impaired tissue maintenance and a shortened lifespan [31]. In vitro studies indicate that cellular senescence occurs after low levels of telomere dysfunction [31]. Telomerase is an enzyme that adds or restores the nucleotides that were removed from telomeres after cell division to counteract senescence. Telomerase has a catalytic subunit named telomerase reverse transcriptase (TERT) that is capable of translocating from the nucleus to the mitochondria during times of oxidative stress [32]. Stress-induced TERT transport to the mitochondria increases the inner membrane’s potential and reduces the production and level of peroxides. Therefore, cells under oxidative stress overexpress TERT to reduce mitochondrial DNA damage through increasing antioxidant defense mechanisms [32].

Extrinsic and intrinsic stresses can lead to DNA damage, and to repair stress, DNA repair mechanisms are employed in the form of the DNA damage response (DDR) pathway. Two biomarkers involved in the DNA damage response signaling pathway that researchers oftentimes use to detect senescence are ataxia-telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) kinases. The DDR pathway consists of effectors, signal sensors, and transducers [33]. These kinases are found mostly upstream in the DNA damage response pathway and work together to regulate downstream processes after DNA damage [33]. ATR is activated by many types of DNA damage, whereas ATM is activated by double-stranded DNA breaks [33]. It was found in cells with replication stress that ATM inactivation was able to overcome senescence, thereby reprogramming the cell’s metabolic capabilities in comparison to cells with functioning ATM [34]. Other studies have noted that ATM signaling is highly activated in cellular senescence and in cases of accelerated and natural aging, and inhibition of this kinase rescues senescence by suppressing nuclear factor kappa B (NF-κB) activation [35]. It was also found that ATR activation, in the absence of DNA damage, can promote cell cycle arrest, leading to senescence [36]. This is thought to be implicated in the G2 phase of the cell cycle, showing a direct link between this phase and DNA repair in order to protect non-transformed cells’ genomic stability [37].

High mobility group box 1 (HMGB1) is a nuclear protein that aids in the maintenance of chromosomal structure and function. A variety of factors ranging from stress and cell death to post-translational modification can lead to the release of HMGB1 into the extracellular space [38]. After its release, HMGB1 functions as a damage-associated molecular pattern (DAMP) molecule, which activates the immune system. Although this helps in the cell’s defense mechanisms, it also promotes pathological inflammatory responses by activating the NF-κB pathway [39]. There have been studies that link the depletion of HMGB1 in cells to the shift from senescence to apoptosis, and the increase in this protein to the shift in cell fate from apoptosis to senescence in doxorubicin-treated cells [40]. This was also proven by the same authors using HMGB1 knockout models under genotoxic stress. Another study was able to link HMGB1 to stimulator of interferon genes (STINGs), which selectively regulate p21 into causing cell cycle arrest in senescence [41].
NF-κB signaling is thought to be one of the major signaling pathways that stimulate the induction of SASP [42]. This family of proteins regulates the expression of over one hundred fifty genes and is part of the DNA damage response. It is thought that with the stimulation of NF-κB, DNA repair and cell cycle checkpoints are activated in an attempt at restoring the normal cell life cycle [43]. There are two main signaling pathways, the canonical and non-canonical pathway cascade. In the canonical pathway, cytokines, free radicals, and other pathogen-associated molecular patterns (PAMPs) bind to cell surface receptors to activate NF-κB, whereas the noncanonical pathway selectively responds to ligands of the TNFR receptor superfamily [44]. Once activated, NF-κB is translocated to the nucleus, where it binds to specific DNA sequences to control the gene expression of many inflammatory genes, cell surface receptors, growth factors, etc. It is believed that oxidative stress has a major role in activating NF-κB in aging [45]. NF-κB is considered a central mediator for inflammatory and immune responses, due to its activation by a variety of inflammatory signals [46]. Suppression of NF-κB in fibroblasts causes the evasion of immune recognition and allows cells to bypass senescence in cooperation with p53 inactivation [47]. In more advanced cases of aging, a phenomenon called immunosenescence in which a pro-inflammatory characteristic with a decline in immune mechanisms causes something called inflammaging [45]. Inflammaging refers to chronic inflammation related to an age-related increase in pro-inflammatory markers. Researchers believe that NF-κB signaling is to blame for this phenomenon [45].

γH2AX is a histone variant that works to stabilize the genome by recruiting and localizing DNA repair proteins [48]. This histone is phosphorylated by kinases like ATM after DNA damage forms double-stranded breaks, and is one of the earliest markers of damage [49]. Unpaired double-stranded breaks can result in either cell death or cellular senescence [50]. Studies have shown that γH2AX is formed close to double-stranded breaks at a ratio of 1:1 [51]. γH2AX is a component of senescence-associated heterochromatin foci (SAHF), which are implicated in senescence-associated structural changes that take place in the nucleus. It was found that γH2AX allows DNA time to repair by regulating checkpoint responses, such as the recruiter for p53, during low levels of DNA damage; however, this was not the case with higher levels of damage [52]. This histone is also an effective marker of telomere shortening due to the correlation of shortened telomeres with the accumulation of γH2AX foci in cells [51]. γH2AX is considered the second most common biomarker of senescence after senescence-associated β-galactosidase [51].

2.1.4. Cell Cycle and Proliferation Markers

One of the most defining features of senescent cells is cell cycle arrest. Therefore, the expression of genetic markers relating to the cessation of the cell cycle is widely used when identifying senescent cells. In vitro studies of replicative senescence have identified that senescent cells experience growth arrest in the G1 phase of the cell cycle [53]. The inhibitors of this phase of the cycle are inhibitors of cyclin-depending kinases, p16[^INK4a], p21[^CIP1], p53, and p27[^KIP1], which are encoded by tumor suppressor genes [54]. The most popular cell cycle arrest marker of cellular senescence is p16[^INK4a], which is encoded by the CDKN2A gene [10]. p16[^INK4a] has been shown to be dramatically increased in a variety of tissues in old rodent models [55]. The p16[^INK4a] protein binds to cyclin-dependent kinase 4 and 6 (CDK4/6) to limit cellular proliferation in the case of DNA damage and oncogenic stress. Since senescent cells can no longer proliferate, it is expected that this protein will be overexpressed [56].

p21[^CIP1] is another commonly studied cyclin-dependent kinase inhibitor in the context of aging. This protein binds to and inhibits cyclin-dependent kinase 2, 1, and 4,6 complexes. p21[^CIP1], if upregulated, causes cell cycle arrest at the G1 phase or G2 phase of the cell cycle [57]. This protein was found to have both anti-apoptotic and pro-oncogene qualities [58]. With extensive DNA damage, this protein’s expression decreases, and the cell can undergo apoptosis. In the case of senescence, an overexpression of this protein triggers cell growth arrest and the upregulation of other genes and transcription factors that are associated
with senescence, such as NF-κB [59]. Experiments have also shown that upregulation of p21\textsuperscript{CIP1} increased intracellular levels of ROS, and by blocking ROS, cells with p21-induced senescence are able to avoid permanent growth arrest; whereas p16\textsuperscript{INK4a} was not found to be associated with ROS levels [60].

