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Cellular Senescence in Health, Disease and Aging

Blessing or Curse?

Edited by

Markus Riessland

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Editor

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Cover image courtesy of Markus Riessland

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About the Editor

Riessland, Markus is a trained molecular biologist with a background in neuroscience, human genetics and neurodegenerative diseases. Dr. Riessland received his PhD from the Clinic of the University of Cologne, Institute for Human Genetics, Germany. Early in his career, Dr. Riessland was involved in several internationally funded projects, where he performed and published studies on epigenetic modifiers as a potential therapy for the neurodegenerative disease spinal muscular atrophy (SMA). His research is particularly focused on the identification and characterization of neuron-specific disease-modifying factors that may facilitate the development of novel therapeutic strategies for degenerative disorders of the central nervous system. Dr. Riessland focuses on the understanding of cellular senescence. Cellular senescence is a common biological process in which mitotic cells may shut down the cell cycle when they recognize they have suffered DNA damage during division. This process causes a generation of "undead cells" (also known as "Zombie Cells"). This helps to prevent damaged cells from growing uncontrollably and causing problems such as cancer. Undead cells are, in fact, common and they are found all over the body. However, senescence is not typically seen in the nerve cells of the brain. Unlike most other cells in the body, neurons stop dividing once they are fully formed. In the lab of Nobel Laureate Paul Greengard at Rockefeller University, Dr. Riessland discovered that, surprisingly, post-mitotic dopaminergic neurons—which regulate motivation, memory, and movement by producing the chemical messenger dopamine—can nevertheless become senescent. This finding could have widespread implications for the understanding of many age-related neurodegenerative disorders (e.g., Parkinson's disease) and the aging process itself. Currently, his lab in the Center for Nervous System Disorders at the Department of Neurobiology and Behavior at Stony Brook University uses stem cell-based approaches as well as mouse models and next generation sequencing techniques (TRAP-seq, RNA-seq, ATAC-seq, scRNA-seq, etc.) to tackle the questions where and how cellular senescence in the brain could occur and spread, which cell types are involved and what the molecular triggers are. Additionally, research in the lab focuses on the identification and molecular characterization of genetic modifiers that influence the vulnerability of neuronal subtypes. The knowledge of molecular modifiers helps us to understand the underlying reasons of vulnerability that could be leveraged to protect cells from neurodegeneration. Moreover, the lab's research aims to interfere with the aging process by ameliorating the unwanted negative effects of cellular senescence.

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Editorial

Cellular Senescence in Health, Disease and Aging: Blessing or Curse?

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Sixty years ago (1961), Hayflick and Moorhead reported that primary cells terminate their growth and stop dividing after ~50 passages or one year in culture. This seminal study described the phenomenon that we now refer to as "cellular senescence" [1]. More specifically, the description by Hayflick and Moorhead unraveled "replicative senescence", which is caused by cell-division-dependent telomere attrition. Since then, increasing numbers of additional senescence-inducing factors have been identified. In parallel, a plethora of cell types have been recognized to possess the ability to enter a state of cellular senescence. These studies revealed diverse senescence-related cellular phenotypes and identified various metabolic changes, gene-activity alterations and other molecular markers [2–4]. Although some gene expression changes are characteristic hallmarks of cellular senescence, a single molecular marker has not been identified. Accordingly, the univocal identification of a senescent cell remains challenging. To address this problem, the International Cell Senescence Association (ICSA) assembled a list of key features observed in senescent cells [2].

A particularly interesting feature of senescent cells is the so-called senescence-associated secretory phenotype (SASP), which remodels the gene epression profile of a senescent cell causing the secretion of proinflammatory molecules to signal to the immune system "come here and remove me". During development, and in organisms with fully functional immune systems, senescent cells are usually detected and cleared from the tissue [5]. In case where immune cells do not remove the senescent cells, they remain in the tissue and continue to express the SASP. In turn, this would cause a damaging local inflammation and could also induce remodeling of the surrounding tissue as well as the spreading of senescence. Aged organisms possess a significantly reduced regenerative potential and immune function resulting in the accumulation of senescent cells [5]. Interestingly, this accumulation has also been observed in age-related disorders, neurodegenerative diseases, cardiovascular diseases, and others [6,7]. Because of its detrimental effect on the surrounding tissue, the accumulation of senescent cells is not just a consequence, but can instead be understood as a major driver of aging. Accordingly, recent studies described that the removal of senescent cells showed beneficial effects on healthspan and lifespan [8]. This exciting research led to the discovery of "senolytics", drugs which can kill senescent cells. Moreover, because of the heterogeneity of cell types that show senescence-like phenotypes, including cardiovascular cells and post-mitotic neuronal cells [6,9,10], further research is required to unravel the molecular background that renders a cell type vulnerable to senescence and to determine the pathways that induce senescence in a cell type-specific manner.

Given that there are many open questions in the field, this Special Issue of *Life* was created to shed light on the molecular pathways of cellular senescence, inflammaging, and the possible strategies to interfere with these processes. The work published in this Special Issue of *Life*, entitled "Cellular Senescence in Health, Disease and Aging: Blessing or Curse?", mirrors the broad interest in the field of cellular senescence since the presented

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studies highlight quite diverse aspects of senescence and related pathways from various areas of research.

The manuscript by Panchanathan et al. reports observations that identify the interferon inducible POP3 PYHIN protein as a potential negative regulator of the AIM2 inflammasome and SASP in senescent human prostate epithelial cells. This study provides insight into the age-related development of prostatic inflammatory diseases [11].

Senescence DNA damage foci (SDF) and telomere-dysfunction-induced foci (TIF) can be identified by the histone marker γH2AX for cellular senescence and DNA damage, respectively, which makes γH2AX a useful tool for the identification of these traits in diverse tissues [12]. In this Special Issue, Siddiqui and colleagues determine the feasibility of using γH2AX as a molecular biomarker of DNA damage in Alzheimer's disease (AD). The authors report a protocol that employs laser scanning cytometry (LSC) to measure endogenous γH2AX in buccal cell nuclei from mild cognitive impairment (MCI) patients, AD patients, and healthy controls [13].

Secreted protein acidic and rich in cysteine (SPARC), a molecule that has been described to be overexpressed in senescent cells [14], was the topic of an Opinion manuscript by Ghanemi et al. [15]. The authors emphasize that SPARC not only acts as a regeneration factor but also counteracts the aging-related decrease in regeneration ability, and thus can be seen as a potential factor for preventing age-related conditions.

$p16^{INK4A}$, which is often highly upregulated in many types of cellular senescence, acts as a tumor suppressor and is frequently reduced in human cancers. In this Special Issue, Leon et al. review the potential role of p16 in the regulation of immunological surveillance. In brief, the authors discuss the hypothesis that a p16-positive tumor would foster immunosurveillance by inviting immune cells into the tumor microenvironment, whereas a p16-null tumor would reduce immunosurveillance and promote tumor growth [16].

Finally, two reviews from the Orr lab highlight the importance of cellular senescence in the human brain. Gillispie et al. summarize the role of mitotic cells in brain senescence and discuss implications in neurodegenerative diseases and cancer [17]. The second manuscript reviews the recent discovery of post-mitotic senescence in the brain. In short, Sah et al. provide a comprehensive overview of the current knowledge of the cellular senescence of brain cells, including neurons [18]. Additionally, this manuscript gives an elegant introduction into the field of cellular senescence.

Generally, I hope that this Special Issue of *Life* will capture the attention of both specialists and non-specialists who are interested in understanding the molecular processes involved in cellular senescence and inflammaging. As seen in the diverse articles in this Special Issue, cellular senescence and the molecules that are crucial in its underlying pathways are of high interest in many areas of research. The rising interest in a more thorough understanding of cellular senescence is reflected by the fact that the National Institutes of Health (NIH) have recently established the Common Fund's Cellular Senescence Network (SenNet) Program to identify and characterize the differences in senescent cells within the body, across various states of human health, and throughout lifespan. It is an exciting time for researchers working on senescence and aging, and overall, there is great hope that the outcome of this research can translate into strategies that provide beneficial effects on healthspan and lifespan in humans.

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Article

Human Prostate Epithelial Cells Activate the AIM2 Inflammasome upon Cellular Senescence: Role of POP3 Protein in Aging-Related Prostatic Inflammation

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Abstract: Increased levels of type I (T1) interferon (IFN)-inducible POP3 protein in myeloid cells inhibit activation of the AIM2 inflammasome and production of IL-1β and IL-18 proinflammatory cytokines. The *AIM2* mRNA levels were significantly higher in benign prostate hyperplasia (BPH) than the normal prostate. Further, human normal prostate epithelial cells (PrECs), upon becoming senescent, activated an inflammasome. Because in aging related BPH senescent PrECs accumulate, we investigated the role of POP3 and AIM2 proteins in pre-senescent and senescent PrECs. Here we report that the basal levels of the POP3 mRNA and protein were lower in senescent (*versus* young or old) PrECs that exhibited activation of the T1 IFN response. Further, treatment of PrECs and a BPH cell line (BPH-1) that expresses the androgen receptor (AR) with the male sex hormone dihydrotestosterone (DHT) increased the basal levels of POP3 mRNA and protein, but not AIM2, and inhibited activation of the AIM2 inflammasome. Of interest, a stable knockdown of POP3 protein expression in the BPH-1 cell line increased cytosolic DNA-induced activation of AIM2 inflammasome. These observations suggest a potential role of POP3 protein in aging-related prostatic inflammation.

Keywords: prostate; senescence; inflammation; AIM2 inflammasome; POP3

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1. Introduction

Molecular mechanisms that contribute to the development of aging-related prostatic inflammatory diseases, including benign prostate hyperplasia (BPH), remain largely unknown [1–4]. Notably, studied using biopsies from patients with BPH and informative animal models have indicated a role for prostatic inflammation (PI) in the development of BPH [5–9].

Prostatic infections induce production of T1 interferons (IFN-α/β) through activation of the cGAS-STING pathway as a part of innate immune response [4,10,11]. The T1 IFNs, upon binding to a cell surface receptor, activate the JAK/STAT signaling in cells, resulting in stimulation of the expression of T1 IFN-inducible proteins [12]. The T1 IFN-inducible PYHIN protein family includes human IFI16 proteins, pyrin-only protein 3 (POP3), and AIM2 protein [13–15]. The proteins in the family share the *N*-terminal PYRIN domain (PYD) and the C-terminal HIN domain [13]. The PYD allows homotypic protein-protein interactions and the HIN domain allows sequence-independent binding to DNA [13,14]. The POP3 protein lacks the HIN domain [15].

We have reported earlier that treatment of human normal prostate epithelial cells (PrECs) and normal prostate stromal cells (PrSCs) with T1 IFN increased the levels of the androgen receptor (AR) and stimulated the transcription of AR-regulated gene [16]. Activation of AR in PrECs and PrSCs by the male sex hormone, dihydrotestosterone (DHT), regulates cell proliferation and survival [17,18]. Further, we noted earlier that activation of

the AR in human prostate cancer cell line PC-3 stimulated the expression of IFI16 PYHIN proteins [19]. Increased expression of the IFI16 proteins in human normal PrECs, PC-3 prostate cancer cell line, and human normal diploid fibroblasts (HDFs) associated with the onset of cellular senescence [20–23]. Of interest, AR also drives human PrECs to cellular senescence [24]. Although the senescent cells exit the cell cycle permanently and do not divide, these cells secrete proteases and proinflammatory cytokines (e.g., IL-6, IL-1β, and IL-18) [25,26]. This phenotype of senescent cells has been termed senescence-associated secretory phenotype (SASP) and the phenotype is thought to contribute to aging-related chronic inflammation [25,26]. Notably, senescent PrECs accumulate in BPH [27] and their SASP promotes BPH [28]. However, the molecular mechanisms that contribute to the development of SASP in the senescent PrECs remain unclear.

The AIM2 protein senses cytoplasmic dsDNA in a variety of cell types and recruits an adaptor protein ASC through its PYD to form the AIM2 inflammasome [13,14]. The activated AIM2 inflammasome through activation of caspase-1 protease proteolytically cleaves the gasdermin D protein, pro-IL-1β, and pro-IL-18 [29]. Activated gasdermin D induces cell death by pyroptosis [29]. Proteolytic cleavage of pro-IL-1β, and pro-IL-18 promotes the secretion of the mature IL-1β and IL-18 proinflammatory cytokines [13,14,29]. Notably, increased levels of the POP3 protein in macrophages bound with PYD of AIM2 protein and the binding diminished the ability of the AIM2 protein to bind with ASC adaptor protein and to form AIM2 inflammasome [15].

Given that human prostatic infections are associated with chronic inflammation [4,5], and the development of BPH is associated with an accumulation of senescent PrECs with SASP [27,28], we investigated the role of POP3 and AIM2 proteins in senescent PrECs. Here we report that levels of the POP3 protein decreased in senescent PrECs as compared with pre-senescent proliferating or old cells. Further, DHT-mediated activation of the AR in human PrECs and in a benign prostate hyperplasia (BPH) cell line (BPH-1) up-regulated the expression of POP3 protein and inhibited cytosolic DNA-induced activation of the AIM2 inflammasome. Further, a knockdown of POP3 protein expression in BPH-1 cells activated the activity of the AIM2 inflammasome. Our observations have important implications for the development of aging-related prostatic inflammatory diseases.

2. Materials and Methods

2.1. Reagents

Synthetic double-stranded DNA (Poly (dA:dT)) in complex with transfection reagent (LyoVec) and LyoVec were from InvivoGen (San Diego, CA, USA) and EDTA-free protease inhibitor cocktail was from Roche Applied Science (Indianapolis, IN, USA). Dihydrotestosterone (DHT) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and a stock (100 mM) was prepared in 100% ethanol and stored at −20 °C.

2.2. Primary PrECs, Prostate Cell Line, and Treatments

Human primary prostate epithelial cells (PrECs; at passage 2) from different donors (age 19 to 37) in culture (or frozen vials) were purchased from Lonza (Houston, TX, USA). Cells were maintained in culture as suggested by the supplier in the presence of medium supplements that were provided by the supplier as a part of the PrEGM™ Bulletkit™. Immortalized BPH-1 cell line was originally provided by Dr. Simon Hayward (Vanderbilt University Medical Center, Nashville, TN, USA) [30].

When indicated, cells in culture were treated with the indicated concentrations of dihydrotestosterone (DHT; stock in 100% ethanol) in phenol-free culture medium that was supplemented with charcoal/Dextran-treated fetal bovine serum (to decrease the endogenous levels of the steroid hormones) from the US source (from HyClone).

The asynchronous onset of cellular senescence in the primary cultures of human PrECs in late passages (passage 7 and higher) was assessed using well-described criteria for cellular senescence, including cell morphological changes and positivity to senescence-associated acidic β-galactosidase (SA-β-gal) as we have described [20]. In senescent

cultures of PrECs, >90% cell population tested positive for the SA-β-gal, exhibited a large and flat cell morphology, and stopped cell proliferation [20].

2.3. Antibodies

Following antibodies were used to specifically detect proteins in immunoblotting: AR (sc-816), IFI16 (sc-8023), ASC (sc-22514), IL-1β (sc-7884), and IL-18 (sc-7954) from Santa Cruz Biotech (Santa Cruz, CA, USA); Caspase-1 (AHZ0082) from Invitrogen (Grand Island, NY, USA); Anti-STAT1 (cat # 9172), p-STAT1 (cat # 9171), and β-actin (cat # 4967) from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibodies that we raised against the C-terminal AIM2 peptide that specifically detected two human hAIM2 isoforms have been described [31]. Specific custom anti-peptide rabbit polyclonal antibodies were raised against a peptide (REEQETGICGSPSSARSV) in the POP3 protein, which detected an IFN-inducible POP3 protein of an expected size (~18 kDa) in total cell extracts from IFN-treated THP-1 cells as described [15]. Horseradish peroxidase (HRP) conjugated secondary anti-mouse (NXA-931) and anti-rabbit (NA-934) antibodies were from GE Healthcare Biosciences (Piscataway, NJ, USA).

2.4. Immunoblotting

Total cell lysates were prepared in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) that was supplemented with complete mini EDTA-free protease inhibitor cocktail and phosphatase inhibitors (Cell Signaling, Danvers, MA, USA) as described [31]. The lysates containing approximately equal amounts of total proteins (~25−50 μg) were subjected to immunoblotting [31]. When indicated, actin protein was used as an internal control (because levels of actin did not change after DHT-treatment of cell types that we used). Enhanced chemiluminescence (ECL) signals of proteins were measured by the Molecular Imager Gel Doc XR$^+$ System (Bio-Rad, Hercules, CA, USA). Immunoblots that were used for quantification of protein levels are shown in Supplementary Figures and the quantification values in an accompanied Table.

2.5. Inflammasome Assay

Activation of inflammasome activity in PrECs or BPH-1 cells was assessed using the criteria described earlier [32]. In brief, we subjected the total cell lysates or proteins from cell culture medium to immunoblotting and assessed (i) a decrease in the cellular levels of pro-caspase-1 (p45); (ii) an increase in the cellular levels of activated caspase 1 (p20) and/or (p10); (iii) a decrease in the cellular levels of pro-IL-1β (p31); and (iv) an increase in the cellular levels of the mature IL-1β (p17). Notably, in contrast to macrophages [32], in PrECs and BPH-1 cell line, activation of the inflammasome activity was accompanied by moderate to appreciable changes in the cellular levels of pro-caspase-1 (p45) and pro-IL-1β (p31) under our experimental conditions as described [31]. Further, when indicated, we detected the secreted levels of the mature IL-1β and IL-18 in the culture medium after precipitation of the proteins from the medium.

2.6. RNA Isolation and PCR

Cells were collected by centrifugation and the pellets were suspended into the Trizol reagent (Invitrogen) to isolate total RNA as described [31]. cDNA synthesis and semi-quantitative RT-PCR were performed as described [31]. The following primers were used for RT-PCR: the human AR (forward: 5′-CATCTGTGAAATAGAGCCTATCATATCCAC-3′; backward: 5′-TAACGCCTGCCTAGTGGCTTTGGAG-3′), IFI16 (forward: 5′-CCAAGACT GAAGAC TGAA-3′; backward: 5′-ATGGTCAATGACATCCAG-3′), POP3 (forward: 5′-ATGGAGA GTAAATATAAGGAG-3′; backward: 5′-TCAACATGCATTCCCA GAAAT-3′), AIM2 (forward: 5′-ATGTGAAGCCGTCCAGA-3′; backward: 5′-CATCATT TCTGATGG CTGCA-3′), and actin (forward: 5′-GCTCGTCGT CGACAACGGCTC-3′; backward: 5′-CATG ATCTG GGTCATCTTCTC-3′). Levels of actin mRNA were used as an internal

control. To determine the fold change (FC) in the levels of an mRNA following a treatment, the intensity of the actin DNA band (an internal control) on the agarose gel and the DNA band of a gene of interest were measured by the Molecular Imager Gel Doc XR$^+$ System (Bio-Rad, Hercules, CA, USA) with Image Lab Software. Next, the ratio was calculated using the DNA band intensity value for the gene of interest and actin DNA band. This ratio in control cells was indicated as 1 and the FC for DHT-treated samples was calculated by calculating the ratio between the value from treated samples (calculated as in the case of control sample) and the control value 1.

For quantitative real-time TaqMan PCR assays, Applied Biosystems's (Foster City, CA, USA) technique was used [31]. The PCR cycling program consisted of denaturing at 95 °C for 10 min and 40 cycles at 95 °C for 15 s, and annealing and elongation at 60 °C for 1 min. The TaqMan assays for *IFI16* (assay Id #Hs00194216_mL), human interferon-β (*IFNB*; assay Id # Hs01077958 _s1), and for the endogenous control β-actin (assay Id# Hs99999903_mL) were purchased from Applied Biosystems (Foster City, CA, USA) and used as suggested by the supplier. The POP3 TaqMan assay was custom designed: (forward: 5′-AGCACGAGTAGCCAACTT GATT-3′; backward: 5′-GGTCTTCCTCACTGCAGACA-3′).

2.7. Transfection

Sub-confluent cultures of PrECs or BPH-1 cells were either treated with vehicle (ethanol) or with the indicated concentrations of DHT (in ethanol) as noted. Following the treatment, cells were "primed" with TNF-α for 3 has described [31]. Control or "primed" cells were either transfected with LyoVec (control) or poly (dA:dT)/LyoVec (5 μg/mL) for the indicated time. At the end of incubations, cells were harvested to prepare total cell lysates.

2.8. Stable Knockdown of POP3 Expression

To knockdown POP3 protein expression in BPH-1 cell line, cells were either transfected with an empty vector (pcDNA3.1) or pcDNA3.1-POP3(AS) plasmid (a PCR fragment was cloned in the multiple cloning site in the vector in the reverse orientation), thus, allowing the expression of an antisense mRNA. The transfected cells were selected using G418 (500 μg/mL) for two weeks and the G418-resistant colonies (>300 colonies) were pooled. To maintain cells in culture, a reduced concentration (250 μg/mL) of the G418 was used. The transfected cells were cultured without G418 in the medium for two days prior to the experiments.

2.9. Statistical Methods

Experiments involving immunoblotting and semi-quantitative RT-PCR techniques were repeated at least 3-times. A representative result is shown. For quantitative PCR, the assays were performed in triplicates. Fold-changes in the levels of certain proteins and mRNAs are indicated based on the quantitation of signal in independent experiments. The statistical measurement values, when indicated, were presented as means ± SEM. The statistical significance of differences in the measured mean frequencies between the two experimental groups was calculated using the Student two-tailed *t*-test.

3. Results

3.1. Activation of Type I Interferon Signaling in Senescent PrECs Differentially Regulated the Expression of POP3 and AIM2 Proteins

Senescent human diploid fibroblasts (HDFs), as compared with young proliferating or old HDFs, expressed higher basal levels of the IFN-β and activated the type I IFN-signaling [22]. Further, activation of the type I IFN-signaling in senescent HDFs increased the levels of AIM2 protein but decreased IFI16 protein levels [22]. Therefore, we examined the expression of IFN-β and the IFN-β-inducible PYHIN-family proteins in proliferating (passage 2), old (passage 5), and senescent PrECs (passage 8). As shown in Figure 1A, the levels of IFN-β mRNA were significantly higher in senescent vs. proliferating or old PrECs.

Consistent with our previous observations [22], senescent PrECs exhibited activation of T1 IFN response as compared with proliferating or old PrECs as determined by increases in the levels of type I IFN-inducible STAT1 protein and its activating phosphorylation on Tyr-701 residue (Figure 1B). Interestingly, in contrast to senescent HDFs, the levels of type I IFN-inducible IFI16 proteins were higher in senescent PrECs than the young or old PrECs. Expectedly [16], the levels of AR were also higher in senescent PrECs than the young or old PrECs. Further, the levels of AIM2 protein were higher in senescent vs. young or old PrECs. However, the levels of POP3 protein were lower in senescent vs. young or old PrECs. Because POP3 protein inhibited activation of the AIM2 inflammasome [15], we also examined the levels of the mature IL-1β (p17) and IL-18 (p18) in the culture medium. We found that the levels of IL-1β and IL-18 were higher in the culture media of the senescent PrECs than young proliferating cells. These observations thus suggested activation of an inflammasome in senescent PrECs.

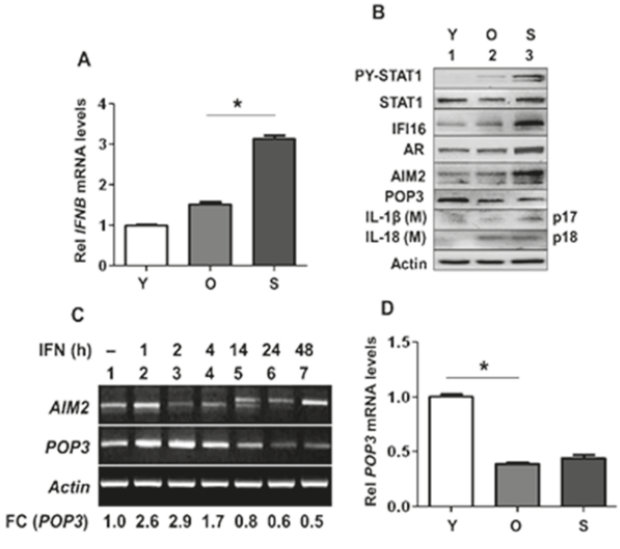

Figure 1. Activation of type I interferon signaling in human senescent PrECs differentially regulated the expression of POP3 and AIM2. (**A**) Total RNAs isolated from young proliferating (Y; passage-2), old (O; passage-5), or senescent (S; passage-8) human PrECs were subjected to quantitative real-time PCR using the TaqMan assay specific for the human *IFNB* mRNA. The RNA levels were normalized using *ACTIN* mRNA. The relative levels of the *IFNB* mRNA in young PrECs are indicated as 1. The values indicated as SEM (* $p < 0.05$). (**B**) Total cell extracts prepared from young (Y; passage-2), old (O; passage-5), or senescent (S; passage-8) human PrECs were analyzed by immunoblotting using the antibodies specific to the indicated proteins. The IL-1β(M) and IL-18(M) indicate the cleaved forms of the pro-IL-1β and pro-IL-18 that were detected in the culture medium. The experiments were repeated at least two times from cells derived from two different donors of different ages. Immunoblots that were used for quantification of protein levels are shown in Supplementary Figure S1 and the quantification of protein levels in an accompanied Table. (**C**) Young proliferating (Y; passage-2) PrECs were either left untreated or treated with 1000 u/mL of IFN-β for indicated times (h). Total RNA was isolated and subjected to RT-PCR for the indicated mRNAs as described in Material and Methods. Fold changes (FC) in the levels of POP3 mRNA were calculated as noted in Materials and Methods. (**D**) Total RNAs isolated from young, old, or senescent PrECs, as described in the panel (**A**), were subjected to the quantitative real-time PCR using the TaqMan assays (in triplicates) specific for the human *POP3* mRNA. The RNA levels were normalized using *ACTIN* mRNA. The relative levels of the *POP3* mRNA in young PrECs are indicated as 1. The values indicated as SEM (* $p < 0.05$).

IFN-β treatment of human macrophages for increasing length of time (0 to 48 h) differentially regulated the expression levels of POP3 and AIM2 mRNA [15]. Therefore, to investigate the potential role of AIM2 inflammasome activation in SASP of PrECs that activated the T1 IFN-signaling (Figure 1A), we treated proliferating PrECs with increasing length of time (0–48 h) and compared the levels of POP3 and AIM2 mRNA levels. As shown in Figure 1C, the treatment of PrECs with IFN-β increased the levels of AIM2 and POP3 mRNAs within an hour. However, the levels of AIM2 mRNA decreased after an hour of treatment but increased again after 48 h of treatment. In contrast, the levels of POP3 mRNA stayed higher after an hour of the treatment of PrECs but stayed lower after 14 h of treatment. Accordingly, a quantitative PCR revealed that old and senescent PrECs expressed lower basal levels of POP3 mRNA than proliferating cells (Figure 1D). These observations are consistent with a chronic activation of the T1 IFN-signaling in senescent PrECs, contributing to an increased AIM2/POP3 protein ratio through a transcriptional mechanism.

3.2. Androgen Receptor Activation in Proliferating PrECs Increased the Expression of POP3

Human primary PrECs express detectable levels of the androgen receptor (AR) [16]. Further, treatment of primary PrECs with type I IFN increased the levels of AR and stimulated the transcriptional activity of AR [16]. Because senescent PrECs exhibited activation of T1 IFN response and expressed higher basal levels of AR (Figure 1B), we tested whether activation of the AR in proliferating PrECs could regulate the expression of POP3 and AIM2. Consistent with our previous observations [19], treatment of proliferating PrECs with the male sex hormone DHT (10 nM) for 14 h increased the levels of *IFI16* mRNA (Figure 2A). Further, the treatment increased the levels of POP3 mRNA ~ 4-fold. However, the levels of AIM2 mRNA remain unchanged. Therefore, we performed quantitative PCR to assess the extent of increase in the levels of POP3 mRNA by DHT in PrECs. As shown in Figure 2B, treatment of cells with DHT significantly increased the levels of POP3 and IFI16 mRNAs. Accordingly, we also noted measurable increases in the levels of IFI16 and POP3 proteins in extracts from DHT-treated proliferating PrECs (Figure 2C). Consistent with these observations, treatment of LNCaP human prostate cancer cells, which express abundant levels of AR (as compared with normal proliferating PrECs) [33], with 10 nM concentration of DHT also increased the levels of POP3 mRNA (Figure 2D). Similarly, treatment of human benign prostate hyperplasia cell line BPH-1 with 10 nM DHT also increased the levels of IFI16 and POP3 proteins, but not AIM2 protein (data not shown). However, treatment of androgen independent human prostate cancer cell line PC-3 with 10 nM DHT did not increase the levels of POP3 protein (data not shown). Together, these observations are consistent with activation of T1 IFN-signaling in human senescent PrECs potentiating stimulation of the AR-mediated increases in the levels of IFI16 and POP3 proteins, but not the AIM2 protein.

3.3. Androgen Treatment of PrECs Inhibited Cytosolic DNA-Induced Activation of the AIM2 Inflammasome

To determine whether activation of androgen receptor in human proliferating PrECs, which increased the levels of POP3 protein (Figure 2), could inhibit AIM2 inflammasome activity, we compared the inflammasome activation in proliferating PrECs after vehicle (alcohol) or DHT treatment. As shown in Figure 3, DHT treatment of human primary PrECs (passage 2), as compared with control cells (vehicle treated), appreciably increased the levels of the POP3 protein (compare lane 3 with 1). Further, the basal levels of procaspase-1 (p45) were lower in control cells that were stimulated with the synthetic DNA poly [dA:dT] (compare lane 2 with 4). Accordingly, levels of the activated caspase-1 (p20) were higher in the control cells than DHT-treated cells that were stimulated with synthetic DNA. Similarly, the secreted IL-1β (p17) and IL-18 protein levels were higher in the culture medium of control cells than DHT-treated cells. Together, these observations indicated that activation of AR by DHT in proliferating normal PrECs up-regulated the levels of POP3 and the

up-regulation associated with a decrease in cytosolic DNA-induced activation of the AIM2 inflammasome activity.