### 2.1.5. Inflammatory Markers

The most prominent cytokine of SASP is interleukin-6 (IL-6) [61]. IL-6 is produced early at sites of inflammation and tissue injury by various types of cells [62]. It is a multifunctional cytokine that can act on targets such as T cells, B cells, certain stem cells, hepatocytes, keratinocytes, among others [63]. IL-6 has been studied extensively in both mice and humans because of its significant role in many chronic inflammatory conditions, including metabolic diseases [64]. Despite its classification as pro-inflammatory, studies have shown that this cytokine may also possess anti-inflammatory properties through the inhibition of other pro-inflammatory markers such as tumor necrosis factor (TNF), the stimulation of matrix metalloproteinase (MMP) inhibitors, and the ability to reduce intracellular superoxide production [62]. To produce its anti-inflammatory effects, IL-6 binds to a membrane-bound receptor called IL-6R. This newly formed complex dimerizes with a second receptor protein to initiate signaling through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway [65]. The JAK/STAT cascade is then able to influence different cellular responses related to proliferation, apoptosis, differentiation, and cell survival [66]. The pro-inflammatory activity of IL-6, on the other hand, is due to IL-6 binding to soluble IL-6R, termed trans-signaling. This new complex is capable of activating IL-6 signaling pathways in gp130 expressing cells. Signaling of IL-6 has been shown to trigger and cause premature senescence [67]. IL-6 as a pro-inflammatory cytokine is one of the main soluble factors in SASP along with IL-8 [68]. It was also found that IL-6 has a role in the NF-κB positive feedback role, as well as the reprogramming of p53 and p21 [68].

Interleukin-8 (IL-8) is another prominent cytokine produced by a variety of cells that has been linked to the process of cellular senescence through SASP. IL-8 binding to its receptors is implicated in many diseases related to angiogenesis, tumorigenicity, and cellular proliferation through the activation of the MAPK and phosphoinositide 3-kinase (PI3K) pathways. However, IL-8 is mostly known for its key role in the inflammation process through the activation and recruitment of neutrophils to a site of infection [69]. IL-8 along with IL-6 and TNF are products of the pro-inflammatory toll-like receptor (TLR) signaling pathway. It is believed that circulation of both IL-6 and IL-8 signifies a silent inflammatory process linked to aging [70]. In another study, it was discovered that human serum levels of IL-8 did not significantly increase with age or fall beyond normal reported ranges [71]. It is possible that the older adults in this study were rather healthy, and that IL-8 overexpression is seen more often in cases of chronic illnesses and inflammaging.

Interleukin-1β (IL-1β) is a cytokine member of SASP that is a mediator of pro-inflammatory responses. This cytokine is produced by a variety of cell types in response to DAMPs and is crucial for infection and injury responses [72]. Several mechanisms in which IL-1β is secreted are thought to occur and are dependent on the stimulus’s strength. When IL-1β is activated, it also works downstream to activate NF-κB; NF-κB also controls IL-1β transcription, giving rise to a feedback loop [73]. It was also found that increased expression of IL-1β can induce SASP and IL-6, whereas inhibition of the cytokine can affect their production [74]. Low levels of IL-1β induce inflammation, and with auto-inflammatory diseases, there is an increased release of the cytokine [75]. Therefore, although it is essential for immune responses, there is also the potential to exacerbate damage in cases of chronic inflammatory diseases.

TNFα is a cytokine that is responsible for a wide range of inflammation-related signaling events within cells [76]. It plays several therapeutic roles, including tumor and infection resistance, as well as injury response. Excessive signaling of TNFα is associated with chronic inflammation, and diseases like inflammatory bowel disease, psoriasis, and
rheumatoid arthritis [77]. TNFα can activate transcription factors such as NF-κB, protein kinases such as extracellular signal-regulated kinases (ERKs), phospholipases, reactive oxygen species, and nitrogen radicals [76]. TNFα is another component of SASP and is thus overexpressed in senescent cells. This factor is able to accelerate and reinforce senescence through a signal transducer and activator of transcription (STAT)-dependent positive feedback loop [78]. This feedback loop locks cells into senescence and leads to DNA damage, further cytokine secretion, and sustained expression of interferon response genes [79].

Sirtuin-1 (SIRT1) is a protein encoded by the SIRT1 gene and expressed in most parts of the body. This protein is implicated in different neurodegenerative, metabolic, cardiovascular, and age-related diseases. SIRT1 activation can modulate downstream pathways including FoxOs, NF-κB, mammalian target of rapamycin (mTOR), poly adenosine diphosphate ribose polymerase-1 (PARP-1), peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1α), and Notch, which are involved in age-related mitochondrial biogenesis, inflammation, and fibrogenesis [80]. SIRT1 also protects organisms from oxidative stress and cancer and promotes DNA stability. SIRT1 is reported to be involved in the regulation of cellular senescence by delaying senescence and extending an organism’s lifespan [81]. Therefore, levels of SIRT1 are decreased in senescent cells, leading to the increase in DNA damage and aging phenotypes [82].