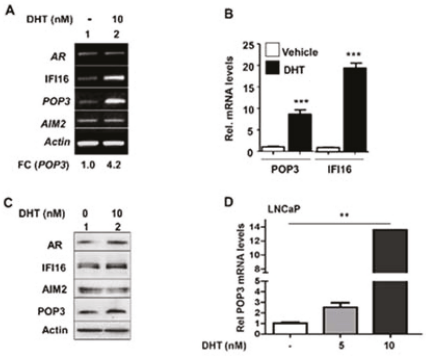

Figure 2. Androgen receptor activation in human proliferating PrECs increased the expression of POP3. (**A**) Sub-confluent cultures of proliferating young (passage 2) human PrECs were either treated with vehicle (ethanol) or 10 nM DHT for 18 h. After the treatment, total RNA was isolated and subjected to RT-PCR for the levels of mRNAs for the indicated genes. The fold change (FC) in the levels of POP3 mRNA in response to DHT-treatment of cells as compared with vehicle treated cells was estimated as described in methods. (**B**) Total mRNA isolated in panel (**A**) was subjected to quantitative real-time PCR (in triplicates) using the TaqMan assay specific for the indicated mRNA. The mRNA levels were normalized using *ACTIN* mRNA. The relative levels of the mRNA in vehicle treated PrECs are indicated as 1. The values indicated as SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (**C**) Cultures of young proliferating (passage 2) PrECs were either treated with vehicle (lane 1) or 10 nM DHT for 18 h as described in methods. After the treatment, total cell lysates containing equal amounts of proteins were subjected to immunoblotting using the antibodies specific to the indicated proteins. Immunoblots that were used for quantification of protein levels are shown in Supplementary Figure S2 and the quantification of protein levels in an accompanied Table. The experiment was repeated two times using cells derived from a single donor. (**D**) Cultures of the LNCaP cells were either treated with vehicle or the indicated concentration of DHT for 18 h. Total mRNA was isolated and subjected to quantitative real-time PCR using the TaqMan assay specific for the *POP3* mRNA. The *POP3* mRNA levels in all samples were normalized using *ACTIN* mRNA. The relative levels of the mRNA in vehicle treated LNCaP cells are indicated as 1. The values indicated as SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.4. A Stable Knockdown of POP3 Protein Expression in BPH-1 Cell Line Increased Cytosolic DNA-Induced AIM2 Inflammasome Activation

We also investigated whether a knockdown of POP3 protein expression in BPH-1 cells could increase activation of the AIM2 inflammasome without or after DHT treatment. As shown in Figure 4A, stable transfection of BPH-1 cells with an expression vector that allowed the expression of the antisense POP3 mRNA appreciably reduced the basal levels of POP3 mRNA in cells as compared with cells that were transfected with an empty vector. Further, androgen-treatment of vector transfected control cells and their stimulation with cytosolic synthetic DNA did not result in appreciable activation of the inflammasome activity as determined by the lack of detection of proteolytically cleaved caspase-1 (p20) in cell lysates and secreted mature IL-1β (p17) and IL-18 (p18) in the culture medium (Figure 4B). However, a knockdown of the POP3 protein expression in cells and their treatment with DHT did not result in a measurable increase in POP3 protein levels. Importantly, treatment of cells with the synthetic DNA robustly activated the inflammasome activity in cells, as measured by increases in the proteolytically cleaved and activated caspase-1 (p20) levels in cell lysates and the secreted levels of the mature IL-1β (p17) and IL-18 (p18) in the culture medium. Together, these observations indicated that a stable knockdown of

POP3 protein expression in BPH-1 cell line activated cytosolic DNA-induced activity of the AIM2 inflammasome.

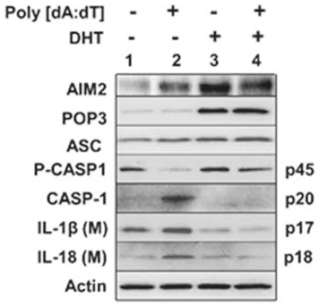

Figure 3. Androgen treatment of PrECs inhibited cytosolic DNA-induced activation of the AIM2 inflammasome. Cultures of human proliferating PrECs were either treated with vehicle (lanes 1 and 2) or with 10 nM DHT (lanes 3 and 4) for 18 h. Cells treated with either vehicle or DHT were further treated with 10 ng/mL TNF-α for 3 h to "prime" cells. The primed cells were either treated with LyoVec (lanes 1 and 3) or poly(dA:dT)/LyoVec (5 μg/mL; lanes 2 and 4) for 4 h. After the treatment, total cell lysates and culture medium (after precipitation of proteins) were subjected to immunoblotting using the antibodies specific to the indicated proteins. The IL-1β (M) and IL-18 (M) indicate the cleaved forms of the pro-IL-1β and pro-IL-18 that were detected in the culture medium. The experiment was repeated using proliferating PrECs from a single donor. Immunoblots that were used for quantification of protein levels are shown in Supplementary Figure S3 and the quantification in an accompanied Table.

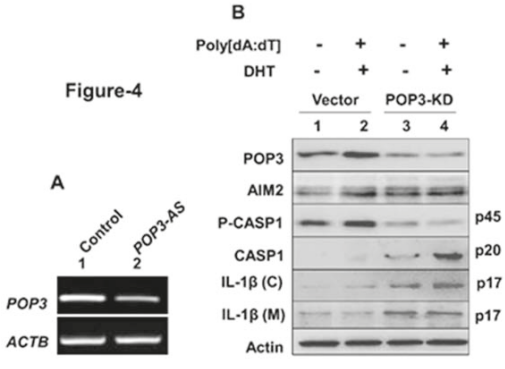

Figure 4. A stable knockdown of POP3 protein expression in BPH-1 cell line increased AIM2 inflammasome activation. (**A**) Total RNA isolated from vector transfected control BPH-1 cells (lane 1) or cells transfected with the pcDNA3.1-POP3(AS) vector allowing the expression of the antisense POP3 mRNA were analyzed by RT-PCR for the indicated genes. The experiment was repeated two times. (**B**) Control BPH-1 cells in panel (**A**) or cells transfected with pcDNA3.1-POP3(AS) vector were either treated with vehicle (lanes 1 and 3) or 10 nM DHT (lanes 2 and 4) for 18 h. Cells were further treated with 10 ng/mL TNF-α for 3 h to "prime" cells. The primed cells were incubated with either LyoVec (lanes 1 and 3) or poly (dA:dT)/LyoVec (5 μg/mL; lanes 2 and 4) for 4 h. After the treatment, total cell lysates and cell culture medium (after precipitation of proteins) were subjected to immunoblotting using the antibodies specific to the indicated proteins. The IL-1β (C), the cleaved IL-1β (p17) within the cell; IL-1β (M), cleaved IL-1β detected in the culture medium. The experiment was repeated two times. Immunoblots that were used for quantification of protein levels are shown in Supplementary Figure S4 and the quantification in an accompanied Table.

4. Discussion

Senescent PrECs accumulate in BPH [27]. Further, SASP is associated with activation of an inflammasome activity and an increase in the production of proinflammatory cytokines [34,35]. Therefore, our observations that (i) human senescent PrECs expressed higher basal levels of the AIM2 protein and lower basal levels of POP3 protein (Figure 1); and (ii) reduced basal levels of the POP3 protein in senescent PrECs (Figure 1) associated with activation of cytosolic DNA-responsive AIM2 inflammasome are consistent with a role of POP3 protein in the suppression of SASP in senescent PrECs.

Serum androgen levels decrease in men with aging [36,37]. Further, androgen receptor levels increase in PrECs in certain parts of the prostate [37]. Therefore, our observations that senescent PrECs that activated type I IFN signaling expressed higher basal levels of the AR (Figure 1B) and activation of androgen receptor in human PrECs increased the levels of POP3 protein (Figure 2) and increased levels of POP3 in PrECs inhibited cytosolic DNA-induced activation of the AIM2 inflammasome activity (Figure 3) support the idea that aging-related reduced serum levels of androgens in men contribute to a decrease in the levels of POP3 protein in PrECs, thus leading to an increase in activation of the AIM2 inflammasome. Because our observations implicate a role for the POP3 protein in aging-related prostatic inflammation, further work will be needed to examine the role of androgen-AR/POP3/AIM2 axis in the development of aging-related prostatic diseases.

POP3 protein also bound with the IFI16 proteins and inhibited activation of the IFI16 inflammasome [15]. Notably, androgens mediated activation of AR in human normal proliferating PrECs also increased the expression of *IFI16* gene [19]. As increased levels of IFI16 proteins in human proliferating PrECs potentiated the p53-mediated cell cycle arrest that is associated with cellular senescence [23], it is conceivable that androgens-mediated up-regulation of the POP3 protein in human PrECs also affects the cell cycle inhibitory functions of the IFI16 proteins. Because AR also drives human PrECs to cellular senescence [24], further work is needed to determine whether androgens-mediated increased levels of POP3 protein in human PrECs modulate the p53-mediated functions.

Activation of certain inflammasomes contributes to the development of prostatic diseases in animal models and humans [38–41]. These diseases include chronic prostatitis and chronic pelvic pain syndrome [38], BPH associated prostatic inflammation [39], and prostate cancer [40,41]. However, it remains unclear whether androgens-mediated activation of the AR in PrECs regulates the activity of the inflammasomes. Therefore, our observations that activation of AR in human PrECs suppressed activation of the AIM2 inflammasome are likely to serve the basis for further studies.

Androgen deprivation therapy (ADT) in prostate cancer patients is often associated with increased production of pro-inflammatory cytokines (e.g., IL1β) [28]. However, it remains unknown whether ADT in prostate cancer patients promote prostatic inflammation through activation of inflammasomes. Therefore, our observations that activation of AR in human PrECs up-regulated the expression of POP3 protein, an inhibitor of the production of inflammatory cytokines (IL-1β and IL-18) through activation of inflammasomes, are of significance.

The 5′-regulatory region of the *POP3* gene remains uncharacterized. Although the expression of *POP3* gene is induced by type I IFN [15], the IFN-responsive *cis*-element(s) remain unknown. Therefore, our observations that treatment of human PrECs (and BPH-1) cell line with androgen DHT increased levels of the POP3 mRNA and protein will require further work to identify the molecular mechanisms through which AR activation in PrECs increases the levels of POP3 mRNA and protein.

In summary, our observations identify the IFN-inducible POP3 PYHIN protein as a potential negative regulator of the AIM2 inflammasome and SASP in human senescent PrECs. These observations also suggest that aging-related reduced levels of androgens in men through reduced basal activation of the AR in PrECs increase the activation of the AIM2 inflammasome. Thus, our observations have important implications for aging-related development of prostatic inflammatory diseases.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/life11040366/s1, Figure S1: Protein levels in immunoblots in Figure 1B were quantified and fold changes in protein levels were calculated as noted in the Materials and Methods. The table shows the values for the protein band intensity fractions using the GelQuant.NET software. The values in the parenthesis in the table indicate fold changes in protein levels, Figure S2: Protein levels in immunoblots in Figure 1C were quantified and fold changes in protein levels were calculated as noted above. The values in the parenthesis in the table indicate fold changes in protein levels, Figure S3: Protein levels in immunoblots in Figure 3 were quantified and fold changes in protein levels were calculated as noted above. The values in the parenthesis in the table indicate fold changes in protein levels, Figure S4: Protein levels in immunoblots in Figure 4B were quantified and fold changes in protein levels were calculated as noted above. The values in the parenthesis in the table indicate fold changes in protein levels.

Author Contributions: Conceptualization, D.C.; methodology, R.P., V.R. and H.L.; writing-original draft, D.C. and R.P.; writing—review and editing, D.C. All authors have read and agreed to the published version of the manuscript.

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Evaluation of GammaH2AX in Buccal Cells as a Molecular Biomarker of DNA Damage in Alzheimer's Disease in the AIBL Study of Ageing

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Abstract: In response to double-stranded breaks (DSBs) in chromosomal DNA, H2AX (a member of histone H2A family) becomes phosphorylated to form γH2AX. Although increased levels of γH2AX have been reported in the neuronal nuclei of Alzheimer's disease (AD) patients, the understanding of γH2AX responses in buccal nuclei of individuals with mild cognitive impairment (MCI) and AD remain unexplored. In the current study, endogenous γH2AX was measured in buccal cell nuclei from MCI (n = 18) or AD (n = 16) patients and in healthy controls (n = 17) using laser scanning cytometry (LSC). The γH2AX level was significantly elevated in nuclei of the AD group compared to the MCI and control group, and there was a concomitant increase in *P*-trend for γH2AX from the control group through MCI to the AD group. Receiver-operating characteristic curves were carried out for different γH2AX parameters; γH2AX in nuclei resulted in the greatest area under the curve value of 0.7794 ($p = 0.0062$) with 75% sensitivity and 70% specificity for the identification of AD patients from control. In addition, nuclear circularity (a measure of irregular nuclear shape) was significantly higher in the buccal cell nuclei from the AD group compared with the MCI and control groups. Additionally, there was a positive correlation between the nuclear circularity and γH2AX signals. The results indicated that increased DNA damage is associated with AD.

Keywords: γH2AX; Alzheimer's disease; DNA damage; mild cognitive impairment; senescence

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that is characterised clinically by severe memory loss, cognitive deterioration, and behavioural changes [1,2]. AD is the most common cause of dementia in old age, representing approximately 60–80% of all dementia cases [3–5]. According to

the World Health Organization, 46.8 million people were affected by dementia in the year 2015 [6]. It has been estimated that by the year 2030, 74.7 million people will be affected by AD unless effective interventions are implemented [6]. This increase in the prevalence of AD not only reduces the quality of life, health, and wellbeing of those affected, but also causes a significant financial burden at both the social and economic levels [7].

The classic neuropathological lesions in AD consist of (i) aggregated amyloid plaques containing extracellular hydrophobic deposition of amyloid β peptides (Aβ) in the neuronal body, and (ii) neurofibrillary tangles composed of aggregates of hyperphosphorylated and misfolded tau protein (a microtubule-associated protein) that appear within the neurons [8]. AD patients are usually identified by neuropsychological assessment when the disease has progressed to an advanced stage of cognitive impairment when it is already too late to cure [9,10]. Currently, the ability to detect the early stage of AD and track the different stages of AD progression to guide the choice of therapy is limited. The Mini-Mental State Examination (MMSE) is a validated research-based set of 30 questions assessing memory loss, cognitive decline, and visuospatial and language impairment that is currently used as a standard tool for the clinical diagnosis of AD [11,12]. However, the test lacks accuracy for the diagnosis of AD in living subjects, and diagnostic confirmation can only be achieved post-mortem by the examination of the senile plaques and neurofibrillary tangles in the cerebral tissue [13,14]. The most validated AD disease-related established diagnostic biomarkers are from cerebrospinal fluid (CSF) (aβ1-42, total tau, and phosphorylated tau), structural magnetic resonance imaging (MRI) (e.g., hippocampal volumetry), and amyloid positron emission tomography and fluorodeoxyglucose positron emission tomography imaging [15,16]. Mild cognitive impairment (MCI) is an intermediate state between the cognitive changes of normal ageing and the earliest clinical signs of dementia and is represented as a declining cognition that does not meet the diagnostic criteria of dementia [17]. Individuals affected by MCI have a higher risk of developing AD with an annual conversion rate of approximately 10–15% per year [18–20]. Recent evidence indicates that AD is a systemic disorder that can be mirrored by subclinical pathologies in various peripheral tissues other than the brain, thereby rationalising the grounds for investigating cellular biomarkers in peripheral tissues for the diagnosis of MCI/AD risk [21–25]. A recent study has shown that salivary Aβ42 levels can be used to diagnose AD as well as to predict the risk of its future onset [26]. There is a need for non-invasive biomarkers and inexpensive diagnostic approaches with high specificity and sensitivity to identify individuals at increased risk of developing MCI and AD so that early diagnosis and the initiation of preventative therapy is commenced to halt progression to irreversible neurological impairment.

Human buccal mucosa has considerable potential as an easily accessible source of cells that can be collected in a minimally invasive manner. Defects in buccal mucosa cells may reflect systemic changes in pathology in other tissues of ectodermal origin, such as the nervous system [27–29]. It has been suggested that the ubiquitous presence and different expression of β-amyloid precursor protein (APP) in the buccal mucosa could be a useful means to estimate the regenerative status of tissue [30]. Accumulation of tau protein in the brain is the major component of neurofibrillary tangles, and is the hallmark of AD pathogenesis [31,32]. The amount of buccal cell tau protein was observed at higher levels in AD subjects and correlated with the levels of tau protein in the CSF [33]. AD is associated with genomic DNA damage, and lack of DNA repair capacity could potentially lead to genomic instability [34–39].

The buccal micronucleus cytome assay has been developed to score the cytological markers of DNA damage, cell death, and regenerative capacity of buccal mucosa cells [34,40]. Individuals who had just been diagnosed with AD, but had not yet taken medication for their condition, had significantly reduced basal buccal cell frequency compared to unaffected age-matched controls suggesting reduced regenerative capacity. Aneuploidy (abnormal chromosomal number) has been investigated in buccal cells of AD patients in comparison with respective controls, with the results showing a higher aneuploidy level in chromosomes 17 and 21, which are known to encode tau and APP, respectively [34,41,42]. A recent study showed abnormal DNA content (e.g., hyperploidy in nuclei; a marker of aneuploidy)

in buccal mucosa cells of AD patients [28]. The same study also demonstrated decreased amount of neutral lipids as measured by Oil Red-O staining in buccal cells from MCI patients [28]. Buccal samples of AD patients were tested for telomere shortening and displayed a significantly shorter telomere length when compared to healthy older controls [43]. A previous study suggested that DNA strand breaks may be increased in lymphocytes of MCI and AD patients [44].

In response to double-stranded breaks (DSBs) in chromosomal DNA, H2AX (a member of histone H2A family and part of the chromatin structure) becomes phosphorylated to form γH2AX [45]. γH2AX has also been found to be increased in neuronal cells of AD and with ageing in lymphocytes [46–48]. While H2AX is distributed uniformly throughout chromatin, only H2AX molecules located in close vicinity to DSBs become phosphorylated [45,49,50]. The association of astrocyte degeneration and DNA damage with AD has been elucidated by investigating γH2AX signals in astrocytes from the hippocampus, which is known to be the most vulnerable region affected by AD [46]. The results showed a significantly increased number of γH2AX-immunopositive nuclei in the astrocytes of AD patients in comparison to healthy controls, suggesting that astrocytes may be associated with impaired neuronal function and contribute to the pathogenesis of AD [46]. Additionally, a recent study reported elevated γH2AX levels in the hippocampal tissue of individuals with both AD pathology and clinical dementia than those seen in a normal ageing group [47]. γH2AX has been used as a DSB marker in irradiated human buccal cells and was found to be dose responsive in different buccal cell types [51,52]. However, buccal cell DNA damage involving γH2AX, an important marker of DNA damage and DNA damage response, has not been reported in neurodegenerative disorders such as AD.

Taken together, the evidence outlined above forms the basis of the hypothesis we tested that buccal cells from individuals with MCI and AD exhibit elevated levels of γH2AX compared to buccal cells from healthy controls. To test this hypothesis, the endogenous levels of γH2AX in buccal cells from participants in the Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing (AIBL) who were either healthy controls, MCI cases, or AD cases were measured. An automated laser scanning cytometry (LSC) γH2AX protocol was used to measure multiple parameters (area, integral, MaxPixel) of γH2AX signals, as well as the ploidy and nuclear shapes and senescent cells in thousands of buccal cells per subject.

2. Materials and Methods

2.1. Human Ethics and Clinical Assessment of the Participants

Approval for the Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing (AIBL) was from the institutional ethics committees of Austin Health (Parkville, Vic, Australia), St Vincent's Health (Fitzroy, Vic, Australia), Hollywood Private Hospital (Nedlands, WA, Australia), Edith Cowan University (Perth, WA, Australia), and CSIRO Australia. All volunteers were informed of the purpose of the study and gave written consent before participating in the study. The demographic and health characteristics of participants included in this study have been well characterised and reported previously [53]. Diagnosis of MCI and AD was performed and confirmed by experienced AIBL clinicians using a battery of neuropsychological tests that were selected on the basis that together covered the main domains of cognition that are affected by AD and other dementias [53]. Data reported in this study are from a total of 51 randomly sub-sampled participants, including: (1) the cognitively healthy control group (n = 17); (2) the MCI group (n = 18) clinically diagnosed with MCI; and the (3) AD group (n = 16) clinically diagnosed with AD. Full blood pathology testing was conducted as described previously [54,55]. There were no blood pathology data available for 10 participants.

2.2. Buccal Cell Collection and Microscope Slide Preparation

Prior to buccal cell collection, each participant was first required to rinse their mouth twice with water. Small flat-headed toothbrushes were rotated 20 times against the inner part of the cheeks in a circular motion. Both cheeks were sampled using separate toothbrushes. Heads of the brushes

were transferred into a 25 mL tube containing 20 mL of Saccomano's fixative solution and agitated vigorously to dislodge cells into the solution. Cells were then centrifuged at 1000× *g* for 10 min before discarding and replacing supernatant with fresh 5 mL of buccal cell buffer (10 mM Tris (hydroxymethyl) aminomethane, 0.1 M ethylenediaminetetraacetic acid, 20 mM NaCl, pH 7.0. The cell suspension was drawn up and down five times into a 10 mL syringe using a 21G needle in order to maximise the likelihood of dispersing cell aggregates into a single cell suspension. The cell suspension was then passed through a 100 μm filter in a Swinex filter holder to remove clumps of cells. Cell concentration was assessed using a haemocytometer and cells were then cytocentrifuged for 5 min at 600 rpm onto microscope slides to a final number of 3000 cells per cytospot using a Shandon CytospinVR 4 (ThermoFisher Scientific, Waltham, MA, USA). Slides were washed once with distilled water and air-dried for 1 h and subsequently transferred to ethanol:acetic acid (3:1) fixative for 10 min. The slides were air-dried for 1 h and stored in sealed microscope boxes with desiccant at −80 °C until the staining procedure was performed.

2.3. Preparation of Buccal Cells for Immunofluorescence

A circle was drawn around each cytospot using a hydrophobic PAP pen (Dako, Australia) and air-dried at 22 °C for 10 min. Slides were rinsed in Dulbecco's phosphate-buffered saline (DBPS) for 15 min at 22 °C, incubated in 70% ethanol (4 °C) for 20 min and washed in DPBS for 15 min at 22 °C. Buccal cell cytospots were then treated with 150 μL of prewarmed (37 °C) pepsin solution (containing 750 U/ml of porcine gastric mucosa pepsin) in 0.01 M HCl and then covered with parafilm for 30 min at 37 °C in a humidified box. The slides were then washed twice with DPBS for 5 min. Buccal cells were then permeabilised with 1% Triton X-100 for 15 min at room temperature. Slides were then rinsed three times in DPBS, and a blocking step was performed by incubating cells in 10% goat serum for 1 h at room temperature before being washed once with DPBS. The anti-γH2AX antibody was added to each cytospot at a dilution of 2 μg/mL in DPBS containing 10% goat serum and covered with parafilm overnight at 4 °C in a humidified box. Slides were washed three times in DPBS for 5 min and a secondary antibody Alexa Fluor 488 Goat antimouse IgG was added to each cytospot at 2 μg/mL in DPBS containing 10% FBS and covered with parafilm for 1 h at room temperature. Slides were washed three times in DPBS for 5 min and nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 1 μg/mL for 10 min at room temperature. The excess DAPI was removed by rinsing the slides with a solution containing 300 mM NaCl and 34 mM sodium citrate. Slides were then mounted with coverslips and DPBS: glycerol (1:1) medium. The edges of coverslips were sealed with nail polish to prevent drying prior to performing laser scanning cytometry.

2.4. Laser Scanning Cytometry Measurements of γH2AX

Laser scanning cytometry (LSC) measurements were carried out with an iCyte® Automated Imaging Cytometer (Thorlabs, Sterling, VA, USA) with full autofocus function as well as 405 nm and 488 nm lasers for excitation of DAPI and Alexa Fluor 488, respectively. Fluorescence from DAPI (blue) and Alexa Fluor 488 (green) was collected with a photomultiplier tube. Samples were scanned in separate passes (consecutively) to prevent spectral overlap. The nuclei and γH2AX events were contoured using empirically determined thresholds to exclude the scoring of false positives (e.g., small fluorescent debris). The frequency (%) of nuclei containing γH2AX signal was recorded as well as multiple parameters within each nucleus, including the total γH2AX integral (a function of γH2AX intensity and size) and the MaxPixel value (the value of the most intense γH2AX signal/pixel within nuclei). These parameters were generated using the iCyte® 3.4 software and subsequently transferred into excel, then GraphPad Prism for further statistical analyses. Nuclei were also classified into round, long, or oval shapes by utilising the iCyte software parameters which included area, circularity, perimeter, and diameter as described in the legend of Figure 1.

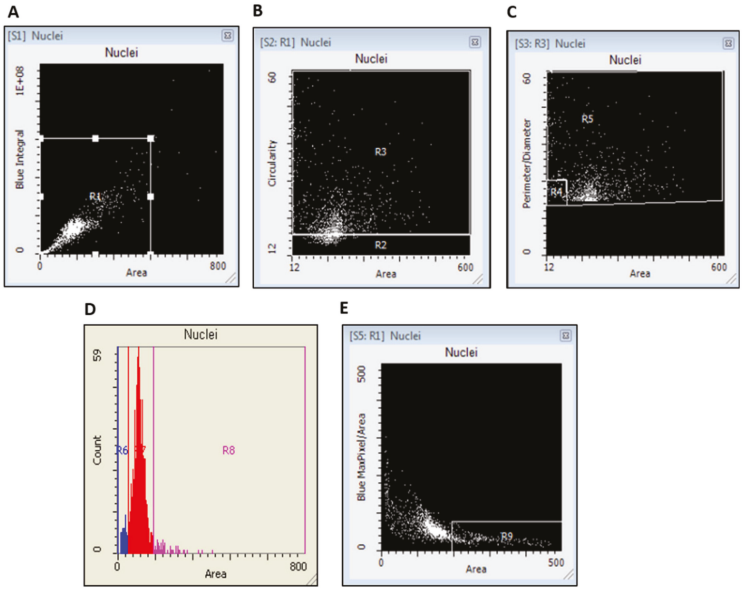

Figure 1. Scattergram and histogram for separation of buccal cell nuclei types by laser scanning cytometry (LSC). A representative example of DNA content scattergram and histogram for a participant from the control group. (**A**) A scattergram was generated to separate cells based on differences in nuclear staining and area by plotting their blue integral versus the area. Nuclei having area values that ranged from 0 to 600 μm^{-2} and blue integral values that ranged from 0 to 4×10^7 (arbitrary units) were separated in Region 1 (R1). (**B**) Nuclei in R1 were analysed by plotting their circularity (*y*-axis) versus nuclear area (*x*-axis) where "round" nuclei were identified in Region 2 (R2). (**C**) Nuclei from Region 3 (R3) were further analysed by plotting their perimeter/diameter ratio (*y*-axis) versus nuclear area (*x*-axis). Two new groups were identified from R3; long nuclei were identified in R4 and oval nuclei in R5. (**D**) A histogram plot of the same data in R1 showing the <2N, 2N, and >2N peaks as represented in R6, R7, and R8, respectively, and the respective frequency of DNA content events scored, showing majority of buccal cells being scored as 2N. (**E**) Nuclei in R1 were plotted against nuclear area versus the ratio of the maximal pixel intensity/area of DAPI fluorescence per nucleus. The cells in R9 had morphometric characteristics of cellular senescence (i.e., increased nuclear size (area) combined with decreased intensity of MaxPixel of DNA-associated fluorescence per nucleus, after DNA staining with DAPI).

2.5. Statistical Analysis

GraphPad Prism 6.01 (GraphPad Prism, San Diego, CA, USA) was used to statistically analyse the data. LSC γH2AX data were checked for normality using the D'Agostino and Pearson omnibus normality test. Differences in relative γH2AX signals in the lymphocytes from control, MCI, and AD groups were compared using the Kruskal–Wallis test for non-Gaussian distributed data followed by Dunn's multiple comparisons test. Correlation coefficients were obtained using Pearson's correlation coefficients for Gaussian distributed data and Spearman's rho for non-Gaussian distributed data. Analysed data are reported as mean ± standard error of the mean (SEM) with $p < 0.05$ considered statistically significant. Receiver-operating characteristic curves (ROC) were prepared for selected γH2AX parameters between the control and MCI or AD groups to obtain the area under the curve (AUC), sensitivity, specificity, confidence interval, and *p*-value.

3. Results

3.1. Clinical Characteristics of Participants

The mean age, gender distribution (male/female), body mass index (BMI), and MMSE score of AIBL participants in the control, MCI, and AD groups is shown in Table 1. There were no significant differences for gender ratio and BMI between the groups, while there was a significant difference in age ($p = 0.0039$) between control and AD group; however, there was no correlation of age with γH2AX ($r = 0.08$). As expected, there was a significant decrease in the MMSE scores of both the MCI ($p = 0.0126$) and AD ($p = 0.0001$) groups compared with the control group.

Table 1. Clinical characteristics of participants.

	Control n = 18	**MCI n = 17**	**AD n = 16**
Sex (M:F)	12:6	11:6	9:7
Age (years)	72.2 ± 1.5	78.7 ± 1.9	81.0 ± 1.8 **
BMI	27.0 ± 1.3	23.4 ± 1.3	24.8 ± 1.1
MMSE score	29.1 ± 0.2	26.0 ± 0.8 *	12.8 ± 1.8 ***

Means and standard error of the mean (SEM) are reported for each group. Significance was accepted at $p < 0.05$. Abbreviations: AD, Alzheimer's disease; F, female; M, male; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination score. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

3.2. Scoring of γH2AX Signals in Buccal Cells by LSC

All nuclei were separated and analysed according to their ploidy status (DNA content) as follows; <2N, 2N, and >2N as well as different nuclear shapes (round nuclei, long nuclei, oval nuclei) and cellular senescence status (see Figure 1). For 2N nuclei, the peak of the nuclei count coincided with the mean DAPI integral.