Monocyte chemoattractant protein-1 (MCP-1) or CCL2, a cytokine produced by a variety of immune cells in response to pro-inflammatory stimuli and oxidative stress, is another SASP factor. This cytokine is thought to be an intervention point for different autoimmune diseases [83]. CCL2 binds to CCR2 receptors and can activate PI3K, AKT, NF-κB, Ras, as well as other signaling pathways [84]. Different senescent cells were shown to strongly secrete CCL2 [85], and this cytokine has been implicated in immune homeostasis of aging tissues [86]. Serum levels of CCL2 was determined to be positively correlated to an individual’s age [87]. Mouse models overexpressing CCL2 were found to age quicker and experience premature death [88]. Another study determined that increases in CCL2 levels were also associated with a decrease in memory function in older adults [89]. Due to CCL2’s potential to measure mammalian biological age, it can be used for the identification of the risk of age-related morbidities and early-onset frailty [90].

2.1.6. Structural Change-Related Markers

Senescent cells are known for their morphological differences in comparison to healthy cells. Senescent cells appear much larger, flattened, and contain multiple nucleuses, vacuoles [91], and lysosomes that exhibit progressively deteriorated function [92]. There have also been some reports linking the morphological enlargement of the nucleolus to cellular senescence [93], as well as abnormalities in the morphology of their mitochondria [94].

As previously mentioned, senescent cells undergo structural changes that make them distinct from other cells. Major alterations take place in the nucleus, and in senescent cells, chromatin rearranges into dense structures, known as senescence-associated heterochromatin foci (SAHF) [10]. It is believed that SAHFs contribute to senescence’s irreversible cell cycle arrest [95] by using chromatin reorganization as an attempt at silencing genes that promote proliferation. These heterochromatin foci were discovered to target and silence genes that are required for cellular progression into the S-phase of the cell cycle, such as cyclin A [96]. It is also suggested that SAHFs prevent apoptosis of senescent cells by limiting the extent of DNA damage signaling [96]. Different inducers of senescence can lead to the formation of SAHF, but the presence of SAHF does not always occur during senescence [96]. Heterochromatin markers present in SAHF include HIRA, H3K9Me2, H3K9Me3, HP-1γ, γH2AX, and macroH2A, among a few others [10]. As previously mentioned, γH2AX is commonly used in assays to detect senescence and plays a significant role in the DNA damage pathway.

Another hallmark of senescence related to the structural changes of the nucleus is the downregulation of lamin-B1 (encoded by the LMNB1 gene). This filament protein con-
tributes to the structural component of the nuclear lamina, and with senescence, structural integrity of the nucleus is lost. As a result, many cytoplasmic chromatinis released from the nucleus, which fuels SASP [10]. Lamin-B1 plays roles in DNA replication, cell proliferation, aging, oxidative stress response, and gene transcription [97]. Mutations or decreases in lamin-B1 lead to diseases known as laminopathies, premature aging being among them [98]. It is believed that a decrease in lamin-B1 is brought on by the activation of the pRb or p53 pathways and occurs in vivo due to exposure to radiation [6]. However, it is still unknown whether lamin-B1 reduction causes or is a result of senescence [99].

The extracellular matrix (ECM) is a structure created from proteins with different functions. These proteins and components connect and organize into a stable structure that contributes to tissues’ mechanical properties [100]. Commonly known components of the ECM include collagen, elastin, and gelatin. Matrix metalloproteinases (MMPs) are enzymes involved in the control of extracellular matrix remodeling. MMPs are excreted from different pro-inflammatory cells and connective tissues and are regulated by growth factors, hormones, and cytokines [101]. There are twenty-three different types of MMPs that can be categorized into five subgroups based on structure and specificity [102]. MMP-1, MMP-3, and MMP-9 expression is most studied in the context of cellular senescence; however, many other MMPs are also related to aging. Many MMPs are induced due to skin cell exposure to radiation and are linked with dermal collagen fragmentation [103]. For example, in addition to the three MMPs mentioned, MMP-13, MMP-2, MMP-10, MMP-7, and MMP-12 become upregulated with UV-A and UV-B cell exposure [104]. An increase in the expression of these MMPs can lead to the degradation of the ECM and thus the initiation of different diseases, like cancer and aging.

2.2. Senescence Detection Methods

Common detection methods for senescence include the use of light microscopy, flow cytometry, immunofluorescence, Western blotting, polymerase chain reaction (PCR), immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), enzymatic staining, histochemistry, etc. [11]. Many of these assays are based off more than one technique for analysis but will only be discussed under one section for the flow of information.

2.2.1. Colorimetric Assays

There are different biomarkers that can be used to observe hallmarks of senescence using cytochemistry and histochemistry. Those mentioned in this section are among the most popular for colorimetric assays.

Chromogenic substrates of β-galactosidase can be used to identify senescent cells in vitro and in vivo due to the production of a colored dye that appears once it is cleaved by β-galactosidase. X-gal, one of the chromogenic substrates of β-galactosidase, is commonly used in assays to identify senescence in vitro and in vivo. Once cleaved, X-gal produces an insoluble blueish green dye. It was found that the activity of β-galactosidase correlates with chronological aging, and therefore, staining intensity increases with age [105]. There are a few complications that occur with the use of SA-β-Gal staining that can influence the result of the experiment. For example, slight differences in the pH conditions of the stain can result in false negatives or false positives depending on whether the pH is higher or lower than 6.0. In addition, when dealing with cells, confluence of senescent cells can increase staining, whereas cells maintained at a low density tend to have less frequent SA-β-Gal staining [106].

β-galactosidase is commonly used in assays to identify senescence in vitro and in vivo. However, staining this enzyme’s byproduct is not possible with archival tissues. Sudan Black B serves as an alternative that works with archival tissues. Sudan Black B stains lipofuscin and has been proven to be negative in β-galactosidase-negative tissues and positive in β-galactosidase-positive tissues [107]. Lipofuscin is made up of pigmented granules composed of lipids and is the byproduct from failed catabolism in lysosomes of aging post-mitotic cells [108]. Once stained with Sudan Black B, lipofuscin produces a
black/purple dye. Lipofuscin itself is also auto-fluorescent and can be visualized using fluorescent microscopy [24]. It is agreed that the presence of lipofuscin correlates with chronological aging, and therefore, staining increases with age [105].