Fluorescence images of buccal cell nuclei containing discrete or diffuse γH2AX foci within nuclei were categorised into round, long, and oval nuclei as shown in Figure 2 [51]. Figure 3 summarises the data for the different γH2AX parameters measured (integral, MaxPixel, area, and foci/nucleus) for all nuclei from the control, MCI, and AD groups. Cells were also scored by their ploidy status (i.e., the data for <2N nuclei, 2N nuclei, >2N nuclei, round nuclei, long nuclei, and oval nuclei are shown in Supplementary Table S1). There was a significant increase in the γH2AX integral ($p = 0.0332$) in AD cells compared to control cells in all nuclei (Supplementary Table S1). Consistent with the increase in the γH2AX integral, a significant increase in the γH2AX MaxPixel value ($p = 0.0199$) and the number of γH2AX foci/nucleus ($p = 0.0234$) were also observed in AD cells compared to control cells (Figure 3A,B) and MCI vs. AD ($p = 0.0458$) as shown. Additionally, there was also a significant increase in the linear trend for the γH2AX MaxPixel value ($p = 0.0124$) across the groups (i.e., AD > MCI > control) in all nuclei (Figure 3A). However, there was no significant difference in the area of γH2AX foci (Supplementary Table S1).

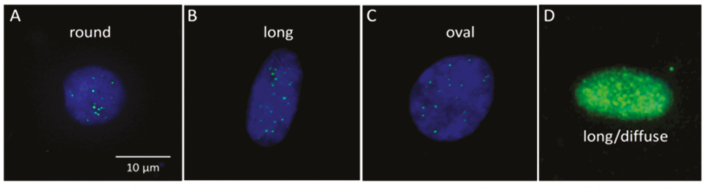

Figure 2. Representative shapes of buccal nuclei and γH2AX foci. Example of buccal cell nuclei visualised (stained with DAPI) with a fluorescence microscope. Nuclei were classified into three categories, i.e., round nuclei (**A**), long nuclei (**B**), and oval nuclei (**C**). Discrete γH2AX foci (green signal) were observed in (**A**–**C**) in these representative images. (**D**) A diffuse γH2AX signal within a nucleus.

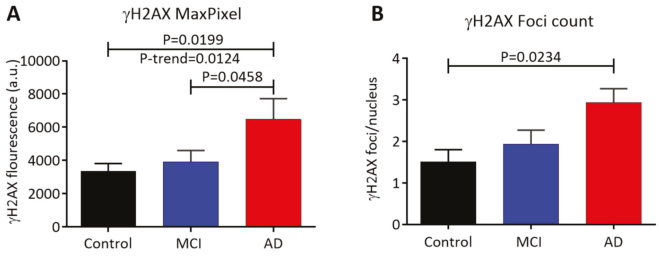

Figure 3. γH2AX MaxPixel and number of foci/nucleus in all cells. (**A**): γH2AX MaxPixel; (**B**): γH2AX foci/nucleus. These parameters were measured by LSC for control (n = 17), MCI (n = 18), and AD (n = 16). Abbreviations: a.u., arbitrary units; AD, Alzheimer's disease; MCI, mild cognitive impairment. Data are means ± SEM. *p*-values are shown.

The frequency (%) of round, long, and oval shaped nuclei was not significantly different between control, MCI, and AD groups (not shown). Supplementary Table S2 shows a significant increase was observed for the γH2AX integral ($p = 0.0123$), γH2AX MaxPixel ($p = 0.0014$), γH2AX area ($p = 0.0062$), and γH2AX foci/nucleus ($p = 0.0015$) in putative senescent cells when comparing AD versus control cells. The significant increase was also observed for the γH2AX integral ($p = 0.0349$), γH2AX MaxPixel ($p = 0.0134$), and γH2AX area ($p = 0.0345$) in AD senescent cells compared to MCI senescent cells (Supplementary Table S2). There were no differences in the percentage of senescent cells across the groups (Supplementary Table S3).

3.3. Nuclear (Morphology Characteristics) Circularity, Integral, and Area in Buccal Cells

The circularity of buccal cell nuclei in the control, MCI, and AD groups was also measured using the circularity feature available with the iCyte. A high circularity value indicates more irregular shaped nuclei; in contrast, the lowest circularity value indicates a perfect circle. There was a significant increase in nuclear circularity ($p = 0.0075$) in all nuclei of AD cells compared to control cells. In addition, a significant increase in nuclear circularity ($p = 0.0257$) was also observed in AD cells compared to MCI cells. A significant increase in the linear *p*-trend for the nuclear circularity value ($p = 0.0027$) was observed across the groups (i.e., AD > MCI > control) in all nuclei (Figure 4). For the nuclear integral and area, no significant difference was found between the control, MCI, and AD groups.

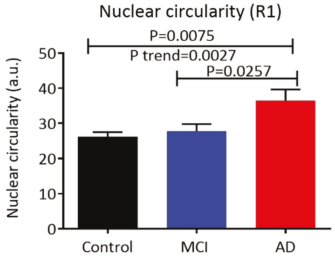

Figure 4. Circularity of buccal cell nuclei. Circularity of all buccal cell nuclei was measured in the control (n = 17), MCI (n = 18), and AD (n = 16) groups. Abbreviations: a.u., arbitrary units; AD, Alzheimer's disease; MCI, mild cognitive impairment. Data are means ± SEM.

3.4. Receiver-Operating Characteristic Curve

Since the γH2AX parameters (e.g., integral, γH2AX MaxPixel, γH2AX foci/nucleus) were significantly higher in AD compared to the control group for each category of nuclei, with evaluation of the diagnostic values of these parameters for discriminating AD patients from controls,

receiver operating characteristic (ROC) curves were generated. The area under the curve (AUC) values for γH2AX integral, MaxPixel, and foci/nucleus were 0.7353 (p = 0.2118), 0.7794 (p = 0.0062), and 0.7684 (p = 0.0086), respectively (Figure 5A–C). Of all parameters analysed using ROC curves, the γH2AX MaxPixel value showed the greatest value for the identification of AD, with 75% sensitivity and 70% specificity.

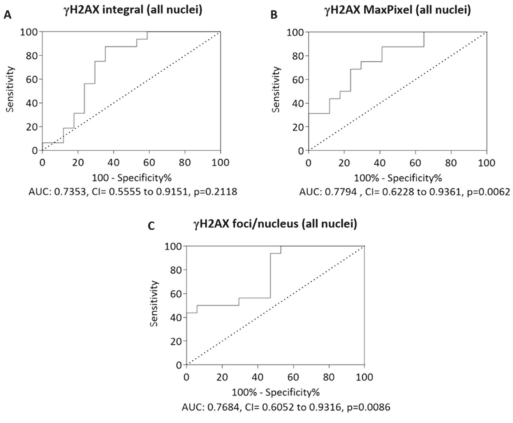

Figure 5. Receiver operating characteristic (ROC) curves for selected LSC-measured γH2AX parameters for control and AD nuclei. ROC curves were generated for the γH2AX integral (**A**), γH2AX MaxPixel (**B**), and γH2AX foci/nucleus (**C**) using measurements in buccal cells from control and AD cells. Abbreviations: AUC (area under the curve), CI (confidence interval).

3.5. Correlation of γH2AX Signals (Integral, MaxPixel) in Different Types of Buccal Cell Nuclei with the MMSE Score

To investigate whether the γH2AX signals in different types of buccal cell nuclei were related to the advancement of cognitive decline in the subjects, the correlations between the γH2AX integral, γH2AX MaxPixel, and MMSE scores were tested. Table 2 summarises the correlation coefficient (r) and p-values for each of the γH2AX parameters analysed in different types of buccal cell nuclei. The parameters highlighted in bold indicate that the γH2AX integral or MaxPixel negatively correlated with the MMSE score, to varying degrees, dependent on cell type analysed. For example, senescent cells had a strong negative correlation of γH2AX integral with the MMSE score (r = −0.5229, p = 0.0002).

Table 2. Summary of correlations between LSC scored γH2AX signals vs. MMSE score.

	Parameters	Correlation (r)	CI	p-Value
All nuclei	γH2AX integral	−0.1899	−0.4014–0.0408	0.0959
	γH2AX MaxPixel	−0.2266	−0.4331–0.0024	**0.0460**
Round	γH2AX integral	−0.3535	−0.5816 to −0.0737	**0.0148**
	γH2AX MaxPixel	−0.4550	−0.6565 to −0.1930	**0.0013**
Long	γH2AX integral	−0.3039	−0.5437 to −0.0183	**0.0378**
	γH2AX MaxPixel	−0.4141	−0.6268 to −0.1440	**0.0038**
Oval	γH2AX integral	−0.3534	−0.5816 to −0.0736	**0.0148**
	γH2AX MaxPixel	−0.4678	−0.6656 to −0.2086	**0.0009**
Senescent	γH2AX integral	−0.5229	−0.7044 to −0.2773	**0.0002**
	γH2AX MaxPixel	−0.5156	−0.6993 to −0.2680	**0.0002**

Parameters highlighted in bold text were considered statistically significant. All are Spearman's rho correlation. CI: 95% confidence interval.

3.6. Correlation of γH2AX Integral with Blood Parameters

Correlation tests were carried out between each of the blood parameters shown in Table 3 and the γH2AX integral values in all nuclei. Of all blood parameters analysed for correlation with γH2AX integral, only total protein was significantly correlated ($r = 0.332$, $p = 0.0389$). In addition, correlation tests were also performed between each of these blood parameters and the γH2AX MaxPixel values. There was no correlation of γH2AX MaxPixel with any blood parameters when data from all nuclei were analysed.

Table 3. Summary of the correlations tested between the γH2AX integral in buccal cells and blood measurements from the Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing (AIBL) cohort.

Parameters	Correlation (*r*)	95% Confidence Interval	*p*-Value
Homocysteine	0.0092	−0.1537–0.4472	0.9541
Serum folate	0.1617	−0.377–0.198	0.3125
Vitamin B12	−0.1295	−0.4205–0.1856	0.4195
Red cell folate	0.0005	−0.3151–0.3161	0.9975
Calcium	0.0422	−0.2770–0.3531	0.7985
Cholesterol	−0.0270	−0.1924–0.4290	0.4261
Triglycerides	−0.118	−0.3397–0.2911	0.8704
HDL	−0.1846	−0.4726–0.1391	0.2606
LDL	0.2371	−0.08484–0.5142	0.1461
Albumin	0.0305	−0.2879–0.3428	0.8539
Bilirubin	−0.2013	−0.4860–0.1220	0.2191
Urea	−0.0181	−0.3318–0.2992	0.9131
Creatinine	0.0134	−0.3035–0.3276	0.9354
eGFR	0.0427	−0.2766–0.3535	0.7964
Glucose	−0.2302	−0.5088–0.09207	0.1586
Total protein	**0.332**	**0.01837–0.5862**	**0.0389**
ALT	0.0088	−0.3077–0.3234	0.9579
AP	0.0101	−0.3065–0.3247	0.9514
GGT	0.0708	−0.2504–0.3779	0.6684
Ceruloplasmin	−0.2476	−0.5224–0.07374	0.1286
Fe	−0.2834	−0.5498–0.03533	0.0804
Transferrin	0.170	−0.1539–0.4608	0.3009
Trsat	−0.2688	−0.5387–0.05111	0.0980
Ferritin	−0.0201	−0.3336–0.2973	0.9031
Insulin	−0.1066	−0.4084–0.2163	0.5185
Testosterone	0.1546	−0.1692–0.4483	0.3472
LH	0.0245	−0.2933–0.3375	0.8822
FT4	0.1808	−0.1429–0.4696	0.2707
TSH	0.1425	−0.1812–0.4384	0.3868
FT3	0.1999	−0.1234–0.4849	0.2223
Cl	0.04746	−0.2722–0.3577	0.7742
AST	−0.1123	−0.4132–0.2108	0.4961
PCV	−0.0888	−0.3933–0.2334	0.5911
Mg	0.1919	−0.1317–0.4785	0.2418
RCC	−0.0009	−0.3165–0.3147	0.9952
MCV	−0.226	−0.5055–0.09647	0.1665
MCH	−0.2427	−0.5185–0.07897	0.1366
MCHC	−0.1327	−0.4303–0.1909	0.4206
RDW	−0.208	−0.4913–0.1152	0.2039
ESR	−0.1164	−0.4167–0.2068	0.4803
Platelets	−0.05805	−0.3669–0.2623	0.7255
MPV	−0.1251	−0.4239–0.1983	0.4481
WCC	−0.2584	−0.5307–0.06222	0.1122
Neutrophils	−0.2226	−0.5028–0.1001	0.1733
Lymphocytes	−0.1001	−0.4030–0.2225	0.5442
Monocytes	−0.2631	−0.5343–0.05722	0.1056
Eosinophils	−0.1277	−0.4261–0.1958	0.4386
Basophils	−0.2012	−0.4859–0.1222	0.2194

Parameters highlighted in bold text were considered statistically significant. Abbreviations: ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; Cl, chloride; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sediment rate; Fe, iron; FT3, free thyroxine; FT4, free triiodothyronine; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LH, luteinising hormone; MCH, mean cell haematocrit; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; Mg, magnesium; MPV, mean platelet volume; PCV, packed cell volume; RCC, red blood cell count; RDW, red cell volume distribution; Trsat, transferrin saturation; TSH, thyroid stimulation hormone; WCC, white cell count.

3.7. Correlation of γH2AX in Control, MCI and AD Nuclei with Blood Parameters

Correlation tests were carried out between each of these blood parameters and the γH2AX integral or the γH2AX MaxPixel values in control, MCI, and AD nuclei. Table 4 summarises the *r* and *p*-values obtained for γH2AX integral with each of the blood parameters. *p*-values highlighted in bold text indicates significant correlations. Albumin, AP, testosterone, and MCV positively correlated with γH2AX integral (Table 4) in MCI nuclei. Total protein, transferrin, LH, FT4, MCH, and MCHC correlated with γH2AX integral in control nuclei. There was no correlation of any of the blood parameters with γH2AX in the AD group.

Table 4. Summary of the correlations tested between γH2AX integral scores in buccal cells and blood parameters in the control, MCI, and AD groups from the AIBL cohort.

	Control	MCI	AD
Homocysteine	$r = -0.070$, $p = 0.804$	$r = 0.514$, $p = 0.106$	$r = -0.175$, $p = 0.518$
Serum folate	$r = 0.193$, $p = 0.491$	$r = 0.256$, $p = 0.448$	$r = 0.134$, $p = 0.635$
Vitamin B12	$r = -0.041$, $p = 0.883$	$r = -0.293$, $p = 0.382$	$r = -0.243$, $p = 0.383$
Red cell folate	$r = 0.288$, $p = 0.299$	$r = 0.003$, $p = 0.993$	$r = -0.149$, $p = 0.595$
Calcium	$r = -0.041$, $p = 0.884$	$r = -0.433$, $p = 0.244$	$r = 0.065$, $p = 0.817$
Cholesterol	$r = 0.467$, $p = 0.079$	$r = -0.279$, $p = 0.467$	$r = -0.072$, $p = 0.799$
Triglycerides	$r = 0.114$, $p = 0.685$	$r = -0.516$, $p = 0.155$	$r = -0.033$, $p = 0.906$
HDL	$r = 0.194$, $p = 0.489$	$r = -0.266$, $p = 0.488$	$r = -0.292$, $p = 0.292$
LDL	$r = 0.465$, $p = 0.080$	$r = -0.016$, $p = 0.968$	$r = 0.292$, $p = 0.802$
Albumin	$r = 0.209$, $p = 0.454$	**$r = 0.724$, $p = 0.027$**	$r = -0.018$, $p = 0.951$
Bilirubin	$r = -0.286$, $p = 0.300$	$r = -0.173$, $p = 0.656$	$r = -0.187$, $p = 0.504$
Urea	$r = 0.500$, $p = 0.058$	$r = -0.181$, $p = 0.640$	$r = -0.326$, $p = 0.236$
Creatinine	$r = -0.276$, $p = 0.320$	$r = 0.407$, $p = 0.277$	$r = -0.038$, $p = 0.893$
eGFR	$r = 0.186$, $p = 0.508$	$r = -0.259$, $p = 0.502$	$r = 0.092$, $p = 0.745$
Glucose	$r = -0.457$, $p = 0.087$	$r = 0.112$, $p = 0.775$	$r = -0.175$, $p = 0.534$
Total protein	**$r = 0.557$, $p = 0.031$**	$r = 0.127$, $p = 0.745$	$r = 0.133$, $p = 0.636$
ALT	$r = -0.224$, $p = 0.421$	$r = 0.109$, $p = 0.779$	$r = -0.035$, $p = 0.901$
AP	$r = -0.189$, $p = 0.498$	**$r = 0.681$, $p = 0.043$**	$r = -0.046$, $p = 0.870$
GGT	$r = -0.108$, $p = 0.700$	$r = -0.087$, $p = 0.824$	$r = 0.025$, $p = 0.931$
Ceruloplasmin	$r = -0.133$, $p = 0.638$	$r = -0.149$, $p = 0.703$	$r = -0.294$, $p = 0.287$
Fe	$r = -0.298$, $p = 0.280$	$r = -0.385$, $p = 0.306$	$r = -0.309$, $p = 0.261$
Transferrin	**$r = 0.628$, $p = 0.012$**	$r = -0.225$, $p = 0.560$	$r = -0.034$, $p = 0.904$
Trsat	$r = -0.344$, $p = 0.209$	$r = -0.294$, $p = 0.442$	$r = -0.282$, $p = 0.308$
Ferritin	$r = -0.252$, $p = 0.366$	$r = 0.025$, $p = 0.949$	$r = -0.100$, $p = 0.721$
Insulin	$r = -0.162$, $p = 0.565$	$r = 0.013$, $p = 0.975$	$r = 0.280$, $p = 0.310$
Testosterone	$r = -0.162$, $p = 0.565$	**$r = 0.684$, $p = 0.042$**	$r = 0.175$, $p = 0.532$
LH	**$r = 0.522$, $p = 0.046$**	$r = -0.235$, $p = 0.542$	$r = -0.177$, $p = 0.527$
FT4	**$r = 0.648$, $p = 0.009$**	$r = -0.078$, $p = 0.842$	$r = 0.155$, $p = 0.582$
TSH	$r = 0.228$, $p = 0.411$	$r = 0.056$, $p = 0.887$	$r = 0.146$, $p = 0.603$
FT3	$r = 0.431$, $p = 0.109$	$r = -0.014$, $p = 0.972$	$r = 0.115$, $p = 0.684$
Cl	$r = -0.173$, $p = 0.650$	$r = -0.269$, $p = 0.485$	$r = 0.173$, $p = 0.538$
AST	$r = -0.173$, $p = 0.536$	$r = 0.032$, $p = 0.935$	$r = -0.185$, $p = 0.508$
PCV	$r = -0.267$, $p = 0.335$	$r = 0.074$, $p = 0.850$	$r = -0.061$, $p = 0.829$
Mg	$r = -0.016$, $p = 0.954$	$r = 0.263$, $p = 0.495$	$r = 0.255$, $p = 0.359$
RCC	$r = -0.081$, $p = 0.773$	$r = 0.279$, $p = 0.467$	$r = -0.071$, $p = 0.799$
MCV	$r = -0.425$, $p = 0.115$	**$r = -0.678$, $p = 0.045$**	$r = -0.045$, $p = 0.871$
MCH	**$r = -0.658$, $p = 0.008$**	$r = -0.657$, $p = 0.055$	$r = 0.054$, $p = 0.848$
MCHC	**$r = -0.689$, $p = 0.005$**	$r = -0.479$, $p = 0.193$	$r = 0.307$, $p = 0.265$
RDW	$r = -0.197$, $p = 0.481$	$r = 0.213$, $p = 0.582$	$r = -0.378$, $p = 0.165$
ESR	$r = -0.157$, $p = 0.577$	$r = -0.209$, $p = 0.589$	$r = -0.186$, $p = 0.507$
Platelets	$r = 0.049$, $p = 0.861$	$r = 0.265$, $p = 0.490$	$r = -0.158$, $p = 0.576$
MPV	$r = 0.057$, $p = 0.844$	$r = -0.143$, $p = 0.713$	$r = -0.438$, $p = 0.103$
WCC	$r = -0.163$, $p = 0.563$	$r = 0.369$, $p = 0.327$	$r = -0.473$, $p = 0.075$
Neutrophils	$r = -0.292$, $p = 0.291$	$r = 0.588$, $p = 0.096$	$r = -0.496$, $p = 0.059$
Lymphocytes	$r = 0.412$, $p = 0.127$	$r = -0.356$, $p = 0.347$	$r = -0.206$, $p = 0.460$
Monocytes	$r = -0.420$, $p = 0.119$	$r = 0.091$, $p = 0.815$	$r = -0.335$, $p = 0.223$
Eosinophils	$r = 0.015$, $p = 0.958$	$r = -0.517$, $p = 0.154$	$r = -0.218$, $p = 0.435$
Basophils	$r = -0.171$, $p = 0.542$	$r = 0.408$, $p = 0.275$	$r = -0.331$, $p = 0.226$

Parameters highlighted in bold text were considered statistically significant. Abbreviations: ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; Cl, chloride; eGFR, estimated glomerular filtration rate; ESR, erythrocyte sediment rate; Fe, iron; FT3, free thyroxine; FT4, free triiodothyronine; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LH, luteinising hormone; MCH, mean cell haematocrit; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; Mg, magnesium; MPV, mean platelet volume; PCV, packed cell volume; RCC, red blood cell count; RDW, red cell volume distribution; Trsat, transferrin saturation; TSH, thyroid stimulation hormone; WCC, white cell count.

4. Discussion

The objective of this study was to investigate whether buccal cells from MCI and AD patients have higher levels of endogenous γH2AX (a biomarker of double-strand DNA breaks) compared with healthy controls, with the ultimate aim of testing whether the buccal cell γH2AX assay might be useful as a diagnostic test for those with cognitive impairment and or AD. The γH2AX assay offers an excellent opportunity to robustly measure the levels of DNA double-strand breaks and cellular response in individuals or populations and test its suitability for clinical purposes [56–58]. The LSC method was used to quantify endogenous γH2AX in buccal cells from individuals who met the clinical criteria for MCI or AD and in healthy controls. The results of this study showed increased levels of γH2AX (thus DNA damage) in the buccal cells of patients with AD compared to those in cells from MCI patients or healthy controls, and there was a concomitant increase with a linear trend from the control group through MCI to the AD group. This result was further supported by the significantly increased negative correlation between γH2AX signals and MMSE scores when the analysis included all subjects. The LSC protocol developed here simultaneously quantifies different γH2AX parameters (integral, MaxPixel, area, foci/nucleus) in cells with different nuclear DNA content (ploidy status) as well as cells with different morphological features such as nuclear shapes, based on their area, perimeter, diameter, and circularity. Nuclear circularity (irregular nuclear shape) was increased significantly in AD cells compared to control cells and there was a concomitant increase with a linear trend from controls through MCI to AD. A significant positive correlation was also observed between nuclear circularity and γH2AX signals in the different types of nuclei analysed. The results of this study demonstrate that buccal cells exhibit increased levels of endogenous γH2AX in AD cells relative to those from MCI patients and healthy controls, and suggest the possibility of using γH2AX as a potential marker for determining those individuals with MCI that may be progressing to AD.

At present, the analysis of Aβ (1−42), total tau, and phospho-tau-181 in CSF allows reliable, sensitive, and specific diagnosis of AD, but the collection of CSF is an invasive procedure with potential random variation in AD-specific biomarker measurements [59–61]. Thus, there is a clear need to search for inexpensive and minimally invasive surrogate markers to diagnose and monitor AD progression. The use of surrogate cells, and particularly exfoliated buccal cells, is of particular interest since buccal cell collection is reliable, fast, relatively simple, cheap, minimally invasive, and painless. Since both the human nervous system and buccal cells are of ectodermal origin, the regenerative potential of the brain, which has been found to be altered in AD, may be mirrored in the buccal mucosa. Studying the buccal mucosa cells from healthy individuals revealed decreased nuclear diameter and cell diameter with increasing age [62]. Another study showed a decrease in the thickness of the epidermis and underlying cell layer with increasing age [63]. It is possible that the lack of regenerative potential of buccal cells from MCI and AD patients may be a consequence of accelerated ageing. A previous study has investigated the formation of micronuclei (a cytogenetic marker of either chromosome segregation or breakage) in buccal mucosa cells. An increased micronuclei frequency was observed in patients with AD compared to age- and gender-matched controls [64]. The same study also reported an abnormal cytome profile characterised by a lower frequency of basal cells, condensed chromatin, and karyorrhectic cells in AD patients, suggesting reduced regenerative capacity in buccal cells from AD patients. Another study showed a significant 1.5-fold increase in trisomy 21 and a significant 1.2-fold increase in trisomy 17 in buccal cells of AD patients compared to matched controls [34], providing further evidence of abnormalities in buccal cells in AD patients. A number of studies have been conducted to assess the association between astrocyte degeneration and DNA damage in AD by investigating the γH2AX signals in astrocytes from the hippocampal region [46,47]. The results from these studies demonstrated increased γH2AX signal in the nuclei of cells from AD patients compared to those from healthy controls. To the best of the researcher's understanding, there are no earlier reports investigating the levels of γH2AX in buccal cells and their ability to distinguish those individuals with MCI and AD from those of control patients. Since the level of DNA DSBs in buccal cells, as marked by γH2AX immunostaining, has not been previously used to investigate the pathogenesis of AD,

the findings from this study support the previous observation of increased γH2AX signals in nuclei of astrocytes from AD patients relative to those of healthy controls [46,47]. In the present study, there was an increasing linear trend in the γH2AX MaxPixel values observed in control through to MCI and AD cells, suggesting that buccal cells from MCI patients may be more susceptible to DNA damage than those from healthy controls. There are no reports investigating γH2AX in buccal cells from MCI patients compared to those from healthy controls; however, the insights from our previous studies carried out in lymphocytes are in line with the observations of the current study, and demonstrate a significant increase in oxidative DNA damage (oxidised DNA bases) in lymphocytes from an MCI group compared with a control group [44]. It is of interest to explore whether MaxPixel γH2AX in AD nuclei represent some unique type of DNA damage (e.g., a site of clustered DSBs).

ROC curve analysis was carried out to assess the diagnostic accuracy of γH2AX assay in identifying individuals with AD from controls. ROC curve for LSC scored γH2AX MaxPixel yielded the area under the ROC curve value of 0.7794 with 75% sensitivity and 70% specificity for the AD ($p = 0.0062$) group, suggesting that measurement of γH2AX MaxPixel in the buccal cell might be useful in discriminating AD and control. Although the good sensitivity and specificity achieved in this study are promising for the value of γH2AX assay in identifying AD from control, given the relatively low number of participants tested within each group, and the lack of defined γH2AX thresholds for determining of test positivity, we cannot currently recommend its routine use in clinical practice. Therefore, it is important to clearly demonstrate its accuracy involving larger numbers of participants tested within each group and standardise the γH2AX assay by validating the stringent cutoff point of test positivity prior to it being widely used routinely for differentiating AD from non-AD and from control.

In this study, irregular nuclear shapes (circularity) were measured using the circularity parameter of LSC in different types of nuclei (e.g., all nuclei, <2N nuclei, 2N nuclei, >2N nuclei). A higher circular value indicates a more irregular nuclear shape, and correspondingly, normal ageing affects nuclear shape that may involve defects in lamins [65]. The results showed a significantly higher circularity in all nuclei of AD cells compared to control cells, as well as in AD cells compared to MCI cells. The higher circularity in AD cells compared to control and MCI cells might be due partly to the accumulation of DNA damage leading to morphometric and cytometric alterations in the buccal mucosa cells of AD patients. Previously, the morphological and cytometric parameters of buccal cells have been assessed using microscopy and ImageJ analysis, respectively, following Papanicolaou staining [66]. The results from that study showed a significant decrease in the number of intermediate buccal cells in the AD group compared to the control group [66]. In addition, evidence of increased levels of DNA damage, indicated by the formation of micronuclei (a biomarker of chromosome mis-segregation) has been previously detected in buccal cells from AD patients and Down syndrome cases who have a high risk of developing AD [64,67]. In our study, the γH2AX integral and MaxPixel values were positively correlated with nuclear circularity in the different types of buccal cell nuclei analysed (data not shown), which may reflect the fact that DNA damage in these cells is associated with an irregular nuclear shape. It is possible that the increased DNA damage in those irregularly shaped nuclei is associated with altered nuclear lamina structure. The nuclear lamina is a filamentous structure under the inner nuclear membrane composed of A-type and B-type lamins [68,69]. Recent studies show that the deficient A-type lamin is associated with altered structural nuclear proteins with a variety of human diseases, including severe premature ageing syndromes. Indeed A-type-lamin-deficient cells have been associated with impaired DNA repair capacity and maintaining telomere localisation, structure, length, and function [70,71]. Moreover, loss of A-type-lamin-leads to localisation of telomeres away from the nuclear membrane towards the center of the nucleus [71]. Colocalisation of γH2AX with a telomere DNA probe allowed visualisation of dysfunctional telomeres [72–74]. A previous report in human buccal cells of AD patients showed significantly shortened telomeres in an older AD group in comparison with older controls [43]. Therefore, it is plausible that the positive correlation between nuclear circularity and γH2AX in buccal cells of AD patients observed in this study may be linked with deficient nuclear lamin contributing to telomere dysfunction. Future studies should explore whether

the γH2AX signals in buccal cells of AD patients are mostly in the nuclear periphery or aggregated centrally and associated with dysfunctional telomeres which may be due to deficient A-type lamin coupled with increased nuclear circularity. It is possible that irregular nuclear shape caused by a defect in lamins lead to telomere dysfunction and/or shortening. Taken together, altered nuclear morphology, cellular structure, and increased levels of DNA damage associated with dysfunctional telomeres in buccal cells may contribute to the irregular nuclear shape observed in buccal cells of AD patients. A further study of changes in nuclear circularity coupled with multiple DNA damage markers (e.g., γH2AX, 8HOdG) associated with telomere dysfunction and AD-specific markers (e.g., putative tau, Aβ) in buccal cells from a large patient cohort will better assess the likelihood of discriminating AD and MCI patients from healthy controls using these tests.