2.2.2. Fluorometric Assays

Reactive oxygen species are commonly assessed using small-molecule fluorescent probes [109]. A common probe for ROS analysis is 2′,7′-dichlorodihydrofluorescein (DCFH), which is oxidized to a fluorescent product by unspecific ROS. Another probe commonly used is dihydorhodamine (DHR) 123, which detects oxidases in mitochondria. Detection of ROS through these probes is limited to the use of in vitro methods, as in vivo measurement of ROS and oxidative damage in vivo is challenging, but not impossible. In vitro quantification of ROS through these methods is much easier, as these probes are added to cell cultures and incubated for a set period before being read in a fluorescent plate reader or imaged by fluorescent microscopy. However, they do have some limitations, such as selectivity and sensitivity to pH and O2 levels [109]. As a result, newer fluorescent probes based on specific detection of hydrogen peroxide, superoxide anion radical, and the hydroxyl radical have been created [110].

A fluorescent alternative to the SA-β-galactosidase assay is another option that can be used to detect senescence in vitro. The premise of this assay is very similar to that of the colorimetric SA-β-galactosidase assay. In this case, β-galactosidase cleaves a fluorescent substrate for β-galactoside. The use of a fluorescent probe allows for an easier form of quantification for senescence using a fluorescent plate reader. The cells can also be imaged by fluorescent microscopy for visualization of fluorescent intensity. Assays using fluorescent substrates as opposed to the colorimetric substrates do not fall into the same issues with human error when it comes to the quantification of stained cells [111]. However, these assays do present similar limitations as the colorimetric counterpart in terms of pH sensitivity and false negatives and positives stemming from cellular confluence.

Although not specific to senescence biomarkers, there are also different fluorescence-based assays that can be used for the visualization of different organelles within cells. Different fluorescent stains are created for individual organelles which can be visualized with fluorescent microscopy [112]. Many of these stains work with live cell imaging, while others require fixed cells. Lysosomes, mitochondrion, and nucleoli are among those organelles that staining can give insight into morphological changes for [113–115]. Although that does not provide a direct link to senescence, it can provide a means for comparison between different samples.

Flow cytometry is another method that can be used for the detection of senescent cells. Flow cytometry uses scattered fluorescent light as signals that are converted and analyzed by a computer. Different fluorescent reagents can be used for flow cytometry that identify biomarkers such as antibodies, proteins, DNA, ions, etc. [116]. Since flow cytometry is not limited to the type of biomarker it identifies, different targets related to senescence can be analyzed. SA-β-galactosidase is one of the markers that can be used with flow cytometry in cellular samples [117]. Other commonly used senescence-related markers studied with flow cytometry include antibodies for p16\(^{INK4a}\) and γH2AX [118]. Essentially, any other biomarker of senescence that has commercially available antibodies can be studied using this method. Oftentimes, these antibody markers of senescence are paired with fluorogenic substrates of SA- β-galactosidase. Flow cytometry also allows for the analysis of cell size, which with senescence, is increased greatly in comparison to normal cells. There is not a lot of in vivo evidence of this increase in cell size, so it is limited to in vitro studies [10].

2.2.3. Immunoassays

Detecting and measuring specific proteins using antigens and antibodies is perhaps the most widely used procedure for identifying and studying senescent cells. Common immunoassays used include ELISA, Western blots, immunohistochemistry, immunocytochemistry, and bromodeoxyuridine (BrdU) assays.
ELISA assays are extensively used to detect antibodies, and there are four major types of ELISA that can be used depending on plate coating and what is being screened. Data that are gathered by this assay can be quantitative, semiquantitative, or qualitative; qualitative results confirm or deny the presence of an antigen/antibody in a sample, whereas quantitative results provide intensity signals to identify concentrations of antigens/antibodies [119]. There are different potential pitfalls with using ELISA that include issues with manufacturing, such as uneven plate coating and substrate quality. Other issues can arise due to human error with contamination of buffers, incorrect volumes, durations, temperatures, and pH of reagents [119]. This assay is also considered one of the more expensive options for immunoassays. ELISA can be used for aging biomarkers, and there are currently a few commercially available ELISA kits for senescence that are precoated with antibodies specific to LIMS1 [120]. SASP factors such as IL-6, IL-8, MCP1, etc., have also successfully been analyzed using ELISA [121].

Other immunoassays that also double as fluorescence-based approaches, also known as immunofluorescence, for the detection of senescence are immunohistochemistry and immunocytochemistry. These two techniques are like each other but differ in what is being stained. Immunohistochemistry uses tissue samples, whereas immunocytochemistry uses cells for imaging. In both techniques, antibodies for senescence biomarkers can be stained to identify locations in which SASP and other proteins, cytokines, etc., are present in samples. Common markers associated with senescence that are used in tissue samples include γH2AX, p16, p15, p53, p21, IL-1β, IL-6, MMP3, and HMGB1 [122].

BrdU is a cell proliferation assay that is used to label cells that are undergoing DNA replication. BrdU is a synthetic nucleoside analog of thymidine that is incorporated in proliferating cells in place of thymidine [123]. This chemical is detected by immunostaining either cells or tissues with anti-BrdU antibodies that are either conjugated with a fluorochrome or other detection method. Since cell proliferation is analyzed in this assay, it is expected that no signal would be visible in samples with senescent cells. Senescent cells no longer have the capability to proliferate, and therefore, the BrdU would not be incorporated into the DNA. There are a lot of limitations to this assay, which stem from its technical difficulty, lack of compatibility with other assays, and the potential in damaging the sample if using tissues [124]. Because of its difficulty, an alternative that uses 5-ethynyl-2′-deoxyuridine (EdU) and applies click chemistry was created [124].

One of the most popular immunoassays is Western blotting. This technique utilizes gel electrophoresis to separate proteins based on molecular weight and uses antibodies to identify proteins [125]. Western blotting can be used with both cells and tissues. Just like all other assays that utilize antibodies, this assay can be used to identify many biomarkers of senescence. There are currently commercially available kits that contain antibodies for specific biomarkers of senescence that can be used for Western blotting and immunofluorescence alike. Since senescence is still not completely understood, many studies are continuing to be performed with a variety of different antibodies to understand what proteins are involved in the process. Essentially, a lot of studies using Western blotting aim at identifying and validating proteins that could be potential markers of senescence [126].

2.2.4. Gene-Based Assays

PCR is a gene-based technique that amplifies a short sequence of DNA present in any biological sample into a larger and more easily detectable number of copies. There are different variations of PCRs which differ based on quantification type. In all PCR variations, fluorescent DNA binding dye is added to samples and emits fluorescence once bound to double-stranded DNA [127]. Simple PCR is mainly used for the detection of genes based on the application of gel electrophoresis. In this assay, DNA products are separated based on size and charge [128]. A more advanced method of PCR includes reverse transcriptase PCR (RT-PCR), which allows for the detection and quantification of the PCR product [128]. This assay can be used to investigate gene expression changes with cell differentiation, drug exposure, and environmental changes [127]. Therefore, genes that are implicated in cellular
senescence can be used with this technique. The main drawback of this method regarding senescence detection is that genes must be decided on manually. Therefore, samples must be validated as senescent, and then genes of interest can be analyzed to identify whether they are involved in the phenomenon or not. Otherwise, samples unknown to be senescent or not are commonly tested using a few genes considered to be senescence biomarkers, such as those coding for p16, p21, IL-6, IL-8, lamin-B1, etc.

Alternative methods to PCR that test for a wide variety of genes and do not require manually selecting them include RNA sequencing and DNA microarray. In DNA microarrays, a solid surface or chip contains a collection of microscopic DNA spots. The genes that are located on the chips are predetermined per the manufacturer and there are different chips for different organisms. Usually, microarrays contain thousands of genes per chip. Currently, there are no gene chip options created with the specificity of testing senescence-associated genes. However, unlike with PCR, microarrays allow researchers to detect changes in thousands of genes for one sample. This can aid in identifying new genes that may be involved in senescence. RNA sequencing is very similar to microarrays in that a lot of genes are being tested. However, with RNA sequencing, there is no limit to the number of genes in the output. Whole cells, whole tissues, etc., are some of the many options of samples that can be used for sequencing after RNA extraction. Therefore, RNA sequencing may be one of the best options for understanding senescence. However, with such a large output of data, data analysis will be time-consuming.

3. Zebrafish as an Aging Model

3.1. Benefits of Using Zebrafish

Cellular senescence is a phenomenon of irreversible proliferative arrest [9]. These cells remain metabolically active and resist apoptosis, and therefore have the potential to result in/exacerbate many age-related diseases. Understanding the underlying genetic role of the phenomenon both from intrinsic and extrinsic aging perspectives is difficult and time-consuming in typical in vivo rodent models. Therefore, in vitro cell models have commonly been used to study senescence and aging [11]. Yet, there are obvious disadvantages to using in vitro approaches. Some of these shortcomings include not being able to mimic the complexity of organ systems and the body, as well as the need to validate results using an in vivo approach post-in vitro studies [129].

Common in vivo approaches rely on rodent models to understand aging. As previously mentioned, these approaches are rather time-consuming and difficult. Just like with in vitro approaches, there are different ways to induce senescence in vivo. Typical aged rodent models include mice or rats chronologically aged, exogenous exposures to xenobiotics, and knockout models. Lab rodents have a typical lifespan of about two-and-a-half years. To use a rodent that is considered “old”, researchers need to wait at least eighteen months after the rodent is born to run experiments. Not only is this time-consuming, but there is an added expense just in the maintenance of the rodents until their use. Laboratory rodents also have a typical litter size of about six to eight pups and the average gestation time takes about nineteen to twenty-one days [130]. Therefore, there is a small turnover in breeding, which can become an issue with genetically modified rodents. To generate a knockout model, at least three generations of rodents need to be bred to produce a homozygous genotype. Multiple groups of rodents would also need to be genetically modified to produce enough rodents homozygous for the gene knockout. This itself is also very time-consuming and can come with a few difficulties.

An animal model that is gaining popularity in the scientific community is zebrafish. One of the main benefits of using zebrafish as an animal model is their rapid development and generation times [131]. A pair of zebrafish is capable of producing over one hundred embryos per clutch [132]. Embryos are developed externally and are produced quickly at onset of light if a male and female are placed in a tank together the evening before [133]. The same fish can be bred multiple times a month, but it is recommended not to breed more than once a week [134]. Zebrafish larvae hatch within forty-eight hours post-fertilization and
can be used for experiments as early as five days post-fertilization due to the development and functioning of all their major organ systems [135]. These fish also reach sexual maturity within three months and are considered adults at that time [136].

Another major advantage of zebrafish over other animal models is their affordability. For example, the cost of zebrafish maintenance is less than 1/1000 the cost of a similar mouse study [137]. As of 2017 at Boston University, a single mouse costed USD 1.05 a day just for housing, whereas an entire tank of zebrafish costed USD 0.25 [138]. Many zebrafish can be housed in a single tank depending on the tank size, which allows for the maintenance of a large quantity of fish in a limited space. Management is relatively easy; however, these fish require a specific water temperature, pH, and conductivity to ensure survivability [139]. Factors such as water quality, feeding, and fish housing density can influence their rate of development [136].

There are several different strains of zebrafish that are used in research. Some of the more popular include AB, Casper, Tubingen, Ekkwill, and absolute. Different strains of zebrafish differ not only genetically, but also morphologically and behaviorally [140]. For example, strains like Caspers and absolutes are transparent in adulthood and allow for research dealing with visualization of organs or tumors that may not be possible with other models. Studies have even revealed that there are differences in shoaling tendency and motion between strains [141]. As a result, experiments should be designed with strain-specific qualities in mind due to the better fit of some strains over others in result reproducibility and morphological applicability.

The entire genome of zebrafish has been sequenced, which has provided researchers with the ability to create mutations in more than 10,000 of their genes [142]. From these data, scientists were also able to determine that zebrafish share around 70% of the same genes as humans, and that 84% of genes known to be associated with human disease can also be found in zebrafish. With the availability of this type of data, genetically modified zebrafish have been established by the knockout or knock-in of specific genes of interest. These data also make it easy to create random mutations in their genome in order to link these mutations to a physical or biochemical change [143].

In addition, many major tissues and organ systems of humans are also found in these fish, including blood vessels, a heart, kidneys, liver, spinal cord, ears, teeth, etc. [142]. The zebrafish immune system is also like that of humans, including innate and adaptive immune cells, which allows researchers to create models that use pathogens that commonly affect the human body [144]. The neuroanatomic and neurochemical pathways of the zebrafish brain also resemble those of humans, allowing them to serve as appropriate models for Alzheimer’s disease [145]. However, the zebrafish respiratory system and reproductive system differ from those of humans, making them unsuitable for the study of diseases linked to these organ systems in humans. Yet, due to the genetic similarities between zebrafish and humans, zebrafish can serve as useful models for the understanding of various human diseases, aging included.