Cellular senescence is elicited in damaged cells and characterised by the presence of γH2AX, and senescence-associated β-galactosidase (SA-β-gal) activity, and is detectable by immunocytochemistry [75,76]. Previous studies have shown increased number of senescent nuclei during ageing and in age-related diseases [75,76]. It is accepted that older animals exhibit more cellular senescence than younger animals as demonstrated by increased p16 (INK4a), senescence associated β-galactosidase activity, and γH2AX positive signals [73,77,78]. The morphological features of senescent nuclei in cultured fibroblasts after methotrexate (Mtx) treatment have been assessed using the features available in the iCyteR software for LSC [79,80]. In a recent study [79], senescent nuclei were isolated based on the criteria of decreased levels of DAPI staining (MaxPixel staining) paralleled by increases in nuclear size (area) and the simultaneous expression of senescence markers (e.g., the p21WAF1, p16INK4a, or p27KIP1 cyclin kinase inhibitors), and demonstrating that senescent nuclei are flattened and larger in size. To date, the morphological features of senescent nuclei in buccal cells have not been assessed using the features available in LSC. In this study, putative senescent nuclei were identified by plotting the ratio of MaxPixel intensity of DAPI fluorescence per nucleus to nuclear area versus the nuclear size (area). A significant increase in the γH2AX signal was observed in senescent nuclei of AD cells compared to control and MCI cells for all individual γH2AX parameters measured by LSC, suggesting that accumulation of DNA DSBs may contribute to cellular senescence and impaired repairing capacity of senescent nuclei may ultimately contribute to the risk of developing AD. Although previous studies in cultured fibroblasts have characterised the morphological features of senescent nuclei using immunocytochemical analysis of the expression of additional senescent markers, such as the p21WAF1, p16INK4a, or p27KIP1 cyclin kinase inhibitors, our study did not confirm this, but rather attempted for the first time to identify senescent nuclei of control, MCI, and AD cells by their morphometric features alone. It is important to note that senescent cells showed the strongest negative correlation for γH2AX integral and γH2AX MaxPixel in relation to MMSE scores. While investigating the morphological features of senescent buccal cells is important, it is also important for future research to simultaneously measure the expression of senescence markers in conjunction with DNA damage markers (e.g., γH2AX) and AD-specific markers (e.g., aβ1-42, total tau, and phosphorylated-tau) in buccal cells in order to discriminate AD and MCI patients from healthy controls.

In the present study, from all of the blood parameters examined, only total protein showed a positive correlation with buccal cell γH2AX signals when all samples were analysed together. Correlations between blood parameters and buccal cell γH2AX signals in the control, MCI, and AD groups were further assessed in three separate tests. Although a significant correlation between buccal cell γH2AX signals and several blood parameters (e.g., albumin, total protein, transferrin, FT4, FT3, MCH, MCV) in control and MCI group was observed, in the AD group, no blood parameters showed a significant correlation with buccal cell γH2AX signals. The negative correlation with MCV and MCH are important because these are biomarkers of anemia, which was previously shown to be a risk factor for MCI and AD in the AIBL study [81]. In this study, the positive correlation between transferrin and γH2AX signals suggests that the plasma transferrin levels may have a role in increasing γH2AX signals in AD. However, a previous study showed lower serum transferrin levels in AD patients compared with controls [82]. These results strongly suggest that the development of pathological features of AD

is not restricted to the brain but is associated with multiple metabolic changes occurring in peripheral cells [27].

5. Conclusions

To date, no studies have assessed the presence of γH2AX in the buccal cells of AD patients relative to control and MCI patients, and the available literature on the use γH2AX as a DNA DSB marker in ageing populations is not yet sufficient to understand the association between DNA DSBs and AD. Identification of reliable biomarkers in non-invasive samples will be useful for early diagnosis and treatment of AD, which may prevent the onset of irreversible AD and reduce the overall economic and human cost of the disease. Buccal cells offer a sample source that is easily obtained in a relatively non-invasive manner. The LSC-based γH2AX protocol may be converted to an ELISA-type format or other simpler analytical technique for cellular γH2AX and therefore may provide a practical tool for assessing DNA DSBs in buccal cells of control, MCI, and AD patients. The levels of γH2AX in buccal cells quantified by LSC may have prognostic implications to understand the pathogenesis of AD better and offer the opportunity to monitor disease progression and the bioefficacy of potential preventative measures (i.e., diet, lifestyle, and therapeutics). Moreover, LSC provides identification and quantification of buccal cell subtypes based on cellular features that were previously not measurable (e.g., nuclear shape, DNA content, nucleus size, nucleus MaxPixel value). Scoring of buccal cell nuclear parameters in conjunction with multiple DNA damage parameters and AD-specific markers will be useful to establish a potential biomarker panel with high specificity for AD patients. Thus, the combination of cytome and proteome approaches to a single sampling of buccal cells may significantly increase the sensitivity and/or specificity for AD diagnosis, which will have relevance not only for future clinical practice but also for the reliable prediction of those individuals who are likely to develop MCI and AD and also to monitor the bioefficacy of a preventative strategy. The buccal cell γH2AX assay may provide a useful method for AD and MCI diagnosis, particularly when sample collection must occur remotely and/or in disadvantaged communities unable to attend more expensive prognostic or diagnostic testing facilities. In this study, a small sample was analysed; therefore, comprehensive studies using large prospective cohorts are warranted in order to validate the suitability of the LSC-based buccal cell γH2AX assay, particularly to identify those in the early stages of AD.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-1729/10/8/141/s1, Table S1. Summary of one-way ANOVA tests for different γH2AX parameters measured using LSC in different types of buccal cell nuclei; Table S2. Summary of the one-way ANOVA tests for different γH2AX parameters in putative senescent nuclei; Table S3. Summary of the one-way ANOVA tests for % of senescent nuclei across Control, MCI, and AD.

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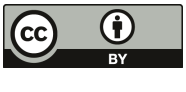

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Review

Loss of p16: A Bouncer of the Immunological Surveillance?

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Abstract: p16^{INK4A} (hereafter called p16) is an important tumor suppressor protein frequently suppressed in human cancer and highly upregulated in many types of senescence. Although its role as a cell cycle regulator is very well delineated, little is known about its other non-cell cycle-related roles. Importantly, recent correlative studies suggest that p16 may be a regulator of tissue immunological surveillance through the transcriptional regulation of different chemokines, interleukins and other factors secreted as part of the senescence-associated secretory phenotype (SASP). Here, we summarize the current evidence supporting the hypothesis that p16 is a regulator of tumor immunity.

Keywords: senescence-associated secretory phenotype (SASP); senescence; cell-cycle; melanoma; pancreatic adenocarcinoma; tumor infiltration; chemotherapy resistance

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1. Introduction

Since the 19th century, immunologists have been speculating about the idea that the immune system may be a strong, efficient, and specific weapon against cancer (reviewed in [1]). However, it was not until the beginning of the 21st century that scientists began to understand the mechanisms behind tumor immunity and to develop immunotherapy regimens [2]. In parallel, the senescence field discovered that senescent cells, although in a stable state of cell cycle arrest [3], are highly active and acquire a pro-inflammatory microenvironment termed the senescence-associated secretory phenotype (SASP) [4,5]. Through the SASP, senescent cells can modify their microenvironment and regulate other cells, including cells of the immune system [5–10].

Among the multiple pathways that are commonly deregulated in cancer and senescence [11], the p16^{INK4A} (hereafter called p16) pathway is particularly intriguing. On one hand, loss of p16 is a common feature of cancer that causes an increase in the proliferative capacity of the cell [12]; on the other hand, upregulation of p16 is a hallmark of senescence that contributes to the characteristic state of cell cycle arrest [13]. Interestingly, recent publications demonstrate that suppression of p16 correlates with decreased activity of immune cells [14–17], and our recent publication shows that p16 suppression decreases expression of the SASP [18]. Altogether these data suggest that p16 may have a, yet unknown, role in the regulation of tumor immunity that might have important implications in the treatment of cancers with low or null p16 expression. In this review, we describe the roles of p16 and the SASP in both senescence and cancer and dissect the latest publications that support the hypothesis that p16 may be a regulator of tumor immunity.

2. p16 in Cancer and Senescence

Cyclin Dependent Kinase Inhibitor 2A (*CDKN2A*) located in chromosome 9p21 is a tumor suppressor gene that encodes p14^{ARF} (p19^{ARF} in mice, hereafter p14 and p19) and p16 proteins using two different open reading frames. While p14^{ARF} is involved in

the regulation of the p53 pathway (reviewed in [19]), p16′s canonical role is to inhibit the assembly and activation of the cyclin-dependent kinases CDK4/6, impairing the hyperphosphorylation of the retinoblastoma (RB) protein and the E2F-mediated expression of proliferation-promoting genes [20]. Due to its role as cell cycle brake, it is not surprising that ~50% of human cancer shows decreased expression of *CDKN2A* [12]. Interestingly, although the most common alterations of p16 are deletions and promoter hypermethylation affecting both p16 and p14 [21–23], cancer-associated mutations are more commonly found in p16 than p14 [24], suggesting a critical regulatory role of p16 in the cell. Importantly, loss of p16 alone is not enough to produce cancer, mainly because normal cells have other mechanisms to abrogate the cell cycle progression (e.g., p53, CHK1/2, APC) (reviewed in [25]). However, it has been shown that the suppression of p16 facilitates malignant transformation of cells upon different hyperproliferative signals and stressors such as oncogenes, oxidative stress, ionizing radiation, and others. All together these data suggest that tight regulation of p16 expression is critical to maintain healthy cellular proliferation.

On the other hand, p16 is known to be highly expressed during cellular senescence [26–28]. Senescence is a stable state of cell cycle arrest acquired upon different stressors such as aberrant proliferative signals or DNA damage among others [29]. Since cells are continuously affected by a wide variety of stressors, it is not surprising that a wide variety of senescence inducers exist, including: oncogenes, ionizing radiation, genotoxic chemicals, reactive oxygen species, chemotherapeutic agents or shortage in dNTPs among others (reviewed in [30]). Interestingly, in the vast majority of cases, the distinctive cell cycle arrest is achieved by upregulation of the p16 protein as a direct consequence of the pathways (e.g., p38 and ERK) [31–34], epigenetic factors (e.g., polycomb) [35–37] and transcription factors (e.g., ETS2 and AP-1) [38–41] altered by the senescence inducers. In this regard, several groups including our laboratory have shown that suppression of p16 can bypass senescence [42–46], indicating that p16 is critical to maintain the senescence phenotype.

In addition to its canonical role regulating the cell cycle, an increasing amount of evidence indicates that p16 has non-canonical, RB-independent roles. For instance, our laboratory found that suppression of p16 bypasses oncogene-induced senescence in part by promoting an increase in nucleotide and deoxyribonucleotide levels in a mechanism mediated by the mTORC1 complex and in an RB-independent manner (i.e., operating outside the cell cycle) [42]. Additionally, p16 has been found to regulate tumor suppressive miRNAs, mitochondria biogenesis, oxidative stress, transcription factors such as AP-1 and NF-κB or protein translation though EEF1A2 (reviewed in [47]). Altogether this shows that p16 is not a simple cell cycle brake, but also a regulator of other processes. Indeed, the observation that the large majority of cancer-associated mutations targeting *CDKN2A* are mainly affecting the p16 open reading frame, reinforces the importance of p16 as a regulator of cellular physiology and stresses the necessity to further investigate p16-mediated regulatory processes.

3. Role of SASP in Senescence and Cancer

Together with the signature cell cycle arrest, the SASP is one of the most prominent phenotypes of senescent cells. The SASP is composed of various soluble and non-soluble factors including cytokines, chemokines, and proteases that are highly expressed and secreted by senescent cells, creating a pro-inflammatory microenvironment that affects themselves and other non-senescent cells in an autocrine and paracrine fashion, respectively [48,49]. In part because of its ability to modify the environment and impact the behavior of other cells, the SASP is tightly regulated at multiple levels. Transcriptionally, various factors (C/EBPβ and NF-κβ) [9,10], upstream regulators (p38, MAPK, GAT4A, p53, or ATM) [5,50–53] and non-coding RNAs (miRNAs, lncRNAs and circRNAs) [54] have been described to regulate SASP expression. Additionally, mTORC1-mediated translational regulation of MAPKAPK2 and IL1A has been shown to impact several SASP factors [55,56]. Finally, during senescence, there is a rearrangement of the genomic architecture leading

to a new TAD (topologically associated domain) landscape [57,58]. This rearrangement is induced by changes in DNA methylation, nucleosome organization and histone modification, giving rise to the so-called senescence-associated heterochromatin foci (SAHF) [59–64]. There is a close relationship between the SAHF and the SASP and multiple epigenetic modifiers such as HMGB2, BRD4, MLL1, macroH2A1 or SIRT1, among others, have been shown to impact expression of the SASP [65–69]. In this regard, recent work from our laboratory shows that increased expression of the histone methyltransferase DOT1L upon oncogene-induced senescence, drives the expression of the major SASP-inducer IL1A though increased deposition of the active histone marks H3K79me2/3 at the *IL1A* gene loci [70].

Due to its inherent arrest of the cell-cycle described above, cellular senescence has been considered a bona fide tumor suppressor mechanism [71,72]. However, in the late 90s and early 2000s new experiments demonstrated that senescent cells can promote cellular proliferation and tumor growth due to acquisition of the SASP [7,73–75]. The SASP is highly dynamic and variable depending on several factors such as genetic background, cell type, the inducer of senescence, and the time of which senescence has occurred [76–79]. Due to its complex and variable nature, physiological roles of the SASP have not been well delineated. On one side, the SASP has been shown to contribute to the immunological surveillance, i.e., the process whereby the cells of the innate and adaptative immune system detect and destroy damaged cells [80–82]. For example, SASP secretion by senescence hepatocytes promotes T-cell mediated immunological surveillance within the liver, promoting the clearance of pre-malignant senescent cells and hence avoiding tumor progression [83]. On the other hand, the SASP has also been shown to contribute to numerous detrimental effects such as tumor promotion and progression and therapy resistance (reviewed in [84]). For instance, it has been described that some SASP secreted by senescent stromal cells promote an immunosuppressive microenvironment increasing the number of myeloid-derived suppressors cells (MDSCs), thus impairing immunological surveillance and promoting tumor growth [85,86]. In this regard, the dynamic nature of the SASP and its time-dependent regulation seems to be key to understanding the interaction with the immune system and hence the positive or negative outcome in the tumoral area [76,87]. Additionally, the SASP is not exclusive to senescent cells, and inflammatory phenotypes similar to the SASP, known as "SASP-like", have been described in tumor cells [67,88]. Indeed our laboratory has found that similar to senescent cells, different tumor types display different SASP (SASP-like) profiles [18], further demonstrating the wide variability of the SASP. Whether this indicates that those tumors bypassed senescence at some point during their malignant transformation is still unknown and more research is needed. However, it is becoming clearer that both the senescent cells in the tumor environment and the tumor cells themselves contribute to the maintenance of a SASP-driven inflammatory microenvironment. Therefore, it is imperative to delineate the mechanisms of SASP expression and secretion as well as map the SASP composition upon different conditions, tissue types and timeframes to design efficient and personalized immunotherapies.

4. p16 Regulation of Tumor Immunity

We previously described that canonical and non-canonical roles of p16 in part regulate cellular homeostasis. However, is it possible that p16 regulates other processes that impact the cellular microenvironment? Recent evidence demonstrates that loss of *CDKN2A* expression in tumor cells correlates with different immunological processes within the tumor that may impair immunological surveillance suggesting that p16 not only regulates cellular homeostasis but also tissue homeostasis. Below we will dissect the current evidence that implicates p16 as a regulator of intratumor immunity.

The first piece of evidence was published by Balli et al., [14]. These authors used a previously published expression signature based on two key cytolytic effectors (*GZMA* and *PRF1*) upregulated upon CD8+ T cell activation [89] to assess the intratumoral cytolytic T-cell activity in pancreatic adenocarcinoma samples from The Cancer Genome Atlas (TCGA).

Interestingly, non-silent mutations and deletions of *CDKN2A* correlated with decreased cytolytic activity. One year later, Wartenberg et al. [15] investigated the immune cell composition within the microenvironment in a series of pancreatic ductal adenocarcinoma and cross compared it with a high-throughput analysis of somatic mutations. They found that high mutation rates of *CDKN2A* correlate with a so-called immune-escape microenvironment, which is a microenvironment poor in T and B cells and enriched in FOXP3+ Tregs. Consistent with this result, Morrison et al. [16] found that loss of *CDKN2A* significantly correlates with immune deserts, defined by a profile of 394 immune transcripts. These pieces of evidence suggest that low *CDKN2A* expression both impacts the number and the activity of the intratumoral immune cells. Moreover, suppression of *CDKN2A* in mesenchymal stromal cells has been shown to decrease CD11b+ Gr-1^{hi} neutrophils, CD11b+ Gr1^{low} monocytes and CD45-CD31-Integrinα7+ satellite cells in a model of chronic inflammatory myopathy [17]. Although all those investigations are based on correlative analysis, these data may indicate that *CDKN2A* is necessary for regulation of the physiological immune response upon different inflammatory events. However, it should be noted that there are some publications that disagree with this thesis [90–92]. Thus, further mechanistic studies are necessary to determine in which circumstances suppression of *CDKN2A* in non-immune cells decreases immunological surveillance.

How does the suppression of p16 abrogate immunological surveillance? A recent publication from our laboratory demonstrates that the specific knockdown of p16 in oncogene-induced senescent cells leads to decreased expression of several SASP genes, including the most well characterized cytokines *IL6*, *CXCL8* and *CSF3*, the proteases *MMP3*, *PLAU* and *PLAT*, the growth factors *AREG*, *EREG* and *VEGFA* and the glycoprotein *ICAM1* [18]. Early suppression of p16 bypasses senescence in vitro and in vivo [18,42,43,93,94]; however, we demonstrated that knockdown of p16 at late time points upon oncogene-induced senescence does not bypass senescence but still decreases the expression of *IL6* and *CXCL8* [18], suggesting that this is uncoupled from the senescence-associated cell cycle arrest. Additionally, we found that low *CDKN2A* expression in tumors of 6 different types, including melanoma and pancreatic adenocarcinoma, correlated with a decreased SASP signature [18], further demonstrating the role of p16 in regulation of the SASP. Consistent with our observation, other articles have found decreased expression of SASP factors upon suppression of p16 in a murine model of intervertebral disc regeneration [95] and liver fibrosis [96]. Interestingly, induction of senescence through p16 overexpression does not increase SASP gene expression [50], suggesting that p16 is necessary but not sufficient to induce the SASP. As we have previously discussed, the SASP has pleiotropic and context-dependent effects that can both promote tumor progression and enhance anti-tumor immunity (Reviewed in [97]); thus, it is plausible that the observed decreased of immunological surveillance in tumors with suppression of *CDKN2A* is mediated by decreased SASP. More studies are needed to understand the exact mechanism whereby suppression of p16 leads to decreased SASP gene expression and whether this leads to decreased immunological surveillance and tumor growth. Additionally, in those tumors where loss of p16 occurs due to deletion of the chromosome 9p21 locus, adjacent genes such as *MTAP* and the interferon α and β cluster may be also affected and lost [98,99]. Previous studies have suggested that melanomas with low *MTAP* expression have decreased cGAS-STING signaling [100], a pathway strongly involved in SASP expression though NF-$\kappa\beta$ regulation [101–103]. Additionally, codeletion of *CDKN2A* and the interferon α and β cluster has been linked with decreased expression of immune cell genes in melanoma tumors [104]. Therefore, it is likely that multiple mechanisms exist in tumors with loss of 9p21 to suppress SASP gene expression and to modulate the tumor microenvironment.

How is immunological surveillance initiated on the tumor microenvironment? A large amount of evidence suggests that intratumoral senescence induction is critical for activation of the immune system and clearance of cancer cells [83,86,105,106], and abrogation of senescence-inducing pathways, mainly p16 and p21, have been shown to be critical to promote immune-checkpoint inhibitors resistance [107,108]. Thus, which is the main

requirement for intratumoral immune system activation: senescence induction or high p16 expression? This is a complicated question since most senescent cells upregulate p16 as a manner to maintain the cell cycle arrest [26,109], and early abrogation of p16 activity overcomes senescence in vitro and in vivo [42,43,93,94], thus likely reducing the intratumoral senescence burden. In this regard, our previous data demonstrate that although etoposide can induce senescence in melanoma cells with stable p16 knockdown, these cells fail to increase the expression of *IL6* and *CXCL8* [18]. Moreover, although tumors with low p16 expression show a significant decrease in SASP factors, we did not observe differences in the amount of intratumoral senescent cells [18]. Interestingly, Novais et al. also found that suppression of p16 in a model of intervertebral disc regeneration decreases the SASP without altering the onset of senescence [110]. Altogether, these data suggest that p16 may regulate the tumor microenvironment and by extension intratumor immunity independently of senescence induction. This indeed, may explain previous observations where overexpression of p16 induces a cell cycle arrest without the SASP [50].

Importantly, the lack of SASP expression observed in cells with p16 suppression indicates that induction of senescence as a mechanism to abrogate malignant proliferation may be a suitable and safe therapy for cancers with null or very low p16 expression. More experiments analyzing the immune response landscape as well as the mechanisms by which p16 decreases SASP are needed to understand this observation and to develop better treatments for the ~50% of human cancers with decreased p16 expression [12].

5. Conclusions

In conclusion, suppression of p16 in tumor cells decreases the expression of interleukins, chemokines and other factors belonging to the SASP that in turn may remodel the tumor microenvironment, thereby impairing immunological surveillance (Figure 1). The observation that suppression of p16 decreases SASP gene expression is sustained by different studies in multiple models including cancer and fibrosis. Additionally, there are multiple high-throughput correlative studies in different diseases suggesting that low *CDKN2A* activity correlates with a decreased number and activity of intratumoral immune cells. Altogether, this suggests that p16 suppression is not only a cell cycle regulator but also a regulator of tissue homeostasis. More research is needed to understand whether this is a direct or indirect effect and whether this is due to canonical or non-canonical p16 roles. This is imperative since currently there are not pharmacological treatments specifically for p16-non canonical pathways [47].

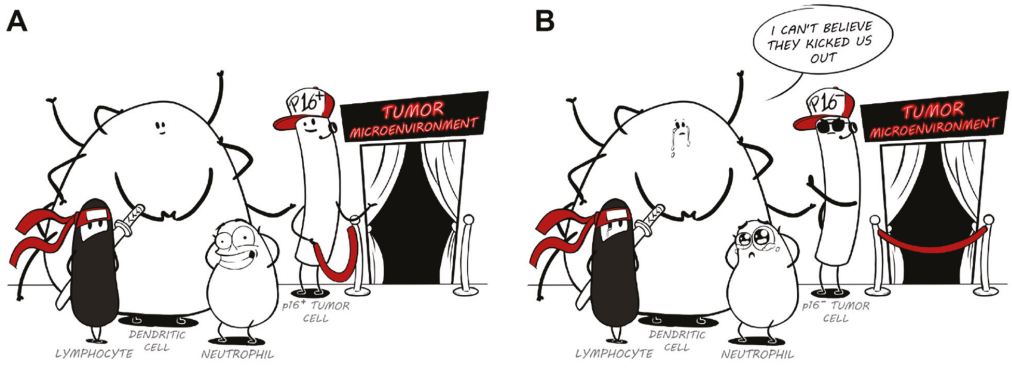

Figure 1. Cartoon representing the hypothesis discussed in this review: the possible role of p16 in regulation of immunological surveillance. (**A**) Represents a p16 positive tumor where cells of the immune system are invited into the tumor microenvironment and hence tumor immunosurveillance is fostered. (**B**) Represents a p16-null tumor, which correlates with decreased number and activity of immune cells, thus impairing immunosurveillance and promoting tumor growth.

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Review

The Cellular Senescence Stress Response in Post-Mitotic Brain Cells: Cell Survival at the Expense of Tissue Degeneration

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Abstract: In 1960, Rita Levi-Montalcini and Barbara Booker made an observation that transformed neuroscience: as neurons mature, they become apoptosis resistant. The following year Leonard Hayflick and Paul Moorhead described a stable replicative arrest of cells in vitro, termed "senescence". For nearly 60 years, the cell biology fields of neuroscience and senescence ran in parallel, each separately defining phenotypes and uncovering molecular mediators to explain the 1960s observations of their founding mothers and fathers, respectively. During this time neuroscientists have consistently observed the remarkable ability of neurons to survive. Despite residing in environments of chronic inflammation and degeneration, as occurs in numerous neurodegenerative diseases, often times the neurons with highest levels of pathology resist death. Similarly, cellular senescence (hereon referred to simply as "senescence") now is recognized as a complex stress response that culminates with a change in cell fate. Instead of reacting to cellular/DNA damage by proliferation or apoptosis, senescent cells survive in a stable cell cycle arrest. Senescent cells simultaneously contribute to chronic tissue degeneration by secreting deleterious molecules that negatively impact surrounding cells. These fields have finally collided. Neuroscientists have begun applying concepts of senescence to the brain, including post-mitotic cells. This initially presented conceptual challenges to senescence cell biologists. Nonetheless, efforts to understand senescence in the context of brain aging and neurodegenerative disease and injury emerged and are advancing the field. The present review uses pre-defined criteria to evaluate evidence for post-mitotic brain cell senescence. A closer interaction between neuro and senescent cell biologists has potential to advance both disciplines and explain fundamental questions that have plagued their fields for decades.

Keywords: cellular senescence; post-mitotic; neuronal senescence; Alzheimer's disease; biology of aging; neurodegeneration; brain; geroscience; amyotrophic lateral sclerosis; tauopathy

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1. Introduction

Many debilitating diseases affecting our modern population have resulted from the deterioration of biological processes suited for a 40-year lifespan. Exceptional examples are neurodegenerative diseases. Age is the single greatest risk factor for the most common neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). Sporadic neurodegenerative diseases, i.e., those not inherited, rarely affect adults before the age of 50 and are nearly absent in adults

younger than 40-years-old. In this way, evolution strongly favored nervous system health. Indeed, adaptation accounts for the success of humans in our recent history, a behavioral flexibility dependent on the nervous system. For these reasons neurodegenerative diseases and central nervous system (CNS) injuries (stroke) are devastating because of the limited regenerative capacity of the mature tissues.

The complexity of the structure and function of the nervous system is achieved through extensive developmental processes and is maintained in part due to the resiliency of the cells to maintain function and resist activating cell death processes. This first evidence of this phenomenon was reported by Rita Levi-Montalcini and Barbara Booker in 1960 in their pioneering experiments demonstrating the critical role of nerve growth factor (NGF) in sympathetic neuron growth and survival [1]. Applying NGF antiserum to newborn mouse neurons resulted in a 97–99% cell loss; the same strategy only resulted in a 34% neuron loss in the adult mouse [1]. Less differentiated cells that fail to interact with their target appear to die by a morphological process (nuclear cell death) that we can now attribute to apoptosis [2]. Apoptosis is an effective and efficient mechanism of cell death involving robust activation of caspases. More mature neurons appear to die in a slower process that was termed cytoplasmic with prominent changes occurring in mitochondria, endoplasmic reticulum (ER), and lysosomes. Several forms of neuronal cell death have been described [3]. Reactivation of cell cycle in post-mitotic neurons has also been reported to be an initiating event for neuronal death (reviewed in [4]). The alterations in mitochondrial function, fission and fusion, ER stress, protein misfolding and aggregations, autophagy, and expression of proteins associated with cell cycle regulators are observed in neurodegenerative diseases and assumed to be processes associated with the neuron's cell death. However, in a post-mitotic system, cellular evolution may have favored a pro-survival response for difficult-to-replace cells in post-mitotic tissues that simultaneously prevented malignancy in mitotically competent cells–senescence.

In 1961, Leonard Hayflick and Paul Moorhead reported a dogma-shifting observation from their cell culture experiments. At that time, primary cells grown in culture were believed to be immortal with indefinite replicative potential. However, Hayflick and Moorhead reported cessation of growth and eventual loss of the lines routinely after ~50 passages or one year in culture. They referred to the phenomenon as "senescence at the cellular level" and hypothesized that it was attributed to intrinsic factors [5]. Subsequent studies revealed that human cells track their cell divisions. Cultured human fibroblasts replicate 50–80 times and then no longer divide, which is referred to as replicative senescence or the "Hayflick limit" [6,7]. By mathematical definition, a "limit" can be approached but not achieved, which is ironically fitting for this phenomenon-translating these in vitro observations to tissues and living organisms have been proposed by many, but a consensus definition has not been reached. Toward this end, we frame senescence as a complex stress response that culminates a change in cell fate.

Replicative senescence, as defined by Hayflick and Moorhead, has clearly defined underlying biology (telomere attrition) and functional phenotypes (inability to divide). Subsequent studies have identified exogenous factors that can cause cell cycle arrest through telomere-independent mechanisms. As the number of exogenous senescence-inducing factors has expanded, so has the number of unique cell types being interrogated. Numerous phenotypes have emerged as a result. Senescent cells have been identified using various morphology markers; gene, protein, metabolic changes; and functional readouts and have been a subject of earlier reviews [8–11]. A specific combination of phenotypes defining senescence currently does not exist [12]; however, most agree that it is a stress-induced change in cell fate which includes a stable cell cycle arrest and cell death resistance.

The identity of the parent cell type and upstream signals has consequences on the post-senescence phenotype [13,14]. The resulting heterogeneity has presented challenges for identifying, defining, and studying senescent cells in vivo and across disciplines. Where biologists agree is that interpreting the senescence phenotype requires integrating various

lines of distinct evidence placed in appropriate context. This is especially true for post-mitotic tissues such as the brain. The intent of this literature review is not to list studies that used an umbrella term "senescence" to describe a physiological response. Rather, it is to critically evaluate results from reports on brain cell senescence using pre-defined senescence-defining criteria: proliferative/cell cycle arrest, apoptosis resistance, senescence-associated secretory phenotype (i.e., cytokines, chemokines, pathogenic proteins, exosomes, miRNA, enzymes, etc.), and senescence associated b-galactosidase activity (SA β-gal) (Figure 1). The International Cell Senescence Association (ICSA) recently provided a list of key cellular and molecular features of senescence [9]. They acknowledged that post-mitotic cells may develop features of senescence, however, their recommendations largely focused on dividing cells. Some of which have major limitation when evaluating brain tissue (i.e., lipofuscin and SA β-gal). Here we provide an overview of senescent phenotypes, as relevant to post-mitotic cells, the assays used to assess them, and the markers relevant to senescence, each in the context of brain cell biology. We then review different brain cell types using these criteria. Overall, the present contribution aims to provide an accessible summary on senescent post-mitotic brain cells with criteria and interpretations relevant to the neurobiology of aging and disease.