Many diseases have been successfully modeled in zebrafish, including different metabolic diseases, cancers, genetic diseases, renal diseases, and behavior-related diseases, among others. These diseases in zebrafish have been determined to be like those found in humans. For example, researchers have been able to clone a mutation in the vHnf1 gene implicated in human polycystic kidney disease [146]. Researchers have also been able to isolate and clone the homolog for Huntington’s disease [147]. Tau transgenic zebrafish models for Alzheimer’s disease successfully present neurofibrillary tangles, neuronal loss, and cell death similar to the expected human pathology [145]. As well, chemically induced models of Alzheimer’s disease symptoms exist using zebrafish [148,149]. Zebrafish embryos of all strains are also transparent, which helps in studying the developmental process from fertilization [142].

Zebrafish models are also beneficial for drug-related testing. Zebrafish larvae as young as five days post-fertilization can be used to study drug-related toxicities and behavioral changes. Due to the fast turnover of zebrafish eggs and the age at which zebrafish can be
used in drug discovery, these animals can be used in a high-throughput *in vivo* alternative for screening drug candidates. Also, due to being aquatic animals, water-soluble drugs can easily be administered for testing, usually in 96-well format. In fact, many drugs have already been tested using zebrafish models of different diseases. For example, drugs have been tested for alleviating symptoms of Alzheimer’s disease [149,150]. The promise of drugs can be shown due to their effects on multiple symptoms and the neuropathology of the disease when tested in zebrafish.

Zebrafish have only recently been explored in the study of aging. These studies are in their beginning stages, as the topic of aging in zebrafish has started to gain attention. Current zebrafish aging studies focus on cognitive and neurobiological changes that occur with age or use mutant models for comparison with wild-type fish. Just like with humans, zebrafish age gradually, but they can also be manipulated to present age-related characteristics like those of older humans. Zebrafish therefore exhibit a promising model and tool for the understanding of age-related processes and mechanisms.

### 3.2. Types of Zebrafish Aging Models

Just like rodent models of aging, zebrafish also can be chronologically aged, exposed to exogenous xenobiotics, and genetically modified to create comparable representations of human aging. Manipulating environmental factors and other genetic tools can easily be utilized to intervene in zebrafish aging processes. With the improvement of molecular techniques with time, using zebrafish as aging models will continue to provide important insight into the process.

#### 3.2.1. Chronological Aging Models

Zebrafish have a typical lifespan of about three to three-and-a-half years but can live up to five years under some laboratory conditions, where mice typically live two to three years. This puts zebrafish on a par with mice regarding lifespan. To create a chronological model of senescence in zebrafish, simply, the fish should just be allowed to grow older with time. In chronological zebrafish models of aging, degenerative changes take place, such as reproductive and regenerative decline, circadian rhythm, sleep, and cognitive function alterations, as well as physical variations [151]. A study aimed at outbreeding zebrafish to identify age-related phenotypes found that the skeletal length of zebrafish increased with age, suggesting indeterminate growth. Although this is not like human aging, a common age-related phenotype these researchers noted was spinal curvature. Further analysis indicated that the result of their curvature was most likely due to muscle abnormalities rather than bone-related changes [152].

Another study was based on staining whole bodies of nine- to thirty-one-month-old zebrafish with SA-β-Gal. The stained fish in the other age groups displayed variable staining, whereas the nine-month-old fish had faint background staining. Upon dissection, the researchers confirmed that staining primarily occurs in the dermis of the thirty-one-month-old fish, whereas no dermis staining was observed in the white parts of seventeen-month-old fish. Immunoblotting of groups of fish between three months and twenty-four months old using anti-DNP indicated that higher levels of oxidized proteins were found in muscle tissue samples of fish older than a year old. Histopathological analysis of these fish found that wasting and spinal curvature increases in fish older than twenty-four months. Other age-dependent changes also found include mucous cell hyperplasia, hepatic necrosis, glomerular tufts in kidneys, and increased melanin deposits in the kidney [153].

A hallmark of human aging is changes in sleep patterns. Like humans, zebrafish also exhibit age-related circadian alterations. A study found that by the age of three years old, zebrafish have desynchronized, reduced-amplitude, and fragmented circadian rhythms in the absence of time cues. Older zebrafish also have a higher arousal threshold, reduced nighttime sleep, and lower activity levels compared to younger fish. Circadian genes such as Bmal1 and Per1 in aged zebrafish were found to be altered, along with progressive
declines in melatonin hormones despite unaltered melatonin receptor expression. This study suggests that the genes identified may play an important role in aging [154].

Another characteristic of aging is the decreased ability to maintain homeostasis in the case of external stresses. Heat shock proteins are highly conserved proteins that have been linked to the modulation of bodily stresses. Expression of these proteins increases as a response to things such as heat, heavy metals, ROS, etc., and is diminished with age [155]. In a study evaluating the effect of aging on heat shock response in three-month-old and two-year-old zebrafish, researchers found that heat shock protein 1α levels were increased and that basal levels of heat shock protein 70 were decreased in older fish compared to younger fish. These results demonstrate that there are age-related differences in heat shock response and that these data can lead to further studies necessary for understanding the mechanisms of age-related stress response [156].

The deterioration of cognitive functions is associated with increasing age. A research study aimed at characterizing cognitive aging in one-year-old, two-year-old, and three-year-old zebrafish used a variety of locomotor tests to assess the fish’s adaptational ability when exposed to spatial, temporal, and visual cues. It was determined that on average, fish in the two older groups traveled shorter distances and at slower speeds when compared to the one-year-old group. It was also established that the one-year-old fish were quicker than the other groups in showing anticipatory behavior around the time of feeding through the increase in locomotor activity. Older fish also took longer to develop a conditioned response when researchers used a positive reinforcement stimulus—with some fish never developing the response during the study, whereas this same behavior was not observed or associated with negative reinforcement stimuli. It is important to note that in the older age groups of fish, there were individuals that were still capable of performing like the one-year-old fish. Overall, these results suggest that cognitive changes are present in aging zebrafish and that using this model can help scientists understand age-related cognitive changes [157].

In one study, researchers aimed at investigating the relationship between learning deficits during aging and oxidative stress. Zebrafish of different ages were studied using behavioral tests that help identify spatial learning abilities. After these tests, zebrafish brain regions relevant to learning were tested to determine levels of lipofuscin and oxidized lipids and proteins. Based on the results, cognitive abilities in older zebrafish were significantly impaired and levels of lipofuscins and oxidized proteins were significantly increased. This indicates that oxidative stress may contribute to cognitive impairments [158].

3.2.2. Genetically Modified Models

Genetically modified zebrafish are gaining attraction due to their ease of genetic manipulation and relatively short generation times [159]. Creating transgenic zebrafish models can aid in studying the effect of different genes on the process of aging and the lifespan overall.

Aging is the extension of biological development, and the findings of some researchers have shown that a senescence phenotype during embryogenesis in zebrafish is associated with cellular senescence and aging in other organisms [160]. These same researchers also created a pool of three hundred and six mutant zebrafish created by insertional mutagenesis. These mutant genomes were then screened for altered expression of SA-β-gal and filtered into eleven candidates. Results indicated that some of the genes with the most SA-β-gal activity were TERFa, SPINS, NRS, and PSM [161].

Telomere biology has been gaining a lot of interest in the context of aging. There have been studies that focus on the aging effect of telomerase-deficient zebrafish. One such study of tert−/− zebrafish noted decreased fertility, premature aging, and a shorter lifespan for first-generation mutant fish. The second generation of these fish showed higher levels of embryonic lethality, abnormal phenotypes, and could not be successfully bred. For example, telomerase-deficient zebrafish started to show signs of spinal curvature by five months of age, and histopathological examination of their livers indicated the
accumulation of lipofuscin. These mutant fish also expressed significantly higher levels of p53 compared to \textit{tert}+/+ fish, indicating that p53 activation is responsible for developmental malformations and mortality in the telomerase-deficient zebrafish [162]. Another study using the same model of fish found that disabling telomerase in zebrafish dropped life expectancy by 70%. These same fish showed drastic changes in their gastrointestinal tracts, and by inducing tissue-specific telomerase expression in their gut, tissue integrity was restored. This telomerase expression also rescued gut senescence and increased fish lifespan by 40% [163].

Another transgenic zebrafish line which expresses Green Fluorescent Protein (GFP) under the promoter of p21, termed p21-GFP, was created as a model for rapid testing of senolytics—senescence-killing drugs [164]. These fish can be induced with oxidative stress, other senescence-initiating methods, or left to age naturally. Cells positive for p21 in the fish will fluoresce green, allowing for quick detection and selection of senolytics.

Recombination activating gene 1 (RAG1), a gene critical in adaptive immunity, has also been knocked out in zebrafish (\textit{rag1}−/−). Although this gene is related to immune function, researchers noticed that \textit{rag1}−/− fish exhibited several senescence- and age-related markers. These fish had a shorter lifespan driven by higher frequencies of cell cycle arrest, apoptosis, oxidative stress, and a decrease in DNA repair gene transcription. Reduced telomere length, upregulated SA-β-gal activity, and an increase in γH2AX were also found in \textit{rag1}−/− fish compared to wild-type fish, indicating that \textit{rag1}−/− fish can be used as a model for senescence. The use of senolytic drugs on these fish further confirmed and validated \textit{rag1}−/− zebrafish as a senescence model [165].

Knockout of SIRT1 in zebrafish (\textit{Sirt1}−/−) confirmed the relationship between SIRT1 and aging. Studies have shown that \textit{Sirt1}−/− zebrafish show no apparent phenotypical changes in early stages of development, but they were found to have intestinal atrophy and inflammatory cell infiltration in the spleen, intestines, and pancreas once older. These fish also experience chronic inflammation and an increase in pro-apoptotic events, leading to a shorter lifespan. \textit{Sirt1}−/− zebrafish have increased levels of ROS and other inflammatory markers, including TNF-α, IL-6, and IL-1β. Other age-associated markers, such as NF-κB and iNOS, were also upregulated, indicating that \textit{Sirt1}−/− zebrafish can be a useful tool in studying inflammation and aging [166].

Another knockout model used to study aging is the knockout for the oxidation resistance gene 1 (OXR1). This gene is important for oxidative stress protection. In \textit{oxr1}−/− zebrafish, oxidative stress in the fish led to increased mortality rates and the downregulation of antioxidant genes. \textit{Oxr1a}−/− zebrafish were also found to display a shortened lifespan, due to the activation of the p53 signaling pathway and an increase in pro-apoptotic events. In fact, mutant zebrafish death rapidly increased after ten months of age, with no fish surviving more than sixteen months. \textit{Oxr1a}−/− fish were also found to be much thinner than wild-type fish, and they became thinner with increasing age. Altogether, the findings of this research demonstrate that \textit{oxr1a}−/− is important for anti-aging and antioxidant defenses in fish [167].

The \textit{klotho} gene has been gaining attention due to its relationship to aging and a shortened lifespan. Knockout models of \textit{klotho} in rodent models have shown accelerated aging. Knockout models of the same gene in zebrafish were not studied until recently. Researchers found that \textit{klotho} knockout zebrafish appeared normal by three months of age, yet experienced premature death, only living up to nine months of age [168]. A similar study noted that these fish started to experience abrupt behavioral and degenerative physical changes by five months of age. Histopathological analysis of these fish showed necrosis in areas of the epidermis and dermis, calcification of blood vessels in skeletal muscles, fibrosis, immune cell infiltration, among other forms of calcification and inflammation [169]. These findings suggest that \textit{klotho} regulates lifespan in zebrafish and can be used to study the process of aging.
3.2.3. Oxidative Stress-Induced Senescence

Oxidative stress is believed to cause aging by damage of different proteins and lipids. In fact, inducing oxidative stress in zebrafish serves as a model for aging. The most common oxidative agent used to achieve ROS and by extension, senescence, in models is H$_2$O$_2$ [170]. Zebrafish exposed to these oxidants can then be genetically tested with assays such as PCR or tested using whole mount or single-cell ROS detection methods [170].