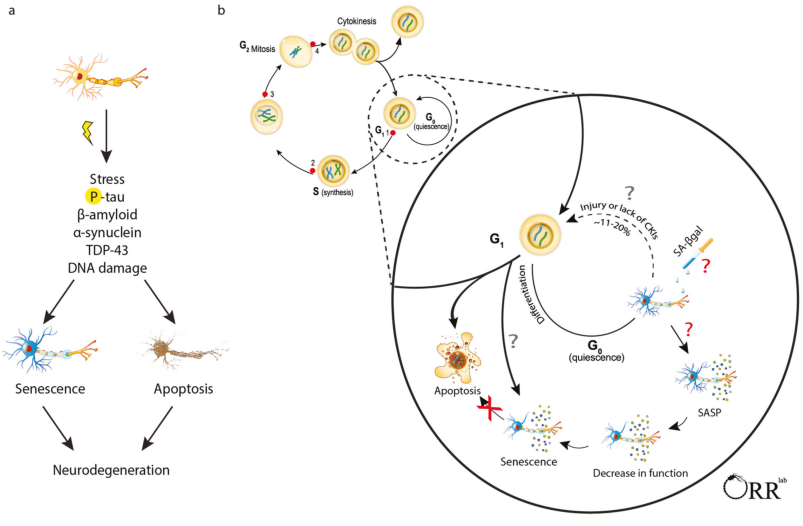

Figure 1. Senescence-associated mechanisms may confer exceptional resistance to cell death, but contribute to pathogenesis through inflammatory SASP. (**a**) The accumulation of stress, including protein aggregates and DNA damage, contributes to both senescence and apoptosis in post-mitotic cells. Protein aggregates including hyperphosphorylated tau, β-amyloid, and α-synuclein are seen in senescent cells, apoptotic cells, and patients with AD, PD, ALS and DLB. (**b**) Mitotic cells exit cell cycle and terminally differentiate into post-mitotic brain cells. Post-mitotic cell cycle re-entry can lead to cell death or senescence. In senescence, post-mitotic cells show stable cell cycle arrest, upregulate SCAPs, and release SASP. Neuronal SASP includes proinflammatory molecules and neurotoxic proteins. The SASP activates glia, drives inflammation, loss of neuronal connectivity, and perpetuates toxicity in a prion-like spread. SA β-gal can detect senescence, but it has a limited ability to discern between quiescent and senescent cells. AD: Alzheimer's disease; PD: Parkinson's disease; ALS: amyotrophic lateral sclerosis; DLB: dementia with Lewy bodies, CKI: cyclin-dependent kinases inhibitors; SCAPs: senescent cell anti-apoptotic pathways; SASP: senescence associated secretory phenotype.

2. Identifying Senescent Brain Cells

Heterogeneity of senescent cells has been revealed through transcriptomic profiling [14–16]. The senescence phenotype is guided by context differences in cell type, upstream stressor and environment. To yield a deeper understanding of cellular senescence signatures across distinct cell types in vivo, a careful examination of multiple key biomarkers is needed and has been the subject of many reviews, for example [17]. Below we provide an overview of this strategy, with specific focus on its utility to identifying senescent post mitotic brain cells.

2.1. Absence of Proliferation/Stable Cell Cycle Arrest

Various indicators of cell cycle arrest such as cell cycle inhibition and telomere attrition have been discussed for mitotically competent brain cells, for example [18]. Despite the persistent dogma that neurons permanently withdraw from the cell cycle upon terminal differentiation, numerous studies have demonstrated the expression of cell cycle proteins in post-mitotic neurons, that can give rise to dysfunctional hyperploid cells [19]. A decrease in telomere length is associated with cell division; notably telomere shortening has been observed in non-replicative neural brain populations in C57BL/6 mice in a cell cycle-independent manner [20]. Telomere shortening may occur in replication-independent scenarios in long-lived post-mitotic cells through oxidative stress and downregulation of telomeric factor *POT1* and shelterin subunit *TRF2* [21]. While markers of proliferation may not necessarily indicate mitotic competency in post-mitotic cells, they may reflect cell cycle re-entry out of G0 into G1 which could make them vulnerable to apoptosis or senescence. Cell cycle re-entry has been estimated to occur in ~11.5% of post-mitotic cortical neurons through DNA content variation and ~20% of post-mitotic neurons in AD through both DNA content variation and expression of cyclin B1 [22,23]. The data collectively indicate a link between aberrant neuronal cell cycle activity and neuronal dysfunction and disease. For example, multiple studies link AD associated Ab [24–26] and phosphorylated tau with aberrant cell cycle activity [27–29]. Cell cycle re-entry in the absence of AD pathology also has been described [30] and put forth as a potential novel therapeutic target for neurodegenerative diseases [31]. In this context, an open question remains whether proliferation markers may also apply to a pre-senescent phase in post-mitotic neurons. Co-expression of cell cycle mediators in post-mitotic cells, such as G1 proteins, in the absence of apoptotic markers (i.e., caspase 3) suggests an arrest of aberrant cell cycle activity, consistent with senescence. Alternatively, post-mitotic quiescent cells may more readily transition to senescence than mitotically competent cells, for example through changes in lysosomal activity [32]. Nonetheless, measuring molecular signatures of cell cycle activity may provide evidence for or against a senescence stress response in post-mitotic cells. Examples of studies looking at stable cell cycle arrest are discussed in Section 3.

2.2. Cell Death Resistance

Senescent cells display enhanced survival over their non-senescent counterparts by activating senescent cell anti-apoptotic pathways (SCAPs) [33,34]. Similarly, post-mitotic cells, including neurons, acquire a greater resistance to cell death as they mature [35,36]. A complete understanding of neuronal cell death resistance is not known; however, some pathways have been identified [37–40]. Similarly, while some SCAPs have been identified for senescent cells, this is a burgeoning research area. It is tempting to speculate that SCAP-mediated degeneration resistance may contribute to (or use similar mechanisms as) post-mitotic cell death resistance. In this way, identifying molecular regulators of cell death resistance in post-mitotic cells may apply to senescence, and vice versa. In response to injury, mitotically competent cells may proliferate; instead post-mitotic cell cycle reentry triggers degenerative processes [41]. In this review, we provide evidence that post-mitotic senescence in neural tissue may preserve cellular integrity by avoiding cell death [42]. Thus, cell cycle inhibitors such as INK4 cyclin-dependent kinases inhibitors (CKIs) (i.e.,

p16, p18, p19) and CK-interacting protein/kinase inhibitor protein CKIs (i.e., p21, p27, p57) may be protective and contribute to cell death resistance in post-mitotic cells [41]. Though a stable senescence cell cycle arrest may confer degeneration resistance to the affected post-mitotic cell, downstream consequences of its preserved survival may include neural network dysregulation and chronic inflammation through secreted factors.

2.3. Secretory Phenotype

Post-mitotic cells can produce a senescence-associated secretory phenotype (SASP) consistent with that of mitotically competent cells. For details on the definition, role, and common factors used to identify SASP in the brain, please refer to previous reviews on this topic [18,43,44]. Briefly, upregulation of NFκb activity and consequential production of canonical pro-inflammatory markers may occur in post-mitotic cells similar to that of dividing cells. Moreover, post-mitotic cells may produce unique SASP factors, like aggregation-prone proteins that impact protein homeostasis and drive neurodegenerative proteinopathies. For example, human postmortem brains from patients clinically diagnosed with AD, PD, and dementia with Lewy bodies (DLB) all show severe hyperphosphorylated microtubule-associated tau, β-amyloid, and α-synuclein loads, although topographical distribution of protein aggregates are different [45]. These protein aggregates may influence each other and synergistically promote the accumulation of one another [46,47]. Thus, SASP is an important component of post-mitotic senescence with implications for neurodegeneration.

2.4. Senescence Associated β-Galactosidase

In-depth details of the SA β-gal assay specific to brain tissue were described in our previous review [18]. Briefly, SA β-gal detects lysosomal-β-galactosidase activity at pH 6.0 [48]. While useful for distinguishing senescent cells in culture, it is detected in brain tissue independently of age or senescence [49]. The *GLB1* gene encodes for lysosomal beta-D-galactosidase and is the origin of the SA β-gal activity, but SA β-gal's cellular roles and mechanism in senescence are not fully understood [50]. In post-mitotic cells, interpretation of SA β-gal requires extra caution. Purkinje neurons in the cerebellum, CA2 neurons, and a subset of cortical neurons all display SA β-gal even in young mice [49]. A recent study demonstrated that lysosomal activity mediates the transition from deep quiescence to senescence [32]. Given that neurons are quiescent, assigning positive staining to senescence versus quiescence becomes especially challenging in static tissue. Even in vitro, SA β-gal positivity has been reported in neurons in the absence of other markers of senescence [51]. A more detailed review of SA β-gal used to identify senescence in post-mitotic cells is described in later sections.

2.5. Concluding Remarks on Identifying Senescent Cells

Evaluating senescence requires in-depth understanding of the cell type and markers of senescence. Post-mitotic cells have different characteristics from mitotically competent cells that should be considered when evaluating for senescence. In the following sections, we review studies reporting senescence in post-mitotic brain cells. We evaluate the methods used by key studies and compare them to our pre-defined criteria presented above and summarized in Figure 1.

3. Neurons

The adult human brain contains an estimated 86 billion neurons [52]. Barring neurodegenerative disease or brain trauma, nearly all cortical neurons (96–98%) remain alive during the lifespan. Their exceptional survival has been attributed to their restriction of apoptotic pathways, though the precise molecular details are not fully understood [16]. An appreciation for dysfunctional, not missing, neurons has emerged over the past decade. For example, age-associated cognitive decline has been attributed to changes in neuronal chemistry, metabolism, and/or morphology, but not necessarily the progressive loss of

neurons [53]. Re-evaluation of the literature and accumulating experimental evidence suggests that age- and disease-induced stressors on neurons initiates a neuronal senescence stress response as a means to avoid active degeneration and cellular loss. However, as we discuss, these neuronal structural and functional changes contribute to pathogenesis in neurodegenerative diseases [49,54–56]. For example, p16 and p21 expression has been reported in neurons and glial cells in postmortem motor and frontal association cortex of ALS patients [57], while microglia express p16, p53, and SASP in late-stage spinal cord of the ALS rat model [58] (please refer to mitotic review for microglia senescence [18]). As terminally differentiated cells arrested in G0, neurons either inherently fulfill one of the key defining features of senescence (near permanent cell cycle arrest); alternatively, they may arrest in G1 after cell cycle re-entry, which has been described in numerous degenerative diseases [22,23] (Figure 1). This phenomenon is not unique to neurons; a recent review provides a discussion on the topic of post-mitotic senescence across tissues [42]. Here we review supporting evidence that neurons, like mitotically competent cells, have the ability to mount a canonical senescence stress response.

3.1. Neuronal Senescence in Tauopathies and Peripheral Neuropathies

Senescent cell heterogeneity, in part, arises from differences in cell biology of the parent cell. Growing experimental evidence demonstrates that the phenotypic diversity of neuronal senescence reflects the heterogeneity of neuronal subpopulations. Historically, neurons have been classified by morphology, anatomical location and/or distinct shapes, and function that can be further classified by direction, action on other neurons, discharge patterns, and neurotransmitter utilization. Recent methodologies, in particular single nucleus transcriptomics, have provided an even deeper insight into neuronal heterogeneity [59]. For example, MAPT encodes the microtubule-associated protein tau proteins, which are often referred to as "neuron-specific" or "axon-specific" proteins. However, the diversity in tau proteins arise from extensive processing at the mRNA and protein levels. Six major tau protein isoforms are expressed in the adult brain which arise through alternative splicing; post-translational modifications further amplify the tau protein diversity by producing dozens of unique forms of tau protein that are differentially regulated and expressed based on developmental age and neuronal subtype [60,61]. Tau protein accumulation is the most common intraneuronal pathology among neurodegenerative diseases, though neuropathology and clinical presentations differ across diseases [62]. Among tauopathies, neurons containing neurofibrillary tangle (NFT) aggregates of heavily post-translationally modified tau are the closest correlate with neurodegeneration and dementia in AD, yet they are long-lived [63]. We recently determined that these neurons display a canonical senescence stress response [49]. Analyzing transcriptomic data from postmortem human brain provided the opportunity for a within-subjects comparison between neurons with or without NFTs. The transcriptomic and pathway analyses revealed expression patterns in NFT-bearing neurons consistent with senescence including upregulated anti-apoptotic/pro-survival pathways and concomitant inflammatory and secretory pathways. Using four independent tau transgenic mouse models, we found evidence for DNA damage; aberrant cellular respiration; karyomegaly; upregulation of cell cycle inhibitors, inflammation and inflammatory mediator NFκB. These phenotypes occurred concomitant with NFT formation and were reduced by genetically removing endogenous tau protein, indicating a molecular link between tau and neuronal senescence. Moreover, intermittent treatment with senolytics (dasatinib plus quercetin) caused ~35% reduction in NFTs that coincided with a reduction in senescence-associated gene signature (cell cycle inhibitors and inflammation). We did not observe neuronal senescence phenotypes in thalamic, midbrain, or cerebellar neurons. It remains unknown whether or not high expression of transgenic tau could ultimately drive neuronal senescence in these other neuronal subpopulations. However, work from other groups suggest that midbrain [56] and cerebellar neurons [54] may utilize other molecular mediators aside from MAPT/tau.

Overexpression of human non-mutated tau and its persistent phosphorylation also contributes to peripheral neuropathy and memory deficits [64]. Long-term and short-term memory were significantly impaired in female transgenic mice expressing all six human tau isoforms [64]. Peripheral neuropathy was evidenced as motor nerve conduction velocity (MNCV) slowing, paw tactile allodynia, paw heat hypoalgesia, and low paw density of intraepidermal nerve fibers in human tau mice compared to wild type mice [64]. Notably, neuronal senescence has been associated with cisplatin-induced peripheral neuropathy (CIPN). Primary DRG neurons treated with cisplatin upregulate SA β-gal activity and expression of *Mmp-9*, *Cdkn1a*, *Cdkn2a*, and display elevated translocation of HMGB1 compared to controls [65]. In a mouse model of CIPN, dorsal root ganglia (DRG) neuronal populations upregulated the DNA damage response pathway and *Cdkn1a* gene expression as determined by single-cell RNA-sequencing. Neuronal senescence was further verified by increased protein expression of p21, p-H2AX, NFκB-p65; SA β-gal; and lipofuscin granules [66]. Clearing p16 and/or p21 positive cells either pharmacologically with ABT263 or by utilizing suicide gene therapy (i.e., p16-3MR ganciclovir/herpes simplex virus thymidine kinase system) reversed CIPN as evidenced by improved mechanical and thermal thresholds [65]. Collectively these studies indicate senescence-associated neuronal dysfunction in the central and peripheral nervous system where tau may be linked.

3.2. Neuronal Senescence in Parkinson's Disease

The H1 MAPT haplotype [67] and single nucleotide polymorphisms in MAPT have been associated with age of onset and progression of PD [68]. Despite the strong genetic association with MAPT, tau pathology occurs only in about 50% of patients with PD [69]. Though cognitive dysfunction may occur, PD primarily affects motor behavior. Neurodegeneration in PD predominantly occurs in the substantia nigra where up to 70% of dopaminergic neurons can be lost in late disease stages [70,71]. These neurons express significantly lower levels of MAPT and tau protein than those in the cortex or hippocampus (~4-fold and ~6-fold difference, respectively) and do not develop tau pathology [72]. Instead, the hallmark protein deposit in PD is α-synuclein. Experiments in cell lines suggest that α-synuclein expression levels differentially regulate the cell cycle [73]; however, conclusive studies demonstrating α-synuclein-mediated neuronal senescence have not been reported. To date, the most comprehensive work on dopaminergic neuronal senescence involves special AT-rich sequence-binding protein 1 (SATB1) [56]. SATB1 functions as a transcription factor and chromatin architecture organizer [74]. SATB1 is overexpressed in various tumors and has been referred to as a T-cell-specific transcription factor given its importance in T cell development. A meta-analysis of genome-wide association studies comparing PD cases with controls identified SATB1 as a candidate risk gene [75]. Neurons in PD-vulnerable brain regions (e.g., substantia nigra pars compacta) display lower levels of SATB1 than neurons from the less susceptible ventral tegmental area [76]. Genetically reducing SATB1 in dopaminergic neurons drives a neuronal senescence response including elevated p21 protein expression, karyomegaly, SASP, and mitochondrial dysfunction [56]. SA β-gal and lipofuscin, hallmarks of mitotically competent senescence that also co-occur in neurons, were also observed. Mechanistically, SATB1 repressed dopaminergic neuron senescence by binding the regulatory region of CDKN1A. In the absence of SATB1, the CDKN1A encoded protein, p21, expression level increased to perpetuate the neuronal senescence stress response. When the authors reduced Cdkn1a in SATB1 knockout neurons, fewer senescent cells (as determined by SA β-gal) were observed, providing evidence for the mechanistic link between SATB1-p21 mediated neuronal senescence. Interestingly, reducing SATB1 in cortical neurons did not modulate Cdkn1a/p21 levels, which was attributed to a more open Cdkn1a locus in dopaminergic than cortical neurons. In contrast, tyrosine hydroxylase expressing neurons require Satb1 expression for their survival and will undergo neurodegeneration within three weeks of downregulated Satb1 [76]. This observation indicated that de-repression of Cdkn1a and concomitant increased p21 expression caused apoptosis and clearance by microglia. Evidence for this was observed by

neuronal SASP production and concomitant microglia co-localization with tyrosine hydroxylase positive neurons. Follow-up experiments to deplete microglia after Satb1 reduction would conclusively demonstrate whether or not Cdkn1a-expressing dopaminergic neurons fulfill the criterion of apoptosis resistance in neuronal senescence. Indeed, elevated p21 expression induced apoptosis in vitro, indicating that it may not confer neuronal apoptosis resistance [77]. Nevertheless, the study by Riessland et al. determined a dopaminergic neuron-specific role of SATB1 in modulating Cdkn1a/p21 expression and downstream senescence phenotypes including karyomegaly, mitochondrial dysfunction, production of SASP, lysosomal dysfunction and presence of SA β-gal and lipofuscin [56].

In PD, disease-related stressors on neurons contribute to defects in several cellular systems ultimately involving alterations in Bcl-2 family signaling, JNK activation, p53 activation, expression of cell cycle regulators [78]. While many of these processes including those addressed above are thought to contribute to neuronal degeneration, some are also hypothesized to reflect survival-promoting mechanisms such as senescence. More recent studies focused on neuronal senescence in PD have revealed that overexpression of mutant p53, p21, or mutant Leucine-rich repeat kinase 2 (LRRK2) increased SA β-gal, and αSyn protein expression and fibril accumulation in vitro [77]. Transgenic mice expressing the same mutant $LRRK2_{G2019S}$ displayed elevated oligomeric αSyn, β-galactosidase and p21 expression. The increase in αSyn was due to impaired degradation, not increased transcription [77]. The results suggest that the $LRRK2_{G2019S}$ mutation may activate the p53-p21 senescence pathway, which is upstream of α-synuclein accumulation. While suggestive of senescence, the study did not evaluate cell cycle activity, apoptosis resistance or SASP production in the affected cells. Nonetheless, future studies to dissect if or how PD mutations may interact with *SATB1* would elucidate whether these pathways converge on a common senescence-associated pathway relevant to PD pathogenesis. An intellectual framework for proteinopathy-induced senescence in neurodegenerative diseases was first proposed in 2009 by Golde and Miller [79]. The idea warrants further studies as the emerging data from mechanistic studies that have directly tested this hypothesis (i.e., tauopathy, α-synucleinopathy, and β-amyloid) indicate that post-mitotic neurons are especially vulnerable to protein aggregation stress as highlighted in these aforementioned studies.

3.3. Neuronal Senescence in Aging

Senescent cells accumulate with advanced age even in the absence of disease. In 2012, Diana Jurk et al. evaluated neuronal senescence in naturally aged mice with or without increased DNA damage by genetically manipulating telomerase [54]. Age-associated DNA damage was associated with neuronal senescence in the brains of 32-month-old mice [54]. DNA damage foci, as determined by gH2A.X immunostaining, was elevated in cerebellar Purkinje and cortical neurons from 32-month-old mice compared to 4-month-old mice. These neurons also displayed activated p38 MAPK (phosphorylated at Thr^{180}/Tyr^{182}) indicative of DNA double strand breaks. Oxidative stress was assessed by visualizing cells with elevated lipid peroxidation product, 4-hydroxynonenal (4-HNE). Immunostaining with 4-HNE revealed cytoplasmic granular accumulation within the same subpopulations of cells. Similarly, these large neurons expressed higher levels of inflammatory protein IL-6 than other cell types. SA β-gal activity and lipofuscin (as measured by autofluorescence) showed similar overlapping patterns. Given the overlapping co-staining of multiple marker combinations, the authors hypothesized that DNA damage (gH2AX) increased with advanced age, which activated the DNA damage response (p-p38 MAPK) to induce a senescence-like pro-inflammatory (IL-6) and pro-oxidant phenotype (4-HNE) similar to mitotically competent cells (lipofuscin and SA β-gal). To begin evaluating mechanistic mediators of the senescence phenotype, they utilized transgenic mice with telomere dysfunction with or without *Cdkn1a*. Neurons from mice with telomere dysfunction (late generation telomerase knockout mice, F4 TERC-/-) displayed elevated levels of gH2AX, p-p38MAPK, 4HNE and IL6 compared to those with one functional copy of TERC. The genetic removal of *Cdkn1a* modulated these phenotypes in mice regardless of telomerase

activity, however genotype and cell type specific phenotypes were observed. For example, in TERC wild type mice, the absence of p21 only significantly altered the 4HNE phenotype and only in Purkinje neurons (not cortical neurons). In contrast, in mice with telomere dysfunction, removing p21 did not modulate 4HNE. Instead, the absence of p21 significantly reduced gH2Ax and IL6 in Purkinje neurons and p-p38 and IL6 in cortical neurons. These results again highlight heterogeneity of the senescence stress response unique to different neuronal subpopulations. Nonetheless, removing p21 robustly reduced inflammation, as assessed through IL6, in both cellular populations to provide evidence that neuronal senescence may contribute to sterile inflammation with advanced age.

Insulin provides trophic support and drives excitatory signaling in neurons [80,81]. A loss of neuronal sensitivity to insulin, referred to as insulin resistance, coincides with their dysfunction and disease. The mechanisms driving insulin resistance in brain cells are not well understood, but risk factors include advanced age, obesity, peripheral insulin resistance, and metabolic dysfunction [82,83]. Recent studies in mice have demonstrated that brain insulin resistance induces neuronal senescence, which leads to synaptic dysfunction [55,84]. In these studies, insulin resistant neurons display several molecular, functional and morphological changes consistent with senescence [55]. Specifically, mice that developed spontaneous peripheral insulin resistance at either young (3-months-old) or old (24-months-old) age also displayed signs of brain insulin resistance (i.e., elevated insulin in the CSF, elevated pIRS1 (Ser^{307} and Ser^{612})], and senescence (i.e., neurite loss, elevated Cdkn1a and Cdkn2a and SA β-gal activity). This finding indicates that insulin resistance, like tau accumulation or loss of *SATB1,* may drive premature neuronal senescence in the absence of advanced age. The insulin resistant mice, regardless of age, behaved poorly on cognitive behavior tasks to indicate that neuronal insulin resistance/senescence co-occurred with poor brain function. Mechanistically, chronic insulin was shown to reduce hexokinase 2, impair glycolysis and increase levels of p25, a potent activator of both CDK5 and GSK3β. The simultaneous signals from CDK5 (neuronal cell death) and β-catenin (cell cycle reentry) pushed neurons to enter a senescence-like state. A detailed signal transduction cascade was elucidated in vitro whereby insulin increased Ccnd1 and Cdkn2a expression, nuclear localization of β-catenin, cyclin D1 and p19ARF. The increase in p16INK4a and PML occurred later. Aberrant β-catenin also induced a parallel p53-p21 senescence pathway. The authors concluded that chronic insulin signaling induced a neuronal senescence phenotype through the over-stabilization and nuclear localization of β-catenin. Tau phosphorylation was not assessed, but given the increased activity of tau kinases Cdk5 and GSK3β and parallels with findings in Musi et al. [49], it is tempting to speculate that aberrant tau may also contribute to insulin resistance-mediated neuronal senescence.

3.4. General Considerations for Evaluating Neuronal Senescence

Observations across the aforementioned studies highlight the complexity of applying canonical senescence measures to post-mitotic cells. For example, we caution the use of lipofuscin and SA β-gal for neuronal senescence as these markers seemingly reflect shared phenotypes among neurons, across age and/or disease, that requires further investigation into their association with other senescence markers. The best example are cerebellar Purkinje neurons that display SA β-gal throughout the lifespan [49]. Jurk et al. noted, "the frequencies of neurons showing multiple markers of a senescent phenotype are very substantial, going well beyond 20% in Purkinje cells already in young mice brains" [54]. A key readout for this conclusion was SA β-gal staining. Given the early stages of defining neuronal senescence in vivo, it remains unknown whether SA β-gal positivity truly reflects senescence in Purkinje neurons, which could become senescent in early life due to their high energetic and metabolic demands. Other studies have demonstrated that cerebellar Purkinje neurons can survive and function as polypoid cells [85]. Neuronal polyploidy suggests that DNA replication occurred, but that neuronal mitosis stalled. Indeed, hyperploid neurons have been reported in preclinical and mild stages of AD as evidenced by immunofluorescence and slide-based cytometry methods cross-validated by

chromogenic in situ hybridization [86]. The neurons avoid apoptosis, upregulate several cell cycle mediators and survive months in the adult mouse brain, which meets several criteria of a senescent cell. Importantly, cerebellar Purkinje neurons are indispensable for motor movement control. Notably, gait speed, coordination, and balance are significant predictors of mortality [87,88]. It is tempting to speculate that senescence of these neurons may contribute to the overall decline in health and increased mortality with advanced age. Alternatively, the physiological function of these neurons may require signaling through cellular and molecular pathways resulting in phenotypes typically attributed to senescence. For example, we routinely observe neuronal lipofuscin throughout the lifespan, though it notably increases with age; similarly, we observed high levels of SA β-gal activity in these same neuronal populations throughout the mouse lifespan [49]. Moreno-Blas et al. also proposed that SA β-gal may not be a reliable marker of senescence by itself [89]. Despite cortical neurons expressing senescence-associated phenotypes such as p21, γH2AX, ruptures of DNA, lipofuscin, SASP, and irregular nuclear morphology, they observed normal nuclear morophology in some neurons with high SA β-gal [89]. Instead, their data suggested that autophagy impairment/dysfunction, perhaps through lysosomal fusion with autophagosomes, critically contributed to the neuronal transition from quiescence to senescence, similar to that reported by [32]. Since SA β-gal positivity overlaps with lysosomal dysfunction, it may be useful to narrow down potential senescent cell candidates; however, as indicated by Moreno-Blas [89] and several studies reviewed here (i.e., [49,51,54]) it cannot be used in isolation. Similarly, neuronal lipofuscin staining was first reported in children in 1903 and has been later confirmed in several studies where it occurs in at least 20% of neurons by 9-years-old [90]. These aggregates of oxidation products of lipids, proteins, and metals autofluoresce non-specifically bind antibodies which can complicate interpretations of immunofluorescence assays and thus requires multiple controls. The pigment granules change with aging by increasing progressively in size, as well as their subcellular localization thus appropriate age-matched negative controls and antibody controls are necessary to interpret results. Within the aging field, the increased rate of lipofuscin formation and accumulation is considered a hallmark of both replicative and stress-induced senescence [91,92] and methods for its specific staining (i.e., Sudan Black B) are increasingly used to detect senescence in vitro and in vivo [93]. It is our opinion that at this time both lipofuscin and SA β-gal require further investigation before using them as decisive markers for neuronal senescence.

3.5. Concluding Remarks

Differentiated neurons are remarkably apoptosis resistant, but their vulnerability to excitotoxicity increases with age [94]. Neurons inherently lack the option to divide, but they upregulate cell cycle proteins in response to stress. The inability to replace these critical cells indispensable for maintaining life may have placed strong evolutionary pressure to favor stress-induced senescence over apoptosis. In this way, neuronal survival would be maintained though the number of dysfunctional cells would increase with advancing age. Indeed, this is what is observed in the human brain [52,53]. As the burgeoning field of neuronal senescence advances, we expect that the next wave of studies will reveal additional molecular regulators, clarify pathways previously identified, and differentiate between shared pathways and neuron subtype specific mechanisms. Additionally, with the increasing use of single cell technologies, we anticipate an increased ability to identify, track and study senescence with greater clarity on the phenotype(s) and how they change across the lifespan and in disease.

4. Astrocytes

Astrocytes are an abundant and heterogenous cell population within the central nervous system (CNS). They comprise 20–40% of the total glial cell population in the brain, depending on region, developmental stage, and species [95–97]. Along with oligodendrocytes, astrocytes originate from the neural tube [98]. Astrocytes differentiate from the

glial progenitor cells proliferating in the forebrain subventricular zone as they migrate outwards to other regions of the brain [99]. The majority of astrocytes are considered post-mitotic, and in the absence of pathology or disease, they display low rates of turnover and proliferation [100].

Astrocytes vary in function and morphology. Distinct types, including radial astrocytes, fibrous astrocytes, and protoplasmic astrocytes have been elucidated within the CNS based on structure, distribution, and function, as well as their expression level of the different isoforms and splice variants of the intermediate filament protein glial fibrillary acidic protein (GFAP) [101,102]. Astrocytes have been implicated in maintaining water and ionic homeostasis, providing metabolic and structural support to neurons, and regulating the blood–brain barrier (BBB) [102,103]. They also cooperate with microglia to control local neuroinflammation and neuronal restoration following damage to the CNS. Similar to microglia, astrocytes prune synapses and remove cellular debris within the synapse in healthy and diseased brains [104,105]. Genes crucial for astrocyte function such as Excitatory Amino Acid Transporters 1 (*EAAT1*) and 2 (*EAAT2*), potassium transporter *Kir4*, and water transporter *AQP4* involved in glutamate, glutamine, potassium, and water homeostasis in the brain have shown to be downregulated when astrocytes become senescent [106]. Thus, their change in function associated with senescence can lead to detrimental effects including the onset of various neurodegenerative pathologies [103,107–110].