In one such study using H$_2$O$_2$ as an aging model, senescence-associated markers were tested after induction. It was found that after treatment, larval zebrafish showed obvious age-associated phenotypes. Researchers found that there was higher SA-β-gal activity along with an increased expression of SERPINE1 levels [171]. SERPINE1 has been identified as being directly involved in the mediation of cellular senescence [172] and its overexpression suggests the presence of senescent cells [173]. Oxidative stress-induced zebrafish also exhibited higher levels of inflammatory genes, such as IL-1β, IL-6, and IL-8, as well as downregulation of SIRT1 and TERT. These same effects were able to be delayed with pretreatment of fish with antioxidants [171].

The induction of oxidative stress is not limited to the use of H$_2$O$_2$. In a study using auranofin to induce oxidative stress in zebrafish embryos, researchers aimed to investigate the effects of oxidative stress during development. Zebrafish were exposed to low doses of this drug from six to twenty-four hours post-fertilization, and then they were raised to adulthood. It was determined that low doses of this oxidant caused upregulation of GSTP1 and PRDX1 genes. With increasing doses, there were developmental abnormalities, such as jaw malformation and cerebral hemorrhaging. When the fish reached adulthood, there were no physical differences, but they experienced lower fertility levels [174].

A different research group created oxidative damage-induced aging by exposing zebrafish embryos to H$_2$O$_2$, ethanol, and 6-hydroxydopamine (6-OHDA). Treatment with these oxidants caused morphological changes to the zebrafish. Some of the changes observed include liver bleeding, spinal curvature, pericardial edema, and heart bleeding. Stress-related genes were also found to be upregulated, including NRF2b, SOD1, and SOD2, and age-related genes MYL2a and SELENBP1 were also upregulated [175]. These findings suggest that oxidants play a role in both inflammation and aging.

3.2.4. Radiation-Induced Senescence

Zebrafish can be exposed to different forms of irradiation to produce DNA damage. Ultraviolet (UV) and gamma irradiation are easily accessible methods for radiation-induced senescence. Ultraviolet light irradiation causes DNA damage and is one of the common causes of skin aging in humans. Therefore, a lot of studies have focused on the effects of UV exposure on DNA damage. This model has also been used to study protective effects of drugs and other chemicals against UVB-induced ROS and cell death [176,177].

Zebrafish are an important model for studying the effects of UV light on repair processes activated to manage DNA damage. In one study, researchers were interested in understanding the effect of UV irradiation in six-day-old and six-week-old zebrafish, as well as embryos. It was determined that the tolerance of six-day-old fish was less than embryos to the same UV dose, possibly due to developmental UV tolerance. At sublethal doses of UVB, zebrafish embryos showed signs of cell death six hours post-exposure. Doses even higher resulted in morphological changes and death. In six-week-old UV-treated fish, γH2AX was detected in the skin and fin tissues, indicating DNA damage. Similar studies on p53 mutant zebrafish indicated that this response was dependent on the p53 pathway [178].

UV irradiation of zebrafish embryos was carried out in another lab to identify biological responses and a novel biomarker for UVB exposure-related biology. Irradiation of zebrafish embryos three days post-fertilization resulted in the activation of the p53 signaling pathway, the release of ROS, and an inflammatory response. Microarray analysis indicated that MMP13a and MMP9 were the most upregulated genes, whereas MMP1-3 was the most downregulated. Gene expression was also found to change post-UVB irra-
Radiation. For example, significant elevation of apoptosis markers occurred around nine hours post-irradiation, whereas MMP gene expression was activated around twenty-four hours post-irradiation. It was also noted that JUNBB gene expression was activated three hours post-irradiation, indicating that this gene may serve as a more sensitive and novel biomarker for UVB response [179].

Another study looking into the inflammatory effects of UV radiation on zebrafish embryos found that upregulation of proinflammatory cytokines was in place to counteract the effect of high UV doses. Twenty-four hours post-fertilization, embryos were exposed to increasing doses of UV. The use of a transgenic NF-κB model validated that exposure to UV leads to the activation of the NF-κB pathway. Transcripts for IL-8, TNF-α, and IL-1β were also found to be upregulated, indicating immune activation post-irradiation. IL-1β was discovered to play a major role in the reparative process by regulating recovery though the induction of various growth factors [180].

Combinations of UVB and UVA were irradiated on zebrafish embryos in a study trying to understand the consequences of UV exposure for the mortality, hatch rate, and malformation of these fish. Zebrafish exposed to increasing doses of UVB were found to have minor or severe spinal deformities and enlarged pericardial sacs, whereas those exposed to UVA had somewhat similar effects but to a lesser degree. This indicates that toxicities are greater for UVB than UVA exposure. Embryos that were exposed to both UVB and UVA showed similar morphology to control groups [181]. This may suggest that zebrafish have a photo repair system, and it is important to take note of it when conducting experiments aimed at understanding the effects of UV exposure on age-related markers.

4. Conclusions

Aging is an inevitable process characterized by the accumulation of bodily changes and the emergence of diseases. The phenomenon of aging is not entirely understood and remains one of the more popular topics of scientific research in recent times. Studies looking to understand aging have started to focus on the cellular aging process, called senescence, in which cells enter a permanent state of cycle arrest. Although this process is acutely beneficial in cases of DNA damage, chronic senescence can cause detrimental effects.

Cellular senescence is oftentimes associated with an increase in inflammation, DNA damage, organelle dysfunction, among other hallmarks of aging. Whether due to external or internal stressors, this process afflicts every living being. Aging can be linked with many diseases, such as Alzheimer’s, osteoarthritis, and cardiovascular disease, among others. There is evidence pointing to the fact that senescent cells are accumulated in aging tissues, which causes dysfunction in physiological processes necessary for the maintenance of health.

A universal marker of senescence has not been found and may never be found. The process of senescence has varying phenotypes and comprises many different markers involved in a wide variety of physiological processes, such as inflammation and cancer. In addition, in vitro markers linked to cellular senescence have not been characterized in vivo at the cellular level [1]. Research on cellular senescence, although rapidly expanding, is still considered to be in its infancy.

Most of the information known to the public regarding senescence is due to in vitro research approaches. Animal and clinical research on cellular senescence has now expanded to understand how these cellular features affect whole-body systems. Although typical in vivo research models to study this phenomenon rely on rodent models, expanding studies to other animals such as zebrafish can aid in advancing age-related research-based knowledge.

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