Astrocyte senescence is often wrongly conflated with astrogliosis or astrocyte reactivity. Reactive astrogliosis involves structural changes to the astrocytes alongside cellular proliferation and migration [100,109]. Reactive astrocytes, also known as A1 cells, have been shown to be induced by activated neuroinflammatory microglia through the secretion of Il-1α, TNFα, and C1q cytokines. Upregulated expression of GFAP is a known marker of reactive astrocytes, and its levels are also increased during aging [111,112]. In contrast, radiation-induced senescent astrocytes demonstrated a downregulation of GFAP [113]. Reactive A1s lose their ability to promote neuronal survival, outgrowth, synaptogenesis, and phagocytosis and induce death of neurons and oligodendrocytes [114]. A1s have also been shown to be present in the brains in many neurodegenerative disorders, including AD, PD, and Huntington's disease [114,115]. The benefit of astrogliosis and subsequent scar formation is the protection of the surrounding neurons and tissue and restriction of inflammation and pathology. However, dysfunction in reactive astrocytes can lead to neuronal dysfunction, and eventually degeneration that can contribute to various CNS disorders. Many of these features are similar with a senescence phenotype, including morphology changes and secretion of pro-inflammatory molecules.

Astrocytes undergo a senescence-like stress response, which has been referred to as "astrosenescence" and described as a functional change from neurosupportive to neuroinflammatory [116]. Oxidative stress, exhaustive replication, inhibition of proteasomes, and an increase in glucose concentration elicit an astrocyte response consistent with senescence, in vivo and in vitro (reviewed: [116,117]). For example, replicative senescent primary human fetal [118] and rat [119] astrocytes displayed an arrest of growth and cell cycle progression; the human fetal astrocytes also upregulated gene expression of *TP53* and *CDKN1A*. Astrocytes do not express TERT [120] and replicative senescence was not avoided with telomerase reverse transcriptase (hTERT) expression [118], indicating that telomere-length independent mechanisms govern replicative senescence in astrocytes. Inhibiting p53 function with human papillomavirus type 16 E6, however, delayed the onset of senescence, implying a p53-dependent mechanism of replicative senescence in astrocytes [118]. Increased SA β-Gal activity, marked by staining kits, was also observed in many of these studies [113,117,121,122]. Strengths and weaknesses of using this method for labeling brain cells have been discussed [18].

Radiation cancer therapy has potential to induce senescence [123]. The effect of radiation therapy on astrocytes in vivo was examined by evaluating human brain from individuals receiving cranial radiation cancer therapy [113]. Senescent cells were identified with immunohistochemical labeling of p16, heterochromatin protein Hp1γ, and expression

of Δ133p53, an inhibitory isoform of p53. Elevated p16 and Hp1γ largely co-localized with astrocytes in patient brains that had received radiation, but not in control tissue. Expression of Δ133p53 was primarily in astrocytes, and its role in senescence was explored in vitro. They found that these irradiated astrocytes in vitro had diminished Δ133p53, and developed a phenotype associated with other senescent cells, such as increased SA β-Gal activity, p16, and IL-6. However, restoration of Δ133p53 expression inhibited and prevented further senescence, promoted DNA repair, and prevented astrocyte-mediated neuroinflammation and neurotoxicity [113]. Collectively, this study [113] and others [106,124] have characterized the radiation-induced senescence phenotype in astrocytes to include decreased proliferation and increased SA β-Gal activity, along with typical increased expression of p53, p21, and p16, which were analyzed using Western Blot [113,121].

Senescent astrocytes downregulate genes associated with activation, including GFAP and genes involved in the processing and presentation of antigens by major histocompatibility complex class II proteins, while upregulating pro-inflammatory genes [121]. Increased expression of p16, p21, p53, and MMP3 have also been associated with astrocytes undergoing senescence and those isolated from aged brains [125]. The downregulation of genes associated with development and differentiation, coinciding with the upregulation of pro-inflammatory genes, manifest as functional changes (i.e., inflammatory stress response). This may perpetuate a pro-inflammatory feedback loop that is stably maintained by senescence-associated changes in gene expression and transcript processing [126].

Astrocyte senescence increases with age in the human brain and in AD [127,128] and PD [129]. The consequences of astrocyte senescence are myriad. Functionally, astrocytes communicate with nearby neurons and the surrounding vasculature to clear disease-specific protein aggregates, including β-amyloid, the accumulation of which has been linked to the progression of AD [96,130]. The release of SASP factors by senescent astrocytes including IL-6, IL-8, MMP3, MMP10, and TIM2 were found to contribute to β-amyloid accumulation, phosphorylation of tau protein, and an increase in NFTs [125,131]. An increased risk for PD has been linked to contact with the herbicide paraquat (PQ), which an environmental neurotoxin. Complementary in vivo and in vitro approaches were used to evaluate mouse and human astrocyte responses to PQ [129]. PQ-treated astrocytes developed several features consistent with senescence, including upregulated *Cdkn2a*/p16. Importantly, senescent cell removal improved neurogenesis in the subventricular zone, reduced neuronal loss and rescued motor function deficits in PQ-treated mice [129]. Collectively their results highlight astrocyte senescence as a mechanism of PQ-associated neuropathology and brain dysfunction, and represents an appealing therapeutic target for the treatment of PD.

Concluding Remarks

"Astrosenescence" is a complex and heterogeneous process that necessitates evaluating astrocyte structure, distribution, function and molecular expression profiles. Measuring the expression level of GFAP [113,125] can help differentiate whether upregulated pro-inflammatory cytokines and chemokines expression reflect astrogliosis or astrosenescence [125,131]. The most consistently shared features across senescent astrocytes were arrest of growth and cell cycle progression, increased expression of p53 and p21, and p16 [113,117,121] and some evidence of increased SA β-Gal activity [117]. Collectively the studies reviewed here indicated that functional changes associated with senescent astrocytes contribute to chronic neurodegenerative diseases and may propagate inflammation and induce senescence to surrounding cells [132,133]. Targeting them for removal represents an opportunity to intervene in neurodegenerative diseases.

5. Endothelial Cells

Endothelial cells form a single layer of cells called endothelium that line the blood vessels of the circulatory system. They have an array of functions in vascular homeostasis such as regulating blood flow, immune cell recruitment, maintaining blood vessel tone, and

hormone trafficking [134,135]. While endothelial cell function is heterogeneous and tissue-specific, several studies have demonstrated that endothelial cells can become senescent in adipose tissue, coronary arteries, and in the human umbilical cord using observations of morphology changes, SA β-gal activity, and SASP through DNA microarray [136–138]. While there is a great literature describing senescent endothelial cells throughout the body, the focus of this section turns to brain microvascular endothelial cells.

Brain endothelial cells are mostly post-mitotic with minimal proliferation [139–141]. They express a high density of tight junction and adherens junction proteins and high transendothelial electrical resistance [142–144]. Functionally, brain endothelial cells contribute to the BBB, regulate local cerebral blood flow as a part of the neurovascular unit (NVU), and thus have important implications for brain diseases [145–147]. The BBB is a highly selective semipermeable barrier with tight junctions that closely regulates the biochemical composition of the brain by restricting the free diffusion of nutrients, hormones, and pharmaceuticals [148]. The tight junctions force molecular traffic to take place through the endothelial membrane through sealing of the paracellular space and by establishing a polarized, transporting epithelial and endothelial phenotype [149]. During aging, endothelial cells experience senescence-associated stressors including oxidative stress, DNA damage accumulation, telomere shortening, increased NFκB signaling and decreased *Sirt1* expression [136,150]. Recent studies suggest that brain endothelial cell senescence could contribute to BBB dysfunction though neurovascular uncoupling and reactive oxygen species [151]. Indeed, increased BBB permeability and vascular dysregulation have been observed in patients with early cognitive dysfunction, cerebral microvascular diseases, and AD [152–154]. However, the co-occurrence of senescent endothelial cells with aging and disease makes it difficult to discern whether they are upstream mediators or downstream consequences of diseases. BBB dysfunction has been observed in patients with AD, Multiple Sclerosis (MS), traumatic brain injury (TBI), and stroke, featuring overexpression of MMP-2 and MMP-9 [155–159]. Molecular cascades such as activation of MMP's have been suggested to induce senescence [160]. Thus, it is possible that brain insult leading to BBB dysfunction causes senescence as well.

There have been several recent studies specifically examining cerebrovascular endothelial senescent cells induced by external stimuli and natural aging. In one study, rat primary cerebromicrovascular endothelial cells were delivered 2–8 Gy of γ-irradiation using a 137Cs gamma irradiator [161]. After irradiation, cerebromicrovascular endothelial cells' DNA damage was examined using the Comet Assay with alkaline single-cell gel electrophoresis followed by fluorescent imaging of the nuclei. Comet Assay visualizes the amount of DNA which leaves the nucleus as a marker for DNA strand breaks [162]. Irradiation caused DNA fragments to migrate out of the nuclei, indicating increased DNA damage [161]. SA β-gal staining was positive in a dose-dependent manner, p16 and p53 upregulation was observed, and a SASP was seen with upregulation of *IL-6*, *IL-1α*, *GM-CSF*, *G-CSF*, *MIP-1α*, *MCP-1*, eotaxin, and *IL-1β* via RT-PCR to verify senescence [161]. In another study discerning cerebromicrovascular endothelial cell senescence, 3-month-old and 28-month-old C57BL/6 mice gene expression profiles were compared by single-cell RNA sequencing [163]. The mean expression of senescence core genes (*Cdkn2a*, *Bmi1*, *Trp53*, *Hmga1*, *Chek1*, *Chek2*, *Prodh*, *Tnfrsf10b*, *Cdkn1a*, *Dao*), senescence effector genes (*Ppp1ca*, *Ahcy*, *Brf1*, *Map2k3*, *Map2k6*, *Smurf2*, *Tgfblil*, *Srsf1*, *Angptl2*), and SASP genes (*Ccl2*, *Ccl24*, *Ccl3*, *Ccl5*, *Ctnnb1*, *Cxcl1*, *Cxcl10*, *Cxcl12*, *Cxcl2*, *Cxcl16*, *Hgf*, *Hmgb1*, *Icam1*, *Igfbp2*, *Igfbp3*, *Igfbp4*, *Igfbp6*, *Igfbp7*, *Il15*, *Il18*, *Il1α*, *Il1β*, *Il2*, *Il6*, *Mif*, *Mmp12*, *Mmp13*, *Mmp14*, *Pgf*, *Plat*, *Timp2*, *Serpine1*, *Ccl3*, *Ccl4*, *Ang*, *Csf2*, *Kitl*, *Serpine2*, *Tnfrsf1a*, *Hgfi*, *Nrg1*, *Ereg*, *Areg*) were used to calculate a running enrichment score [163]. Higher senescence gene enrichment scores were found in brains from aged versus young mice [163]. These SASP factors have potential to promote neuroinflammation and affect BBB integrity [164]. Future studies may further clarify the downstream impact of senescent endothelial cells on their neighboring environment [165].

Other studies also highlight the difficulty of disentangling cause and effect of brain cell senescence, aging and disease. Emerging evidence suggests aberrant angiogenesis, and potentially endothelial senescence, may occur as bystander effects of other cell's SASP. Studies using the rTg4510 mouse model of tauopathy have revealed an increased number of blood vessels and concomitant upregulation of angiogenesis-related genes such as *Vegfa*, *Serpine1*, and *Plau* [166]. Confocal imaging demonstrated aberrant vasculature near neurons with tau-containing NFTs which display a senescence-like phenotype (please refer to Section 3: Neurons) [49]. Together, these studies suggest that factors secreted by senescent NFT-containing neurons may negatively impact surrounding cells, which could drive aberrant angiogenesis. Alternatively, aberrant cerebrovasculature could be upstream of tau accumulation and contribute to NFT formation. To translate these studies to human clinical conditions, postmortem human AD brains with tau pathology were investigated for cerebrovascular senescence [167]. Cerebral microvessels were isolated from 16 subjects with a Braak NFT score of V/VI (B3) and 12 subjects with a Braak NFT score of 0/I/II (e.g., high neuropathology versus low neuropathology). Upregulation of senescence was inferred by elevated expression of *Serpine1*, *Cxcl8*, *Cxcl1*, *Cxcl2*, *Csf2*, and *Cdkn1a*; however, other markers of senescence were not evaluated [167]. Whether tauopathy causes endothelial senescence and induces a leaky BBB and/or endothelial senescence affects the vascular microenvironment will require further investigation [168].

Concluding Remarks

Most of the aforementioned studies examined brain endothelial cell senescence by analyzing expression of senescence-associated genes [161,163,167]. Some studies also examined SASP genes [163,167]. Future studies are needed to evaluate cell cycle arrest, SCAPs, DNA-damage responses, resistance to apoptosis to define and validate senescence in brain endothelial cells [169,170]. Of interest for future studies will be determining brain region-specific differences in endothelial senescence and to better identify their mechanistic impact on the neighboring cells and environment.

6. Oligodendrocytes

Oligodendrocytes (OLs) are derived from oligodendrocyte precursor cells (OPCs) in a highly regulated process [171]. OPCs differentiate into pre-OLs, and later into mature, myelinating OLs in the presence of differentiation-promoting transcription factors [172]. The primary role of mature OLs includes myelination of neuronal axons in the CNS. Additionally, OLs play a role in providing metabolic support to myelinated axons, especially in axons that spike at high frequencies [173]. OLs have also been implicated in information processing, and defects in OL maturation are linked with behavioral abnormalities [173,174]. OLs are highly vulnerable to oxidative stress and mitochondrial injury, and OL loss occurs upon exposure to inflammatory cytokines [171,175,176]. OLs are also highly susceptible to accumulation of DNA damage during normal aging and have been indicated as a potential upstream cause of cellular aging leading to neurodegeneration, illustrated by the involvement of myelin in several neurodegenerative disorders [175,176]. DNA damage is a known mediator of senescence suggesting a potential relationship between senescence of oligodendrocytes and neurodegenerative disorders. Senescent OLs could result in defective myelination as seen in several neurodegenerative disorders [177–179]. For instance, loss of OLs can lead to demyelination as seen in MS.

Only a few studies exist that try to validate senescence in OLs. A rodent model with a novel senescence marker utilizing the p16 promoter, ZsGreen, crossed with the established APP/PS1 AD model was used to look for senescence in different cell types, including OLs [180]. OPCs showed upregulation of p21, p16, and SA-β-gal activity [180]. However, no senescence was observed in OLs (immunohistochemically stained with OL marker, CNP, and ZsGreen p16 senescence reporter) while OPCs were senescent and unable to differentiate into OLs [180]. It is likely that increased susceptibility of OPCs to the microenvironment increase the incidence of senescence in these cells compared to OLs. It may be possible

that senescence in OLs occurs through p16-independent mechanisms. For example, a recent study reported an age-associated increase in p16-positive oligodendrocytes, but they were not cleared using senolytic approaches [181]. Brain cell type specific responses to senolytic clearance [181] highlights the heterogeneity of senescent cells even when in the same tissue, which may (in part) reflect cell type diversity in complex tissues such as the brain [59,182,183].

In a study of white matter lesions of frozen postmortem human brain tissue from patients who were over 65 years old, OLs exhibited elevated SA-β-gal [184]. Immunohistochemistry was used to double-label white matter tissue with SA-β-gal to identify cell types [i.e., astrocytes (GFAP+), microglia (CD68+), and oligodendrocytes (OSP+)]. Additionally, OLs also showed increased levels of 8-OHdG, a marker for oxidative stress, but did not display high levels of p16 [184]. Comparison of mRNA using qRT-PCR revealed a 1.5-fold increase in *TP53*, *H2AX*, and *CDKN1B* [184]. *CDKN1B* encodes p27kip1 and its upregulation results in the induction of a senescent phenotype [185]. Elevated *H2AX* and *TP53* are indicative of increased DNA damage and are also suggestive of a senescent phenotype. However, to confirm true senescence in these OLs, additional inspection of (1) SASP factors, (2) resistance to apoptosis through SCAPs, and (3) the presence of proliferation markers would be beneficial.

Concluding Remarks

Although there are several studies that examine OPC senescence (refer to our other review on senescence in mitotically active brain cells for literature regarding OPC senescence) in multiple disease processes, there are limited data regarding the senescence of OLs in natural aging and other disease models. Limited studies mentioned suggest that SA-β-gal and gene expression analysis may be used to see if OLs have a senescent, but the results are inconsistent. For example, SA-β-gal activity was seen while high levels of p16 was not observed. Further study is required to establish the true senescence status of these cells and their potential role in aging and disease.

7. Summary

Cellular senescence has been best studied and characterized in mitotically competent cells [180]. However, most cells in the brain including neurons, astrocytes, endothelial cells, and oligodendrocytes have very low or no cell turnovers and show mostly post-mitotic phenotypes. Most post-mitotic brain cells that survive brain development will remain throughout the lifespan. While this feature historically precluded the study of senescence in the brain due to the early definition requiring mitotic competency, brain cell types are highly susceptible to acquiring a lifetime worth of damage known to drive senescence (Table 1). These include oxidative stress, DNA damage, and protein accumulation, which impact cell cycle and secretory phenotypes. While senescent cells continue to survive due to their apoptosis resistance, they tend to partially lose (or change) their function and increase expression of pro-inflammatory molecules. SASP from senescent cells can affect the microenvironment in the brain by its paracrine effect, causing other neighboring cells regardless of cell types to go senescent [133]. In the brain, these dysregulations manifest as an increase in neuroinflammation, increased BBB permeability, loss of neuronal synapses, demyelination, and dysregulated metabolism [179,186]. Collectively, these features have been associated with impaired cognition, and clearance of senescent cells as a therapeutic strategy has shown to reduce pathology, inflammation, and neuronal dysfunction [49,65,181,187].

Table 1. Biomarkers previously described to verify senescent cells.

Cell Type	Biomarkers	Reference
Neurons	SA-β-gal	[54], [55], [56], [65], [66], [77], [84], [89]
	H2ax	[49], [66], [89]
	Comet Assay	[89]
	Nuclear morphology	[89]
	Cytosolic HMGB1	[65]
	Telomere-associated DNA damage foci	[84]
	p53	[77]
	p21	[49], [54], [55], [56], [57], [65], [66], [77], [89]
	p16	[49], [55], [65], [84]
	SASP	[49], [54], [56], [65], [66], [89]
	Lipofuscin	[54], [56], [66], [89]
	p38MAPK	[54]
Astrocytes	SA-β-gal	[106], [113], [118], [119], [121], [122], [124], [125], [126], [127], [129]
	Heterochromatin protein Hp1γ	[113]
	Senescence-associated heterochromatin foci	[121]
	BrdU	[118], [129]
	53BP1 foci	[129]
	Lamins	[106], [122], [129]
	GADD45A	[122]
	p53	[118], [121], [122], [124], [125]
	p21	[118], [121], [122], [124], [125]
	p16	[106], [113], [118], [121], [126], [127], [129]
	SASP	[106], [113], [124], [125], [126], [127], [129]
	p38MAPK	[127]
Brain Endothelial Cells	Senescence-associated genes	[161], [163], [167]
	Comet Assay	[161]
	SASP	[161], [167]
Oligodendrocytes	SA-β-gal	[184]

Characteristics needed to positively identify senescent cells that were described in our previous review [18] can be translated from mitotically competent cells to post-mitotic cells, but with caution. For example, absence of proliferation using proliferative markers (i.e., BrdU, Ki-67) should rarely be considered to characterize post-mitotic senescence as most post-mitotic cells show a lack of proliferation and turnover. Instead, presence of these markers may indicate aberrant cell cycle activity consistent with either a fate of apoptosis or, potentially, pre-senescence. In neurons, SA β-gal staining was positive in even young Purkinje neurons. SA β-gal activity may be observed due to metabolic demands rather than as a marker for senescence. Astrocytes present their own challenge

as reactive astrogliosis show senescence-like phenotype with changes in cell shape and secretion of pro-inflammatory molecules. Endothelial cells were evaluated as senescent mainly with gene expression analysis of senescence-associated genes, and there is a lack of verification with DNA damage, cell cycle arrest machinery, and resistance to apoptosis. OLs also showed elevated SA-β-gal activity but no activation of p16, which also questions the validity of the SA-β-gal assay in the brain. Although there is a lack of studies evaluating senescence in OLs, those reviewed here overall did not utilize all of our predefined criteria, similar to studies on dividing cells mentioned in our previous review [18]. Post-mitotic cells discussed in this review highlight the need to critically look at multiple markers of senescence.

Although senescence was initially, and exclusively, studied in mitotic cells, the literature reviewed here (Table 1) provides evidence that post-mitotic cells also undergo senescence as a complex stress response. The emergence of this new field, senescence in the brain, requires clarity of defining features. While tempting to label cells as "senescent" in many of these studies, a thorough evaluation of cell biology placed in the context of the cellular environment must also be considered. As a result, the field of neuroscience has pressured senescence biologists to clarify definitions and labels. As neuroscientists, we are in the early stages of applying methodologies and principles form senescence biology to the brain. In return, neuroscientists have over 60 years of lessons and principles of exceptional resistance to cell death to share with senescence biologists. A closer interaction and sharing of concepts between neuroscience and senescent cell biologists will propel both fields. As these efforts progress, we will continue to clarify definitions and revisit interpretations from the foundational studies reviewed here.

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Review

Evidence of the Cellular Senescence Stress Response in Mitotically Active Brain Cells—Implications for Cancer and Neurodegeneration

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Abstract: Cellular stress responses influence cell fate decisions. Apoptosis and proliferation represent opposing reactions to cellular stress or damage and may influence distinct health outcomes. Clinical and epidemiological studies consistently report inverse comorbidities between age-associated neurodegenerative diseases and cancer. This review discusses how one particular stress response, cellular senescence, may contribute to this inverse correlation. In mitotically competent cells, senescence is favorable over uncontrolled proliferation, i.e., cancer. However, senescent cells notoriously secrete deleterious molecules that drive disease, dysfunction and degeneration in surrounding tissue. In recent years, senescent cells have emerged as unexpected mediators of neurodegenerative diseases. The present review uses pre-defined criteria to evaluate evidence of cellular senescence in mitotically competent brain cells, highlights the discovery of novel molecular regulators and discusses how this single cell fate decision impacts cancer and degeneration in the brain. We also underscore methodological considerations required to appropriately evaluate the cellular senescence stress response in the brain.

Keywords: cellular senescence; Alzheimer's disease; biology of aging; neurodegeneration; brain; geroscience; senolytics; tauopathy; cancer; stress response

1. Introduction

The risk of both neurodegenerative disease and cancer increases with advanced age due to increased damage accumulation and decreased repair capabilities; yet the relative odds of developing one or the other are inversely correlated [1–5]. Molecular profiling studies have identified disrupted genes, proteins, and signaling pathways shared by neurodegenerative diseases and cancer, but in opposing directions. For example, p53 is upregulated in Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease, but downregulated in many cancers (reviewed [6]). Similarly, mutations of the Parkin gene *(PARK2)* have been shown to simultaneously contribute both to Parkinson's disease and tumor suppression [7]. A recent study performed transcriptomic analyses of four different tissues from four different species at ages across their lifespan [8]. Across samples,

the largest number of shared risk single nucleotide polymorphisms (SNPs) were in the genomic locus containing the long non-coding RNA ANRIL which modulates many cell cycle regulators including *CDKN2A/B* [9], which codes for $p16^{INK4A}$ (hereon referred to as p16), one of the best characterized mediators of cellular senescence [10,11]. Notably, SNPs in this locus were identified in the brain, as well as other tissues analyzed [8]. These results point toward aberrant cell cycle, and in particular senescence, as a key age-associated molecular pathway worth further study.

Cellular senescence has emerged as a hallmark biological process that promotes aging (reviewed [12]). The pillars of aging, including cellular senescence, are highly interconnected and do not occur in isolation [13]. For example, epigenetic changes, telomere attrition, DNA damage and mitochondrial dysfunction all may induce cellular senescence, which then contributes to dysfunctional nutrient signaling and proteostasis. Consequences of cellular senescence include stem cell exhaustion and chronic inflammation. Thus, cellular senescence represents an intersection of aging hallmarks [13]. While best studied as an anti-cancer stress response, recent studies highlight its pro-degenerative role in AD and tauopathies [11,14,15]. As such, cellular senescence may contribute to the inverse correlation between the risk for developing neurodegeneration and that for cancer.

Bulk tissue analyses, while informative at a macroscopic level, may not capture important changes occurring in single cells. Senescent cell abundance increases with aging, but the relative contribution to a tissue is relatively low and may be missed in bulk analyses [16] (reviewed [11]). Several laboratories are using single cell technologies to assign cell type specificity to tissue-level observations [17], but to date these analyses have not included senescent cells in the brain. To maximize generalization and interpretation across studies, in this review we only evaluate studies which investigated cellular senescence with cell type specificity, and not bulk analyses. We specifically focus on mitotically competent brain cells; due to space considerations, the topic of postmitotic brain cellular senescence is reviewed in a separate manuscript (Sah et al., *Life*, in review). The present compilation provides evidence on conditions in which cellular senescence may benefit (anti-cancer) or negatively impact (neurodegeneration) brain health. In doing so, this review explores how the cellular senescence stress response may simultaneously distinguish and connect AD and cancer risk.

2. Identifying Senescent Brain Cells

The identities of the parent cell type and upstream senescence-inducing stressors have consequences on the post-senescence phenotype [18,19]. The resulting heterogeneity has presented challenges for identifying, defining, and studying senescent cells in vivo and across disciplines. Senescent cells have been identified using various morphology markers; gene, protein, epigenetic, metabolic changes; and functional readouts and have been a subject of earlier reviews [20–24]. Transcriptomic analyses in particular show significant promise for identifying senescent cells. They have been utilized to study characteristics of senescence common between different cells-of-origin and modes of senescence induction and may help identify senescence markers which are more powerful than current traditional senescent markers such as p16 [25]. However, a specific combination of phenotypes defining cellular senescence currently does not exist [26]. Where biologists agree is that interpreting the senescence phenotype requires integrating various lines of distinct evidence placed in appropriate context [21]. This is especially true for postmitotic tissues such as the brain. We reviewed the literature using pre-defined senescence-defining criteria: proliferative/cell cycle arrest, apoptosis resistance, senescence-associated secretory phenotype, and senescence-associated β-galactosidase activity (Figure 1). As with measuring other biological processes, the interpretation of results requires integrating several lines of evidence. We explain the bases of these chosen criteria in this section.

2.1. Absence of Proliferation/Stable Cell Cycle Arrest

Replicative senescence, as observed by Hayflick and Moorhead [27], may be fundamentally restricted to mitotically competent cells. Brain cells which are susceptible to this fate include neural stem cells (NSCs), neural progenitor cells (NPCs), oligodendrocyte progenitor cells (OPCs), and microglia [28–30]. A subpopulation of astrocytes may be capable of cell division and trans-differentiating into neurons [31], however to what extent this happens in vivo and whether it occurs enough to induce replicative senescence in these rare cells is unclear. Of note, postmitotic cells may also undergo cellular senescence [32], including neurons [15,33–35]; a review of postmitotic senescent brain cells will be available in a separate manuscript (Sah et al., *Life*, in review). A wide range of methods have been validated to measure cell proliferation both in vitro and in vivo, including cell cycle specific markers, BrdU incorporation, ploidy, and the quantity and size of stem-cell containing non-adherent cultures (e.g., neurospheres or brain organoids). A reduction in proliferation at the tissue or cell population level (in situ tissue analyses or in vitro cell culture, respectively) may indicate that a portion of cells have undergone senescence or a general slowing of the cell cycle. To discern these differences and assign a senescence arrest, evaluating single cells is necessary.

Mechanistically, replicative senescence is achieved through intrinsically mediated (cell autonomous) telomere attrition [27] and has been the focus of several recent reviews [21,36,37]. Briefly, telomeres progressively shorten with age due to successive cell divisions and reduced telomerase activity. When chromosomes reach a critical truncated length, cells cease to replicate [36,37]. Average or relative telomere length can be measured using polymerase chain reaction (PCR), terminal restriction fragment (TRF) analysis, single-telomere length (STELA) analysis, and several different fluorescent in situ hybridization (FISH) methods [38]. Quantifying the shortest telomeres, as opposed to average length, may be more beneficial depending on the application and can be accomplished using the telomere shortest length assay (TeSLA) [38,39]. Similarly, telomerase activity can be quantified with a variety of measurement techniques including, most commonly, the PCR-based telomeric repeat amplification protocol (TRAP) [40]. A number of methods have also recently been developed, improving upon the convenience, throughput, sensitivity, and reliability of TRAP [41–43]. These methods provide objective readouts when evaluating potential mechanistic mediators of replicative senescence.

The most broadly accepted and universal phenotype among senescent cells is the change in cell fate that accompanies cell cycle arrest. This change in cell fate goes beyond a reduction in proliferation and is distinct from differentiation. While a reduction in cell proliferation can be reversible and attributable to many non-senescent causes, true senescence involves an irreversible cell cycle arrest. Senescent cells will not re-enter the cell cycle if signaled to do so, if the original stressor is removed, or even if reprogrammed with Yamanaka factors [44–47]. The stressors, pathways, and cell cycle inhibitors involved have been reviewed [45,48–50]. Many senescent cells accomplish cell cycle inhibition by upregulating p16 and/or $p21^{CIP1}$ (hereon referred to as p21); many other cell cycle inhibitors have been reported as well [45]. Based on the prevalence of upregulated p16 across studies, reporter mice using the p16 promoter have been utilized for many of the in vivo experiments (reviewed [51]). A caveat to this in vivo work is its dependence on p16-mediated senescence, overlooking other pathways. Nonetheless, these results have guided the field to date. In summary, a stable cell cycle arrest indicative of cellular senescence includes the evaluation of cell proliferation; expression of cell cycle inhibitors, including p16, p21 and $p19^{INK4d}$ (hereon referred to as p19); telomere attrition; and inability to re-enter the cell cycle when provided relevant stimuli. Additional morphological and functional changes accompanying senescence may include increased cell size; altered nuclei that are either enlarged (karyomegaly) or syncytia (multinucleated) [15,52,53].

2.2. Apoptosis Resistance

The enhanced survival of senescent cells depends on the activation of senescent cell anti-apoptotic pathways (SCAPs) [54,55]. The SCAP molecular pathways which have been identified thus far include BCL-2/BCL-XL, PI3K/AKT, p53/p21/serpines, dependence receptors/tyrosine kinases, HIF-1α and the unfolded protein response. The unfolded protein response in particular may be especially important for proteinopathies which cause many neurodegenerative dementing diseases. The senescence-associated phenotype can be identified by the expression of these SCAP pathways and/or by their resilience when exposed to apoptosis-inducing factors. Senescent cells often utilize multiple, redundant (although not all) SCAPs to survive in an otherwise toxic microenvironment. As such, targeting/evaluating multiple pathways is critical when identifying apoptosis resistance. Additionally, senescent cells should not express markers of apoptosis (i.e., TUNEL and caspase-3 negative).

2.3. Secretory Phenotye

The senescence-associated secretory phenotype (SASP) represents a deleterious effect of chronic senescent cell survival (reviewed [56,57]). Like other stressed cells, senescent cells secrete molecules to communicate to neighboring cells. These include chemokines and cytokines which signal to immune cells to clear them. Other secreted molecules include extracellular remodeling factors, exosomes, miRNAs, growth factors, and proteases that alter the environment and may induce senescence in other cells. The SASP is beneficial for tissue remodeling and wound healing (reviewed [21,58,59]). However, as senescent cells accumulate with age and pathology, the SASP contributes not only to local, temporary inflammation, but also chronic, systemic inflammation. This inflammation, which may have other contributors in addition to senescent cells, has been shown to be disadvantageous for a number of age-related diseases, including those in the brain. SASP factors differ across parent cell type and even within the same cell type exposed to different stressors. The phenotypic diversity of SASP factors represents a major challenge for developing a unifying profile and has been the subject of numerous reviews [56–59]. Moreover, how the SASP may change within a senescent cell over time remains unknown. Nonetheless, some of the common factors used to identify SASP in the studies reviewed here include IL-1α, IL-6, IL-1β, TNF-α, and CXCL1, CCL4, CCL6 and TGF-β.

2.4. Senescence-Associated β-Galactosidase

Senescent cells in vitro retain lysosomal β-galactosidase activity at pH 6.0, which is referred to as senescence-associated β-galactosidase (SA β-gal) activity [60]. The mechanisms surrounding SA β-gal activity are not fully elucidated, although studies have determined that *GLB1*, the gene encoding lysosomal β-D-galactosidase, is required [61]. This finding indicates an absence of a unique senescence-specific enzyme. Instead SA β-gal activity may reflect a change in lysosomal content or function. Toward this end, a recent study determined that lysosomes mediate whether cells remain quiescent or become senescent. As lysosomal function decreased, cells progressively lost the ability to reverse out of quiescence and thus became senescent [62]. However, the relation to SA β-gal staining was not investigated, so it remains unknown whether it coincides with the transition from deep quiescence to senescence. Notably, pharmacologically targeting lysosomal β-galactosidase clears senescent cells as evidenced by reduced expression of cell cycle inhibitor genes associated with senescence [63]. While these studies provide evidence for the utility of the SA β-gal assay to identify senescent cells, appropriately measuring SA β-gal activity requires careful methodological attention.

SA β-gal evaluation is a histological assay that requires enzymatic activity of GLB1. [61]. Due to this need for enzymatic activity, ideal experiments necessitate fresh frozen tissue, yet oftentimes the use of archived frozen or fixed tissues are reported in the literature. Similarly, it is imperative that the precise pH is reported and serial sections are processed at both pH 6.0 and pH 4.0 to discern between global differences in lysosomal activity

from senescence-specific differences. Appropriate controls should include young, age-matched disease-free and/or untreated tissues that are processed side-by-side with the experimental samples.

Given that lysosomal function differences between quiescent and senescent cells are on a continuum and subtle [62], SA β-gal cannot be used as a single surrogate marker. Additionally, not all senescent cells acquire the phenotype. We emphasize the importance of considering the cellular phenotype in entirety when evaluating the senescent phenotype [21]; we used the methodological criteria described above to evaluate SA β-gal interpretations throughout the review.

2.5. Concluding Remarks on Identifying Senescent Cells

Evaluating cellular senescence requires both an appreciation for the phenotypic complexity of cellular senescence, expertise in the parent cell type and an understanding of how they respond to stress. Beyond the pre-defined criteria used in this review, additional markers may be applied to supplement the characterization of senescent cells. Morphological observations such as an enlarged, flattened morphology and a disrupted nuclear membrane and metabolic dysregulation such as increased ROS and mitochondria dysfunction are also commonly employed, among others [24]. In the following sections, we review studies reporting senescent cells of mitotically competent brain cells using the pre-defined criteria presented above and summarized in Figure 1. While reviewing the literature we evaluated the methods used to define senescence and how these phenotypes were placed in the context of brain cell biology.

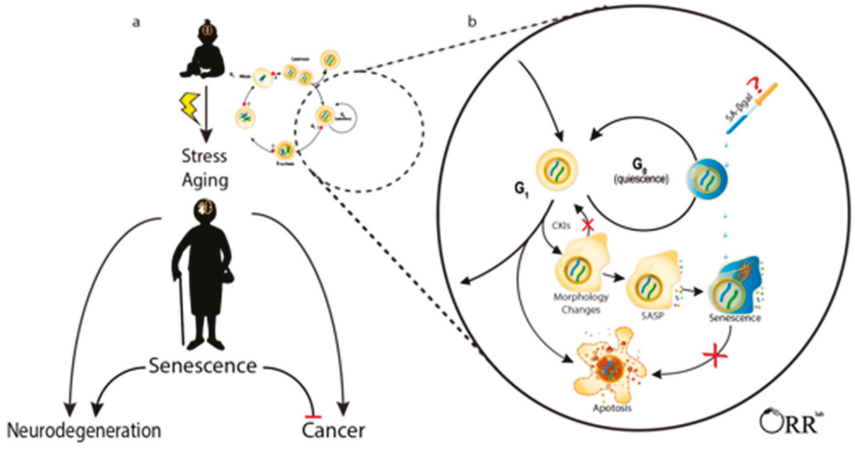

Figure 1. Cellular senescence in mitotically competent brain cells may impact risk for developing cancer and neurodegeneration. (**a**) The accumulation of cellular damage throughout the lifespan induces cell stress responses that impact cell fate decisions. Neurodegeneration increases with age and inversely correlates with risk of developing age-associated brain cancer. Cellular senescence may influence this clinical observation. (**b**) Mitotically competent brain cells may enter quiescence, a reversible G0 arrest. Damaged cells may undergo apoptosis, other cell death processes or senescence. In senescence, the cell permanently exits the cell cycle, upregulates cyclin-dependent kinase inhibitors (CKIs), exhibits morphological changes, acquires a senescence-associated secretory phenotype (SASP), and upregulates senescent cell anti-apoptotic pathways (SCAPs). Senescence-associated beta galactosidase (SA β-gal) staining can be used to identify some senescent cells but also labels some quiescent cells and requires careful methodological techniques and interpretations. For simplicity, senescence was illustrated at G1; however, cells may enter senescence arrest at G0 or G2 as well [64].

3. Neuronal Precursor Cells

Brain maturation continues after birth for three months in mice [65] and 20 years in humans [66]. Even beyond that, the adult brain maintains populations of self-renewing,

multipotent NSCs first identified in rats [67] and later in humans [68–70]. Adult neurogenesis occurs primarily in the subgranular zone (SGZ) in the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) around the lateral ventricles of the forebrain. In humans, the primary progenitor of the SVZ is a subpopulation of specialized, quiescent NSCs known as B cells which give rise to interneurons and OPCs. These are sometimes referred to as SVZ astrocytes due to their morphological structure and their co-expression of the glial fibrillary acidic protein (GFAP), Nestin and carbohydrate Lewis X (LeX) [71]. These specialized NSCs share some physical and molecular characteristics with typical astrocytes, including branched processes, intermediate filament bundles, cell bodies in the cytoplasm, and gap junctions [72]. Thus, precise phenotyping measures are required to identify neurogenic from non-neurogenic astrocytes. Some studies suggest that human NSCs populations persist and contribute to neurogenesis throughout adulthood [73–80]. Others have detected significant reductions during childhood, ranging from simply lower activity all the way to negligible or nonexistent in adults [81–84]. Thus, most studies reviewed in this section were performed in human cells in vitro or in mice.

In healthy adult mammalian tissue, NSCs exist primarily in a quiescent arrest [85]. During quiescence, cells lower their metabolic activity and cell division rate to minimize damage to DNA, proteins, and mitochondria which can lead to cancer, senescence, and exhaustion of the stem cell population [86,87]. Various environmental and behavioral activities have been shown to activate quiescent NSCs (e.g., exercise, sleep, learning) through diverse cell signaling pathways (e.g., neurotransmitters, Notch, neurotrophins, Wnt) [87]. Once activated, NSCs proliferate, migrate, and differentiate toward NPCs and OPCs and terminally differentiate into neurons, astrocytes, and oligodendrocytes [88,89]. Given that aberrant NSC activation may contribute to cancer, the ability for them to utilize the senescence stress response may benefit short-term health and survival.

The incidence of cancer, including in the brain, increases with age [90], and evidence suggests that NSCs are often the cells of origin for brain tumor formation [91,92]. Evolutionarily, the ability for proliferative cells to undergo senescence in response to cell damage provided an advantage over malignancy [93,94]. Some evidence suggests that patient survival rates are favorable when brain cancer cells become senescent [95]. Pilocytic astrocytoma is a slowly growing benign brain tumor derived from astrocytes and is the most common pediatric brain tumor. Genetic mutations in the proto-oncogene B-Raf gene, *BRAF*, cause pilocytic astrocytoma. Human fetal neurospheres transduced with a constitutively active form of *BRAF* were evaluated for senescence-like features. The mutation promoted colony formation and in early passages proliferation did not differ as assessed through BrdU incorporation. However, after five passages BrdU uptake was notably reduced in BRAF mutant cells compared to controls, and was shortly thereafter followed by proliferative arrest. The remaining cells appeared viable as per light microscopy and displayed a 35-fold increase in the percentage of cells with SA β-gal activity with elevated PAI-1 and p16 compared to controls [95]. The *BRAF* mutant NSCs displayed a progressive decrease in SOX2 expression that coincided with the increase in other senescence-associated protein upregulation, suggesting a loss of neural stemness as they progressed into senescence. Telomere length did not differ between NSCs indicating the senescence arrest was independent of telomere attrition. The research team confirmed elevated p16 in 86% (57/66) of patient-derived pilocytic astrocytoma tumors. Moreover, individuals whose tumors were negative for p16 (9 cases) had significantly shorter survival than p16 positive cases. Tumor suppressor TP53 (p53) was detected in 23% of pilocytic astrocytoma samples and significantly correlated with p16 in the cases that were scored. SA β-gal was also observed in >50% of primary tumor cells derived from patients (n = 2) at low passage number. Collectively, these data suggest that elevated p16, and presumably NSC senescence, is favorable for survival in patients with pilocytic astrocytoma.

Human NSCs may undergo cellular senescence in response to non-genetic carcinogens as well. For example, radiation decreased human NSC proliferation and caused cell cycle arrest as measured by BrdU uptake in vitro, acutely increased DNA damage repair (γH2AX

levels returned to baseline by 2 hours) and apoptosis (PARP cleavage returned to baseline by 28 h) [96]. Cellular metabolism was also increased on a per cell basis, as measured by the XTT assay, and neuronal differentiation was impaired, suggesting the surviving cells were senescent rather than quiescent [96]. Irradiation also reduced proliferation of NSCs in the SGZ and SVZ of rats as measured by BrdU incorporation in vivo [97]. Neuronal differentiation was reduced 97% in vivo, but NSCs isolated from these rats were still able to differentiate towards neurons in vitro. Furthermore, non-irradiated NSCs also showed an 81% reduction in neuronal differentiation when transplanted into irradiated rats. Combined, these results suggest the effects of irradiation on NSCs may have been indirectly induced by the damaged microenvironment [97], thus highlighting the importance of healthy cells and environment; both of which are negatively impacted by senescence.

Canonical senescence phenotypes acquired by irradiated NSCs include cell cycle arrest and SA β-gal staining [98]. Unique features include an increased expression of astrocyte markers which has been observed in vitro [98] and in vivo [99]. This differs from astrocytes which downregulate GFAP when undergoing senescence [100]. Development of SASP has not reproducibly occurred in irradiated NSCs across studies. For example, Zou et al., did not detect SASP factors IL-6, IL-8, IL-α concomitant with cell cycle arrest and SA β-gal positive NSCs [98]. However, using a similar cell line, Schneider et al. reported radiation-induced NSC cell cycle arrest via p21 and $p27^{KIP}$, and Rb-dephosphorylation [99]. These NSCs also showed an enlarged, flattened morphology and an increase in SA β-gal staining. In contrast to Zou et al., they found an increased secretion of several cytokines including IL-6 suggestive of a canonical senescence phenotype. Interestingly, irradiated NSCs still lost their markers of stemness (i.e., Nestin and SOX2) even when researchers inhibited the astrocytic differentiation pathway [99], indicating the reduced proliferation of these cells was not simply due to their differentiation into astrocytes. Together, these studies indicate that cell cycle arrest and SA β-gal positivity are not sufficient to label NSCs as senescent; however, these features combined with elevated p21 and $p27^{KIP}$ and morphological changes co-occur with SASP production and more complete senescent phenotype.

Senescence phenotypes in NSCs have been observed in response to chemical carcinogens as well. Independently, Dong et al. [101] using NSCs derived from the SVZ of mice and Daniele et al. [102] using human NSCs derived from embryonic stem cells, observed senescence-like phenotypes after treatment with hydroxyurea (HU). HU is a ribonucleotide reductase inhibitor in the "antimetabolite" family of chemotherapies known to induce DNA damage. Both mouse and human NSCs showed senescent-like phenotypes including reduced proliferation via neurosphere formation, p21, and p16, increased DNA damage via p53 and γH2AX, and an increase in SA β-gal staining. A reduction in apoptosis was also found in NSCs derived from rats [101] and the proinflammatory transcription factor NF-κB was found in human NSCs [102]. Collectively, these in vitro studies suggest that NSCs develop features of senescence in response to HU, although further investigation into the irreversibility of this state, a more in-depth characterization of their resistance to apoptosis, and investigation of SASP production downstream of NF-κB activation would strengthen this assertion.

As so far, the literature indicates that senescence may protect NSCs from becoming cancerous in response to known carcinogens [103]. A long term effect of senescent cell accumulation is chronic inflammation and degeneration. The brain is especially susceptible to age-associated neurodegenerative diseases (reviewed [104]). For example, AD onset prior to the age of 50-years-old is exceptionally rare, with the exception of familial cases. In contrast, 10% of adults aged 65-years-old and roughly 40% over the age of 80-years old have an AD diagnosis. Notably, aberrant NSC behavior has been reported in patients with AD [105,106]. To explore whether AD pathology is a cause or effect of NSC senescence, we evaluated literature from postmortem human brain studies as well as mechanistic studies in vitro.

AD is a progressive neurodegenerative disease characterized by the presence of amyloid-β (Aβ) plaques and tau-containing neurofibrillary tangles [107]. Evidence from neurosphere assays suggests that $A\beta_{42}$ [108] and tau [109] overexpression negatively impact NSC function through senescence-associated pathways. For example, He et al. [108] investigated senescence in response to $A\beta_{42}$ oligomers in vitro and in vivo. Neurospheres derived from mouse hippocampi were exposed to various concentrations of $A\beta_{42}$ for up to 5 days and assessed for proliferation, differentiation, SA β-gal, and toxicity. Apoptosis was increased with 10 μM $A\beta_{42}$, but remained unchanged at lower concentrations. $A\beta_{42}$ also reduced proliferation and increased p16 in a dose-dependent manner. In Aβ producing TgAPP/PS1 mice, the number of Nestin-positive NSCs double stained for p16 increased 1.3-fold and the number of SA β-gal positive cells in the dentate gyrus increased 1.7-fold. FPR2 (a G protein-coupled receptor which mediates the inflammatory response) and p38 MAPK (a stress response protein) were both implicated in the senescence induction of NSCs [108]. These in vivo data were largely driven by the use of p16 antibody staining and SA β-gal, both of which are somewhat unreliable due to lack of specificity (as discussed in [110] and [15], respectively). Nonetheless, the suggestive data are compelling for further investigation.

Tau protein accumulation is the most common intraneuronal pathology among neurodegenerative diseases [111,112]. We recently discovered that tau protein accumulation drives senescence in the brain [15], which was confirmed shortly thereafter by Bussian et al. [113]. Between the two independent studies, several senescent cell types were identified, but neither group looked at the role of tauopathy on NSC senescence. Nonetheless, evidence suggests that tau protein is critical for normal NSC behavior [109] and plays a role in abnormal activity in tauopathies [111]. We generated NSC-containing neurospheres from tau transgenic mice overexpressing either human frontotemporal dementia mutant tauP301L or human tau and compared phenotypes to those generated from wild type mice [109]. We found that mutant tauP301L overexpression resulted in abnormal proliferation and differentiation as indicated by MTT proliferation assays and filopodial/spine morphology measures, respectively. With repeated passaging, the non-adherent neurosphere culture proliferation rate significantly slowed and lost the ability to differentiate into brain cells. Instead, plating the Nestin-positive cells as adherent cultures revealed large, flattened cells with large cell somas containing numerous vacuoles and enlarged nuclei. These adherent cells remained in culture until finally discarding the culture plate after several weeks of attempts to expand the culture or differentiate into brain cells. While we did not examine cell cycle inhibitor expression or SA β-gal, the other defining features of cellular senescence were observed [109]. The Tau Consortium has generated at least 31 induced pluripotent stem cell (iPSCs) lines from 140 MAPT mutation/risk variant carriers and cognitively normal controls [114]. As the scientific community continues to utilize this resource, we anticipate more clarity surrounding the effects of aberrant tau on cellular senescence across brain cell types during early development and in disease.

Similarly, Voisin and colleagues [110] developed human iPSCs from a single patient with Huntington's disease, a rare inherited neurodegenerative disease arising from mutations in the *HTT* gene. In this study, HTT CAG repeat-corrected (C116) cells from the same donors were used as a disease-free control. Using qRT-PCR and a gene ontology approach, they found expression of FOXO3 and its transcriptional targets to antagonize p16 expression. Furthermore, they identified p16 and related genes as key mediators of senescence in Huntington's disease NSCs and medium spiny neurons. Cells derived from the Huntington's disease patient additionally showed decreased proliferation and elevated SA β-gal staining in vitro [110]. These results suggest that Huntington's disease mutations may negatively impact NSC function and perhaps contribute to disease through senescence-mediated pathogenesis; however, senescence phenotypes will need to be confirmed in postmortem human brain tissue from patients with Huntington's disease to validate these intriguing preclinical findings.

Over 200 disease causing mutations to *POLG*, a subunit of the mitochondrial DNA repair protein polymerase gamma (polγ), have been identified—many of which primarily affect the nervous systems. Using fibroblasts from two patients carrying *POLG* mutations, Liang et al. [115] generated iPSCs and then differentiated them into NSCs. While the iPSCs recapitulated some known *POLG* mutation phenotypes, the NSCs especially showed characteristic signs of mitochondrial dysfunction, including reduced energy production, abnormal mitochondrial volume, respiratory chain complex 1 loss, and increased ROS. Other evidence include upregulated mitochondrial uncoupler protein 2 (UCP2, which maintains mitochondrial membrane potential) and reduced phosphorylated Sirtuin 1 (SIRT1, a NAD-dependent protein deacetylase) [115]. In addition to mitochondrial dysfunction, these NSCs showed increased levels of SA β-gal staining and p16 expression suggestive of senescence. While the study did demonstrate SA β-gal staining and potentially a reduction in cell proliferation via p16, their characterization of senescence did not include the cell's resistance to apoptosis or a SASP. Instead the focus of the study was primarily on mitochondrial dysfunction given its strong link with *POLG* mutation disorders. Together these studies highlight the on-going research and approaches utilized by the neuroscience field to understand mechanisms surrounding age-associated neurodegenerative diseases. Cellular senescence is emerging as a potentially significant cell stress pathway worth continued investigation.

While the above studies provided examples of the effects of carcinogens, genetic or pathological variants on NSC function, other studies have investigated sporadic, age-associated phenotypes. Tissues accumulate senescent cells with advanced chronological age. Gao et al. [116] isolated SVZ neurospheres in vitro from 23-month-old mice and compared them to 3-week-old counterparts; an age where developmental neurogenesis has completed but the brain is still maturing. Neurosphere formation, proliferation, and neuronal differentiation were significantly lower in aged NSCs. Further, aged NSCs showed increased p16 expression and shortened telomeres via qRT-PCR. SA β-gal staining, apoptosis, and SASP were not investigated. These changes were partially alleviated by flavonoids of the plant Ribes meyeriy, [116] suggesting the drug's anti-aging and potentially senotherapeutic effects.

Using the senescence accelerated (SAMP8) mouse model at 2, 6, and 12 months of age, Hu et al. [117] showed an age-related increase in NSC senescent phenotypes. NSCs, labelled via Sox2 and either Nestin or GFAP, were found to decrease with age in the hippocampus. Furthermore, SA β-gal staining in the hippocampus increased at 6 months and again at 12 months. The percentage of senescent NSCs, labelled via Sox2 and p16, also increased with age. Isolated NSCs from these mice were less able to form neurospheres or differentiate into neurons. DNA damage-associated cell cycle arrest was evidenced by elevated γH2AX, p53, p16, and p21. Exogenously applied embryonic stem cell extracellular vesicles reduced many of these senescence-associated outcomes both in SAMP8 mice and in vitro. RNA sequencing analyses revealed that *Myt1*, a regulator of neurogenesis, was downregulated with passaging and age and upregulated with the extracellular vesicle treatment. Consistent with these observations, *Myt1* knockout mice developed many of the same senescence-associated phenotypes and were resistant to the beneficial effects of the extracellular vesicles [117]. These data suggest that *Myt1* may be a novel regulator of NSC senescence and that extracellular vesicles from embryonic stem cells have potential to treat age-related neurodegeneration.

A recent study by Xiao et al. [118] investigated senescence phenotypes in aged hypothalamic NSCs. Neurospheres derived from 18-month-old wild type mice were fewer, smaller, and had lower proliferation than those from 3-month-old mice. From these experiments, they identified the long non-coding RNA Hnscr as playing a role in NSC senescence. *Hnscr* null mice displayed similarities to wild type counterparts at young ages, but accelerated aging at 18 months, including reduced NSC proliferation and increased SA β-gal staining. Similar, but less striking, results were seen with viral suppression of *Hnscr* specifically in hypothalamus NSCs. RNA sequencing and bioinformatic approaches revealed cell senescence, apoptosis and inflammatory responses among the altered pathways.

In particular, p16 was upregulated as confirmed by qRT-PCR. Lastly, wild type mice treated with Theaflavin 3-Gallate, an Hnscr mimetic, had lower NSC senescence compared to vehicle treated controls. Outcome measures included SA β-gal staining and RNAseq data consistent with cell cycle arrest, apoptosis resistance, and a SASP suggest a *bona fide* senescence arrest. Collectively their data highlight Hnscr as a critical mediator of hypothalamic NSC senescence. Future studies are needed to determine whether this broadly applies to other NSC populations or SCs in general. Nonetheless, this study highlights a significant advance in senescence biology by evaluating brain cell populations.

Overall, these studies indicate that NSCs acquire senescence-like phenotypes in response to carcinogens and stressors known to promote cancer or neurodegenerative disease. Given the inverse relationship between the risk for developing AD and cancer, it is tempting to speculate that senescence may protect against cancer but promote neurodegeneration. However, further research is needed to draw more conclusive inferences. Through reviewing the literature, we identified important areas for clarifying "senescence" in NSCs. One is the difference between quiescence and senescence. Given that cell cycle arrest is a defining feature of both, the permanency of the arrest needs to be experimentally determined. A gold standard assay to confirm the cell cycle activity in NSCs is their ability to divide and give rise to multipotent clones when provided with cytokine growth factors in the culture, e.g., the neurosphere assay. While useful to identify proliferative arrest ex vivo; cell cycling is not easily discernable in postmortem tissue, especially since similar molecular pathways are used for both quiescent and senescence arrest. For example, the transcription factor Bmi-1 regulates NSC self-renewal by suppressing p16 and p19 [119–121]. p16 regulates whether DG NSCs exit quiescence and undergo neurogenesis in response to running [122] and genetically removing p16 lessens the age-associated neurogenesis decline in the SVZ, but not DG [123]. *Cdkn1a*, which codes for the cell cycle inhibitor p21, expression is required to appropriately regulate NSC proliferation/quiescence during early and middle age [124], and in response to ischemia [125]. These studies highlight the physiological roles of commonly used markers of cellular senescence (i.e., Bmi-1, p16, p19 and p21) in NSC maintenance and behavior in an age, stress and region-specific manner. Thus, their differential expression in histological tissues may represent a snapshot of regulated NSC quiescence, making it difficult to discriminate from senescence. Distinguishing between quiescent and senescent NSCs in particular remains a challenging roadblock to the field.

Other distinguishing markers can help differentiate senescence from quiescence or other causes of decreased neurogenesis. For example, senescent stem cells remain metabolically active whereas a reduction in metabolism is one of the key characteristics of quiescence [87,126,127]. While little is known about the secretome of quiescent cells, limited evidence suggests a potential overlap with the SASP including IL-1α, IL-6, IL-8, CCL4, and CCL6 [128,129]. Apoptosis resistance, to our knowledge, has not been investigated as a marker of NSC senescence. Nonetheless, NSCs naturally display a general resistance to apoptosis [130,131]. Whether or not these pathways change with age or in response to other stressors could be used to help distinguish between senescence and quiescence. A recent study identified a gradual entrance into senescence from quiescence regulated by lysosomal function [62]. While this may complicate the discernment between quiescence and senescence, it suggests that lysosomal content and activity may help decipher these cell states.

Concluding Remarks

In summary, true senescence may have been present in some of these studies, but most have only partially characterized the phenotype. Other studies have taken for granted senescence-related phenomena which occur in other cell types, but have not yet been demonstrated in NSCs. The Hayflick Limit and the SASP, for example, both represent opportunities for future experimentation in NSCs. In addition, while many studies have relied heavily upon SA β-gal staining, few have adhered to proper protocols (see Section 2.4.). Nonetheless, mechanistic studies utilizing brain NSCs are advancing our understanding

of the molecular regulators of cellular senescence, including neurodegenerative disease-associated tau [109], *Foxo3* [110], *Myt1* [117], and lncRNA Hnscr [118]. Whether or not they are cell type specific remains unknown, but they highlight the utility and importance of evaluating senescence in brain cells. Future studies will be needed for more in-depth characterization of senescent NSCs, their effects in brain pathology, and interventions specifically targeting this cell population.

4. Oligodendrocyte Precursor Cells

OPCs form 5–8% of all cells in the adult brain [132]. Developmentally, OPCs arise from multiple progenitor cell pools in the developing spinal cord and forebrain (reviewed [133,134]). Postnatally, OPCs are predominantly found in the SVZ and migrate to white matter regions, where they proliferate and differentiate into oligodendrocytes and astrocytes [134,135]. OPC multipotency provides an opportunity for malignant gliomas [136] including oligodendroglial [137] and astrocytic tumors [138]. Physiologically, OPCs differentiate into oligodendrocytes throughout life and contribute to myelin turnover [139–142], adaptive myelination [141,143,144] and regenerative processes following demyelinating insults [132,145]. The ability of OPCs to respond appropriately to myelin maintenance or stress signals determines whether they positively impact myelination [132,139–142] or potentially divide uncontrollably and become cancerous [136–138]. While cellular senescence protects against the latter, evolutionary pressure for the senescence stress response did not account for the long lifespans of modern day humans. OPC senescence, thus, may contribute to neurodegenerative phenotypes in later life. Evidence for the tradeoff between OPC senescence, cancer and neurodegeneration is presented in this section.

When grown in optimal culture conditions, OPCs challenge the Hayflick limit and replicate indefinitely [146]. Prolonged culture results in elevated expression of many cell cycle inhibitors (Cip/Kip proteins: p21, p27 and p57, and INK4s: p18 and p19) in the absence of cell cycle arrest. This non-senescence profile may be attributed to significant upregulation of positive cell cycle regulators Cdk2, Cdk4 and cyclin D1, D3 and E [146]. These experiments suggest that a healthy brain environment could provide OPCs an opportunity for indefinite replication (e.g., could be beneficial for brain maintenance but contribute to cancer). However, suboptimal culture conditions including prolonged exposure to 15% fetal bovine serum, contact inhibition due to overgrowth/confluency, and low dose genotoxic drugs all produced senescence-like phenotypes. Culture shock conditions resulted in proliferative arrest, including a failure to incorporate BrdU, and flattened morphology [146]. Notably, elevated p16 was not detected in any culture conditions, but viral-mediated overexpression of p16 strongly inhibited BrdU incorporation [146]. While these results indicate that p16 activation can cause OPC proliferative arrest, OPCs do not innately upregulate p16 in response to many known senescence-inducing stressors. Additionally, p21 is required for OPC differentiation to oligodendrocytes [147], which presents challenges for interpreting OPC differentiation from senescence. Thus, two of the most commonly used markers of senescence, p16 and p19, require additional considerations and methodologies and highlight the importance of including measures of DNA damage response pathways, resistance to apoptosis, and the SASP when evaluating OPC senescence.

Age-associated deficits in remyelination often co-occur with a lack of OPCs in the lesion site (e.g., poor recruitment) and/or delayed or failed OPC differentiation. The successful execution of recruitment and differentiation requires a complex interaction between the OPCs and their environment. Thus, poor remyelination may reflect impaired OPCs and/or suboptimal environmental cues. Cell autonomous deficits in OPCs are inferred by increased DNA damage, upregulated *Cdkn2a*, and reduced cellular respiration in OPCs isolated from 20-24 month-old mice compared to 2–3 month-old mice [148]. Given the increase in DNA damage, as evidenced by single cell comet assays, poor OPC proliferation and migration may protect against the development of malignant gliomas. In this way, a senescence response may be beneficial over tumorigenicity. Similarly cultured OPCs from aged mice were less proliferative as shown by decreased BrdU incorporation and cell cycle

arrest [149]. Examining whether these cells are resistant to apoptosis and identifying SASP factors associated with the cellular environment would validate the senescence phenotype in OPCs, and provide much needed information regarding whether they acquire a toxic SASP or remain benignly arrested.

OPCs are present abundantly in grey and white matter, and are believed to survey their microenvironment, migrate to lesioned areas for remyelination through repopulation of oligodendrocytes, and respond to inflammatory cues [132,150]. Although the non-progenitor characteristics of OPCs are less understood, they respond to CNS injury, ischemia, and neurodegeneration, along with microglia [150,151]. Depletion of the endogenous OPC pool, reduced migration of OPCs in a pathological environment, and lack of differentiation of OPCs lead to failure of remyelination in brain lesions, suggesting a critical role for OPCs in neurodegenerative diseases [134]. Interestingly, in post-mortem human brain tissue of patients with AD, elevated levels of p21 were observed in OPCs surrounding Aβ plaques, but not in regions devoid of pathology [152]. The authors reported that >80% of large Aβ plaques (>50 μm) contained cells that co-expressed Olig2 and p21. Interestingly, brain samples from non-demented controls or with mild cognitive impairment (MCI) contained few Olig2 or NG2 positive cells regardless of p21 or p16. This observation suggests that the Aβ plaque environment induces OPCs to proliferate, migrate and/or hone to the plaque environment. Given that p21 is a marker of OPC differentiation, one possible explanation for elevated p21 expressing OPCs near plaques is that they are differentiating into oligodendrocytes, which may be delayed by the plaque environment as described with aging [153]. Similarly, multiple sclerosis brain lesions more often contain OPCs that have failed to differentiate rather than a lack of OPCs [154–156]. In rodent models of these conditions, increasing the number of OPCs does not improve remyelination [157], presumably due to the unfavorable environment. Thus, it is tempting to speculate that the increase in p21 expressing Olig2 cells near Aβ plaques [152] represents a proper OPC response to proliferate and migrate to the lesion, but the unfavorable plaque environment has delayed or prevented their differentiation into mature oligodendrocytes. In this way, identifying the Aβ plaque-associated molecule(s) that impair OPC differentiation could provide an opportunity for therapeutic intervention. Across studies, additional markers are needed to distinguish whether p21 expressing OPCs are actively differentiating into oligodendrocytes (albeit slowly) or senescent.

Mouse models of Aβ plaque accumulation support the notion that OPCs hone to the plaque environment. The 3xTg-AD mouse model develops Aβ and tau pathology characteristic of patients with AD [158]. Hypertrophic OPCs were observed surrounding and infiltrating Aβ plaques in brains of 3xTgAD mice, which again suggests that OPCs hone to regions of Aβ pathology [159]. Similarly, TgAPP/PS1, mimicked the findings of human brain whereby increased *Cdkn2a* mRNA expression, SA β-gal, and IL-6 levels (SASP factor) were found in OPCs surrounding Aβ plaques to suggest that extracellular protein accumulation may negatively impact OPC function [152]. A novel ZsGreen senescence marker utilizing the p16 promoter was crossed to TgAPP/PS1 mice. The TgAPP/PS1 × ZsGreen mice displayed elevated reporter expression compared to wild type ZsGreen mice indicating significant upregulation of p16 [152]. Treatment with senolytics, Dasatinib and Quercetin [54], alleviated senescence phenotypes, reduced the presence of IL-1β, IL-6 and TNF-α (SASP factors), ameliorated cognitive deficits, and reduced neuroinflammation in OPCs of TgAPP/PS1 mice [152]. Although senescent cells were cleared with administration of senolytics, Aβ load remained unchanged in these mice [152], which may suggest that senescent cells alters cognition independent of Aβ. Moreover, these results may suggest that senolytics cleared the negative regulator of OPC differentiation thus allowing them to develop into mature oligodendrocytes. In this case, other senescent cells may be negatively impacting OPC function through their SASP. Indeed other senescent cell types have been identified in AD including neurons with neurofibrillary tangle pathology [15]. Interestingly, enhanced OPC function has been reported in mice expressing mutant human tau that drives senescence in many other brain cell types including neurons and

microglia [15,113,160]. The authors conclude that damaged axons, possibly from senescent tau-containing neurons, promotes OPC differentiation. It is tempting to speculate that neuronal SASP may be responsible for this altered OPC behavior. Indeed, our analyses of transcriptomic differences between neurons with or without NFTs indicate that NFT-bearing senescent neurons [161] differentially express growth factors that may influence OPC behavior.

OPC senescence has been suggested to contribute to disease progression in rodent models of progressive multiple sclerosis (PMS) [162]. High-mobility group box 1 (HMGB1) was identified as a component of the secretome of senescent NPCs [162]. Moreover, HMGB1 induced OPC senescence, as defined by upregulated gene expression of *Cdkn2a*, *Mmp-2*, and *Igfbp2*, and impaired the ability to OPCs to differentiate into oligodendrocytes [162]. The authors interpret that treatment of NPCs with rapamycin, an mTOR inhibitor, reversed the senescence phenotype as evidenced by a decrease in *Cdkn2a* mRNA and protein expression, and decreased SA β-gal intensity [162]. OPCs cultured in media isolated from these rapamycin-treated NPCs were found to differentiate into oligodendrocytes at a higher rate than OPCs cultured in PMS NPC media without rapamycin treatment, further suggesting a role played by senescent NPC environment in successful differentiation of OPCs [162]. Given that senescent cells are stably arrested, we question whether the NPCs in the PMS model are instead in deep quiescence and perhaps not fully senescent [62]. Nonetheless, the beneficial effects of rapamycin on OPC function warrant further investigation for demyelinating diseases.

Concluding Remarks

OPC multipotency offers the brain regenerative capacity throughout life, but at the cost of cancer development. OPCs migrate to lesions, proliferate, and repopulate the area with oligodendrocytes, in an effort to remyelinate and repair. Due to their ability to replicate indefinitely in culture, cellular senescence may play an important protective anti-cancer role in vivo. While OPC senescence-associated phenotypes have been identified in vitro, the in vivo data require further investigation. In particular, the most compelling study in AD used p21 as a marker of OPC senescence [152]. However, given that p21 upregulation is required for OPC differentiation, we cannot discern if these OPCs are experiencing delayed differentiation or if they became senescent and can no longer differentiate. Many of the studies suggest that OPCs hone to sites of injury but fail to differentiate; determining whether these cells represent senescent OPCs requires further investigation. Though several studies have demonstrated senescence-like phenotypes in OPCs, we could not find evidence for SASP or SCAPs. These areas are of primary interest as they will inform on whether future therapeutic approaches should focus on the use of senolytics to treat OPC dysfunction.

5. Microglia

Microglia are known as the primary immune cell of the central nervous system, consisting of 0.5–16.6% of all brain cells in humans [163]. While neurons, astrocytes, and oligodendrocytes originate from the neural tube, microglia are derived from yolk-sac macrophage precursors and migrate to the brain before the blood-brain barrier forms [164,165]. Microglia mostly display postmitotic phenotypes with an estimated turnover rate of 0.08% per day in healthy human cortex with ^{14}C birth dating indicating an average age of 4.2 years [166]. However, they readily proliferate locally in response to injuries [167–169]. Recent studies have also shown that there are latent microglia progenitors that can proliferate and differentiate into microglia in the microglia-depleted brain [170].

Microglia serve a dual function of maintaining the health of the central nervous system and acting as brain macrophages with immune surveillance by monitoring their cellular environment and promoting repair. For example, they remove dying neurons [171], protein aggregates such as Aβ [172] and tau [173], and tissue debris, as well as support synaptic pruning [172]. Through their release of anti-inflammatory cytokines and trophic

factors such as IL-4, IL-10, IL-13, TGF-β, they contribute to neuronal and oligodendrocyte survival [174,175]. However, they also can be activated to secrete pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, MIP-1α, reactive oxygen species, and nitric oxide [176,177]. Opposite to anti-tumorigenic pro-inflammatory cytokines, anti-inflammatory cytokines and trophic factor secretion by microglia are known to be pro-tumorigenic as they promote an immunosuppression of the tumor microenvironment, tissue repair, and angiogenesis that enable tumor invasion and survival [178]. Brain cancers such as gliomas are infiltrated with glioma associated microglia (GAM) that are recruited and reprogrammed by glioma cells [179,180]. These microglia increase glioma growth due to their decreased tumor sensing and immune response, while releasing mitogens and invasion promoting factors [181,182]. Given the role of microglia in neuroprotective and immune-modulatory functions, aberrant microglial function has also been linked to neurological diseases such as AD and PD [165].

Numerous studies have reported that microglia can become senescent and/or dystrophic. In the microglia literature, "dystrophic" and "senescent" are often used interchangeably [183]. However, we note that this is distinct from the cellular senescence criteria we use to evaluate the literature. Specifically, dystrophic microglia show morphological changes such as altered cytoplasmic structures and iron overload, but do not necessarily display the other criteria (i.e., shortened telomeres, cell-cycle arrest, lack of proliferation, resistance to apoptosis, and SASP). Activated microglia secrete proinflammatory cytokines and reactive oxygen species similar to senescent microglia. The key difference between activated and senescent microglia is that senescent microglia are unable to proliferate. Here we review microglia senescence, focusing on the pre-defined phenotypes outlined in Section 2.

Activated microglia are those in a hypersensitive state with exaggerated inflammatory responses. Activating stimuli include natural aging, infections, and other systemic inflammatory episodes [184]. For example, aging microglia display impaired neuroprotective abilities including low motility, reduced phagocytic capacity, and the secretion of pro-inflammatory molecules and reactive oxygen species. Repeated intraperitoneal injection of lipopolysaccharide (LPS) induces similar effects of systemic inflammation both in vivo and in vitro. In response to these stressors, activated microglia secrete pro-inflammatory molecules and reactive oxygen species. To identify different types of activated microglia including the traditionally defined M1 (pro-inflammatory) and M2 (anti-inflammatory) states, mRNA expression or protein levels of inflammatory markers such as *TNF-α*, *IL-1β*, and *TLR2*, and histological analysis of morphology markers such as *Iba-1*, *CD68*, and *CD11b* have been commonly examined [177,185–187]. Single-cell RNA sequencing has recently been used identify a more broadly defined activation response microglia (ARM)/disease-associated microglia (DAM) to examine specific microglial activation gene signatures including *APOE*, *CST3*, and *CD74* [188]. In contrast, various distinctive measures have been used as a biomarker for microglia senescence including detection of senescence-associated heterochromatin foci (SAHF), accumulation of lipofuscin, SA β-gal activity, secretion of matrix metalloproteinase-1 (MMP-1), nitrotyrosine, and other pro-inflammatory molecules, loss of lamin B1 expression, and upregulated p53, p21, and p16 markers [164,189–192]. Altered autophagy and impaired mitochondrial functions were also reported [176,192]. Thus, it is important to not only examine the morphology and secretion of pro-inflammatory molecules of microglia, but also to examine other markers of senescence to distinguish senescent microglia from activated microglia.

Studies in vitro have revealed that microglia can acquire various senescence-like phenotypes. Primary microglia obtained from an amyotrophic lateral sclerosis (ALS) rat model expressing $SOD1^{G93A}$ developed flat morphology, SA β-gal, and increased expressions of p16, p53, and MMP-1 at 12 days in vitro compared to 2 days in vitro. [191]. A similar experiment looked at microglia cells isolated from neonatal mice and compared the cultured cells at 2, 10, and 16 days in vitro [193]. In comparison to culture day 2, day 16 cells revealed altered morphology, increased matrix metalloproteinase (MMP-2) activation,

NF-κB activation, miR-146a expression, SA β-gal activity and decreased autophagy and expression of Toll-like receptor TLR-2 and TLR-4 [193]. Cell culture studies have demonstrated that telomere shortening contributed to microglia senescence. Reduced expression of murine *Tert* and telomere-associated genes were observed in activated primary murine microglia from C57Bl6 mice using mRNA isolation and qRT-PCR [194]. The transcriptomic changes associated with telomerase activity is believed to contribute to microglia senescence as reduced *Tert* mRNA expression is also observed in both ischemia and AD-like pathology in vivo [194]. Experiments in vitro with the BV2 microglia cell line have shown that LPS stimuli repeated 6 times every 48 hours induces acute neuroinflammation, drives heterochromatic foci, increases p53 expression, and increases SA β-gal staining [195]. Thus, chronic inflammation has been suggested as an intrinsic and extrinsic inducer of microglia senescent phenotypes [193,195].

Interestingly, human microglia isolated from Braak stage III or greater AD neuropathology also revealed a higher quantity of microglia with short telomeres compared to that of non-demented individuals using the same assays [196], which provides evidence that microglia from human AD brain with advanced tau neuropathology may be susceptible to cellular senescence. Notably, tau accumulation induces a microglia senescence-like phenotype in transgenic mice [113]. Isolated microglia from 6-month-old TgPS19 transgenic mice that express high levels of mutant human tauP301S showed increased expression of cell cycle regulators p16, p19, and p21, and pro-inflammatory IL-6 compared to control mice through fluorescence-activated cell sorting (FACS) and qRT-PCR analysis [113]. Although this study did not evaluate telomere length, together with the human brain data [196] and other studies on tau-associated senescence [15], the data suggest that neuronal tau pathology may induce a senescence phenotype in microglia.

A relationship between telomere length and senescence has been demonstrated with natural aging as well. Microglia isolated from 30-month-old mice displayed significantly shorter telomeres via telomere flow-FISH and decreased telomerase activity via TRAP compared to the younger controls [196]. Compared to natural aging microglia, LPS injection can generate a model for microglial senescence relatively quickly. Mice from several different studies display strong pro-inflammatory responses to LPS injection, such as large increases in *Tnfα*, Il-6, and Il-1β expression in both a neurodegenerative mouse model of ME7 prion diseases and in middle-aged mice [197,198]. While these pro-inflammatory markers are consistent with SASP, this study [197] did not address other features of senescence such as loss of proliferation, DNA damage, aberrant expression of cell cycle inhibitors, and resistance to apoptosis. Notably, not all markers of senescence fully translate to in vivo studies. An absence of p21/p53 pathway activation, lack of telomere shortening, and increased number of Ki-67 markers were reported for microglia isolated from 24-month-old mouse brains [199]. The authors concluded that the aged microglia may be dysfunctional, but that they do not display a senescent profile [199]. This striking discrepancy between widely accepted in vitro senescent markers and in vivo studies requires better approaches for understanding the differences between induced senescence in vitro and aging in vivo. Furthermore, despite how inflammation can trigger a senescence-like profile in microglial cells, the discrepancy in results between the natural aging and primed microglia studies should perhaps urge researchers to reexamine primed microglia as a model for cellular senescence beyond their pro-inflammatory responses.

Concluding Remarks

Altogether, these studies verified microglial senescence-like phenotypes through observations of morphology [191,193,199], DNA damage [194,196,199,200], lack of proliferation [199], cell-cycle arrest machinery [113,191,195,199], SA β-gal [191,193,195,199], and pro-inflammatory SASP [113,184,193,199]. It is important to note that microglial senescence is complex and heterogeneous. In order to highlight microglial cells as senescent and not simply dysfunctional, we conclude that telomere shortening, activation of cell-cycle arrest machinery, lack of proliferation markers, resistance to apoptosis, and pro-inflammatory

SASP markers are crucial to verify microglial senescent state. The commonly used SA β-gal assay may provide additional insight into microglia lysosomal activity, but cannot be used as a single surrogate to identify senescent cells. In this regard, of the reviewed studies, the study by Stojiljkovic et al. [199] met the predefined criteria outlined in the introduction. As the microglia senescence field moves forward, we recognize that clarity on definitions is needed. Oftentimes, dystrophic and senescent are used interchangeably, but do not imply biological cellular senescence as described in this review. Moreover, activated microglia display many characteristics of senescence. Careful evaluation of the full senescence phenotype is needed to make the distinction.

6. Summary

The accumulation of senescent cells has been reported across tissues in model organisms. Several recent studies have implicated senescent cell accumulation with neurodegenerative diseases [15,34,113,152,201]. Given that age increases both the incidence of neurodegenerative diseases and senescent cell accumulation, determining directionality and causality in the human brain remains unclear [14]. The articles reviewed in this manuscript support the possibility that the cellular senescence stress response may prevent the development of brain cancer. In this way, cells that undergo senescence may evade cancer, but drive neurodegeneration in later life. Thus, the cellular senescence response may contribute to the predisposition of individuals towards either cancer or neurodegeneration. This relationship, however, is complicated by a multitude of factors which have been identified to concurrently increase the risk of neurodegeneration, senescence and cancer. Senescence itself may also increase the incidence of cancer via the SASP [202].

Several concurrent characteristics are needed to positively identify senescent cells. These include proliferative arrest, a permanent change in cell fate, the SASP, a resistance to apoptosis, and possible staining with SA β-gal. Evaluating these criteria in vivo and in situ presents challenges, especially in the human brain. As such, most studies evaluated in this literature review did not measure or meet all of our predefined criteria for defining cellular senescence. While most senescent phenotypes were observed in the brain cell types reviewed, they were rarely observed simultaneously in a single study. By only partially characterizing the phenotype, the results leave room for alternative explanations beyond senescence. A reduction in proliferation and increase in p16 or p21 could indicate quiescent, not senescent NSCs. In particular, whether senescent NSCs exhibit SCAPs has not yet been investigated and their secretory phenotypes have been rarely characterized. Features associated with the senescent phenotype of OPCs are relatively less known. While it has been established that the surrounding environment strongly influence OPCs, further study of the in vivo senescent phenotype is required to reveal SASP or SCAPs associated with OPCs. Current literature utilizes p21 as a biomarker for the senescent phenotype. However, the upregulation of p21 in the differentiation process of OPCs confounds the use of this marker in senescence. In microglia, the SASP was a primary method for identifying senescent cells, but activated microglia also secrete pro-inflammatory molecules and reactive oxygen species. In general, a lack of other methodologies were used to verify that microglia cells were senescent and not simply activated in these studies. Future studies should further verify a resistance to apoptosis in senescent microglia and differentiate between activated, dystrophic, and senescent microglia via proliferative markers.

Staining with SA β-gal is perhaps the most prominent method for identifying senescent brain cells. However, the mechanistic link between SA β-gal staining and senescence remains unclear. The strong staining of quiescent postmitotic neurons additionally calls into question the utility of the method especially in the brain [15,152]. Additionally, many studies discussed in this review used archived tissue and did not utilize proper methods including insufficient controls, replicates, and poor reporting of pH. Lastly, the difficulties associated with dual labeling with SA β-gal makes it difficult to discern which cell type is being observed in vivo, meaning most evidence of cell-specific SA β-gal staining comes from in vitro studies.

Great advances have been made in the past few years regarding the presence of senescent cells in vivo. The use of rodent models carrying reporter transgenes have provided conclusive evidence that senescent cells accumulate with age across tissues. As scientists work to translate these findings to human tissue, there is a need to develop techniques and methodologies to identify, track and study senescent cells at the single cell resolution similar to what has been accomplished in mice. With the advancement of single cell transcriptomics and high-resolution digital profiling, we are on the precipice of developing similar data for human tissues [17,26]. Such data coupled with advanced systems biology approaches [203–206] provide an opportunity to identify robust multianalyte patterns across cells simultaneously and at the single cell resolution. As emphasized throughout this review, no single marker can identify senescent cells. Multianalyte transcriptomic and proteomic data therefore will provide the opportunity to confidently identify senescent cells. Directionality, magnitude and concurrence of senescence-associated pathways will be important in data interpretation. For example, many of the cell types here caution against the utility of using gold standard p16 and p21, especially in isolation, given their critical importance for brain cell function (i.e., NSC quiescence, OPC differentiation, microglial activation). However, a co-occurrence of upregulated cell cycle inhibitors, SCAPs and SASP with concomitant reduction in cell proliferation, differentiation and lysosomal pathways would provide stronger evidence that a cell has entered senescence. We expect comprehensive bioinformatic analyses of these multianalyte measures will also reveal cell-specific SCAPs and SASP with detailed signaling circuits that can be used for drug development. Given that clinical trials for the treatment of AD are underway (NCT04063124 and NCT04685590), results from high resolution studies will be critical in advancing this therapeutic approach.

In conclusion, though additional work is needed to confidently identify, profile and study senescent cells in the brain, this area of research holds great promise. We have highlighted several studies which, while focused on a specific cell type, have broader impacts for senescence in the rest of the body. Novel mechanisms regulating senescence have been explored in brain cells [110,117,118] and senescence in the brain has been shown to have major effects on the entire organism [207]. Future studies focused on senescence in the brain will continue to advance the fields of biology of aging, neuroscience and cancer; and potentially lead to interventions that broadly impact human health and longevity.

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Opinion

Secreted Protein Acidic and Rich in Cysteine as A Regeneration Factor: Beyond the Tissue Repair

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Abstract: Diverse pathologies (inflammation, tissues injuries, cancer, etc.) and physiological conditions (obesity, physical activity, etc.) induce the expression/secretion of the matricellular protein, secrete protein acidic and rich in cysteine (SPARC). SPARC contributes to the creation of an environment that is suitable for tissue regeneration through a variety of roles, including metabolic homeostasis, inflammation reduction, extracellular matrix remodeling and collagen maturation. Such a homeostatic environment optimizes tissue regeneration and improves tissues' repair ability. These properties that SPARC has within the regeneration contexts could have a variety of applications, such as in obesity, cancer, sarcopenia, diabetes and bioengineering.

Keywords: secreted protein acidic and rich in cysteine; regeneration; homeostasis

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Tissue regeneration is a vital process allowing organisms to overcome biological disturbances and adapt to changes and physiological development via the renewal, growth and restoration of diverse cells and tissues. The regeneration ability changes throughout the lifespan, which leads to diverse tissue malfunctions and diseases [1]. The regenerative process could be either normal or limited (abnormal) depending on the biological environment. Indeed, under healthy environmental conditions (stem cells growth ratio [2], growth factors [3], hormones [4,5], pH [6,7], etc.), the regenerative processes are optimized. They allow for regular tissue development and adaptation to the corresponding biological functions. However, under physiological (ageing [8,9]) or pathological (cancer [10], obesity [11], inflammation [12], etc.) conditions, or when impacted by disturbing stimuli or exogenous factors (such as radiations [13]), tissues' regeneration ability and functions could be impaired. To overcome this "negative" regeneration environment, the organism has a variety of tools to compensate or reduce the intensity or the impacts. These correcting or counteracting mechanisms are mediated through what could be considered regeneration factors. Among these molecules, secreted protein acidic and rich in cysteine (SPARC) has a variety of roles and implications. One of the SPARC properties is its ability to optimize the regeneration environment with an improved cellular regenerative capacity from different perspectives (metabolics, tissue repair, oxidation, inflammation, cancer, etc.), as illustrated below.

SPARC, also known as BM-40 or osteonectin (32 kDa [14]), is a matricellular (extracellular matrix-associated) protein. Unlike its name (osteonectin) might suggest, SPARC expression is not limited to bones, but this glycoprotein is also present in diverse tissues including nonmineralized tissues, in platelets [15] and in muscles [16]. Such wide distribution correlates with SPARC roles during embryogenesis [17] as well as during tissue repair, cell turnover, cellular differentiation and remodeling [18–22], which are key steps in tissue regeneration. Therefore, SPARC expression or levels increase following injuries such as myocardial injury [23], myopathies [24] and in situations (either physiological or pathological) where tissues undergo

changes (repair, renewal and remodeling) such as during obesity [18,25], skeletal muscle regeneration [26], cancer [27], systemic sclerosis, hepatic fibrosis [28] and physical exercise. Indeed, SPARC/*Sparc* expression increases in the skeletal muscle during training [29], as well as following electrical pulse stimulation in muscle cells (considered to be the in vitro equivalent of exercise) [30]. Such situations do represent a disturbance of the homeostasis that leads to a "negative" regeneration environment. Therefore, biological processes that overcome such a homeostatic disturbance, restore a suitable environment for regeneration and rescue the affected tissues to allow better developmental patters are required. Interestingly, the situations in which SPARC is overexpressed are mainly those requiring regeneration, either to repair tissues (injury) or adapt to tissue changes (obesity, exercised muscle, etc.). These specific patterns highlight SPARC as a regenerative factor. In addition, the importance of the extracellular matrix in regeneration suggests close interactions between SPARC, the extracellular matrix [31] and matricellular protein components such as thrombospondin-2 [32] during the regeneration process.

Tissue regeneration is a process that requires the implication of numerous cellular organelles and the use of energy. Thus, regeneration has metabolic and biochemical needs to which the cellular machinery has to adapt [33]. In this context, SPARC has been shown to be implicated in a variety of metabolic functions, such as glucose tolerance improvement [34], while it is also required for both glucose homeostasis maintenance and insulin secretion [35]. In the skeletal muscle, SPARC also seems to act towards improved metabolic properties and functions [18,24], including mitochondrial functions [30,36,37], which is of interest knowing the importance of the mitochondria during regeneration [38,39]. Importantly, our latest study suggests that exercise-induced muscle phenotype changes are SPARC-dependent [40]. These SPARC properties are also completed by their important roles in energy balance and storage. For instance, SPARC inhibits adipogenesis [41] and its inactivation leads to an enhancement of high-fat diet-induced obesity [42]. These patterns correlate with the role of SPARC in brown adipocyte activation and lipid usage in white adipocytes [43]. Such energy metabolism effects—in addition to optimizing the regeneration (synchronization)—also lead to increased energy usage, thus reducing the risk of obesity through increased energy expenditure. This represents another illustration of how SPARC counteracts the "negative" regeneration environment, since obesity itself represents a status of impaired regeneration [44]. Indeed, during obesity, many factors lead to such a "negative" regeneration environment due to all the conditions induced by or associated with obesity, such as inflammation, insulin resistance, metabolic disorders [45,46] and even stem cell changes [47,48], that impact regeneration. SPARC is extremely important for bone formation, remodeling and regeneration [14,32,49–51]. This is important as well, not only for the structural homeostasis, but also for both locomotion and, most importantly, the energy metabolism. Indeed, the skeletal muscle that governs most of the energy expenditure [52] is supported by the skeleton with which it forms the locomotor (musculoskeletal) system. Therefore, the good metabolic and contractile function (strength) of muscles would require homeostatic skeleton development due to the close ties between both bones and skeletal muscles, including synchronized development [53].

Furthermore, in addition to such metabolic implications, SPARC is also involved in other growth and homeostasis-related patterns, including cancer homeostasis. SPARC is overexpressed during cancer [27] and has been reported to have anti-cancer properties [54,55]. SPARC has also been shown to have interesting roles within the inflammatory processes [56,57]. It has anti-inflammatory properties [56] and can, for instance, protect from adverse cardiac inflammation during viral myocarditis [58]. These properties of controlling cancer and inflammation development would impact the microenvironment, contributing to an improved homeostasis. Moreover, SPARC is required for the immune system functions [59], which is relevant, for instance, during immune-modulatory therapy to support the regeneration of injured muscles [60] and muscle healing [61]. Importantly, more roles are yet to be explored in terms of SPARC contribution at the physiological levels, such as in cardiomyocyte contraction [23]. This cardiac role would improve the

blood circulation for diverse cells, which are vital for tissue regeneration). In addition, the therapeutic practice of cardiac regeneration [2,62] could benefit from SPARC properties in cardiac regeneration [19,63] as well. Beyond the cardiac properties, SPARC has roles in the cardiovascular properties, as suggested by its production by both bone-marrow-derived cells during myocardial fibrosis (in left ventricular pressure overload) [64] and pericytes, with a possible role in postinfarct healing [65], which is supported by the possible classification of SPARC as a marker for vascular complications in pre-diabetics [66].

All these highlighted properties point to SPARC as a regeneration factor. It not only has significant roles in tissue repair or development but contributes directly and indirectly to generating a "positive" biological environment that optimizes regeneration, as summarized in the graphical abstract. Moreover, other factors that work towards reducing the regeneration ability, such as ageing [1,67] and oxidative stress [68,69], are also counteracted—at least indirectly—by SPARC effects. For instance, SPARC-induced increased muscle functions (including via interactions with actin in skeletal muscle [24]) and metabolism would increase the antioxidant effect induced by exercise [70]. This contributes to the improvement in the regeneration environment by decreasing the oxidative stress. Furthermore, an improved muscular function (including during exercise) would lead to reducing the accumulation of the lactic acid and, therefore, better control of the pH, which both impacts muscle fatigue [71,72] and represents another important factor for different cellular functions [73], including those related to regeneration [74,75]. In addition, ageing-induced collagen loss [76] would be counteracted via the roles of SPARC in collagen properties [77–80]. Moreover, many SPARC effects counteract ageing impacts. In this context, ageing is a factor that decreases the regeneration ability [67], and with which we see an increased risk of obesity [81], sarcopenia [82,83], osteoporosis [84], etc. This points to SPARC not only as a regeneration factor that counteracts the ageing-related decrease in regeneration ability, but also as a factor with key roles against ageing-induced conditions that lead to health problems including sarcopenia, obesity (a health problem that could increase with the ongoing COVID-19 crisis [85]) and osteoporosis, through metabolic, structural and functional roles, and the impacts SPARC has on the corresponding tissues and organs (muscles, adipose tissue, bone, etc.). Therefore, SPARC remains worth exploring in the ageing process and geriatric research. These examples represent additional illustrations of SPARC's contribution to creating the optimal environment for regeneration, and further point to it as a regenerative factor.

These patterns show complimentary roles in terms of the implications of SPARC in tissue repair, and the diverse metabolic and homeostatic effects it mediates [86]. Importantly, the fact that SPARC is overexpressed during pathological situations such as obesity and cancer, as well as during physical activity (physiological adaptation), further indicates that it could represent feedback. Rather than a damaging factor, SPARC would aim to counteract/correct the negative impacts induced by the pathological situations such as inflammation and tissue damage through properties including regeneration ability, as illustrated for the skeletal muscle [87]. Indeed, conditions (pathological and physiological) that lead to impaired regeneration by creating a negative environment are the same conditions under which SPARC overexpression has been reported. Such overexpressed SPARC improves the regeneration ability and reduces the negative environment by inducing functional and metabolic enhancement at different tissues. These actions reverse, correct or reduce the impacts those initial conditions had on regeneration, which will lead to a SPARC-induced corrected regeneration ability.

This paper presents SPARC as a promising therapeutic tool in a variety of health conditions, ranging from metabolism and inflammation to obesity and sarcopenia. Importantly, SPARC could also be an option in the area of tissue engineering based on its involvement in and impacts on the regenerative processes, especially with the known implications of SPARC in the functions of stem cells [88,89], as well as other types of cells such as erythroid progenitors [90]. Thus, SPARC-related pathways also represent a potential pharmacologi-

cal target to optimize therapies in regenerative medicine as an adjuvant to optimize the regeneration environment of the targeted tissues and organs.

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