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Does senescence play a role in age-related macular degeneration?

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Abstract

Advanced age is the most established risk factor for developing age-related macular degeneration (AMD), one of the leading causes of visual impairment in the elderly, in Western and developed countries. Similarly, after middle age, there is an exponential increase in pathological molecular and cellular events that can induce senescence, traditionally defined as an irreversible loss of the cells' ability to divide and most recently reported to also occur in select post-mitotic and terminally differentiated cells, such as neurons. Together these facts raise the question as to whether or not cellular senescence, may play a role in the development of AMD. A number of studies have reported the effect of ocular-relevant inducers of senescence using primarily in vitro models of poorly polarized, actively dividing retinal pigment epithelial (RPE) cell lines. However, in interpretating the data, the fidelity of these culture models to the RPE in vivo, must be considered. Fewer studies have explored the presence and/or impact of senescent cells in in vivo models that present with phenotypic features of AMD, leaving this an open field for further

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Author contributions

GM, JC, KK, AL, DSK contributed to conceptualization, manuscript drafting and editing, and figure design. CW contributed to manuscript drafting.

Ethics

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Declaration of competing interest

None.

investigation. The goal of this review is to discuss the current thoughts on the potential role of senescence in AMD development and progression, considering the model systems used and their relevance to human disease.

Keywords

Age-related macular degeneration; Senescence; Aging; Retinal pigment epithelium

1. Introduction

Amongst the bevy of retinal degenerations, age-related macular degeneration (AMD) remains a major contributor to vision loss in the elderly in many parts of the World (Zhou et al., 2021). Since the first descriptive reports of the aging macula, in the middle of the 19th century (de Jong, 2016), significant progress has been made in characterizing the pathobiology, and genetic, epidemiological, and environmental risk factors associated with the disease. And though drusen, extracellular material that accumulate outside the retinal pigment epithelium (RPE), characteristic of dry AMD, have also been identified in some young individuals (Pedersen et al., 2018), this is more often than not, an exception to the rule, as age is an established risk factor for the disease (Heesterbeek et al., 2020). That AMD is a neurodegenerative disease of the aging retina (Hadziahmetovic and Malek, 2020) raises the question as to whether or not senescence plays a defining role in its occurrence and progression. On first glance it would seem that senescence, a biological consequence of aging, would be central to AMD and therefore an established contributor to disease. However, how and the extent to which senescence may either impart a positive or negative affect in AMD remains an unanswered question and is currently the focus of investigation in many research groups. The goal of this review, written following the 2021 Stephen J. Ryan Initiative for Macular Research meeting by members of the sub-group focused on discussing “Cellular and Organelle Aging in AMD”, is to discuss the current state of the literature and begin to tease out the potential role of senescence in AMD. Throughout, we have embedded discussion points on impeding factors that have held up progress in this field, while focusing on identifying relevant future avenues of research to be pursued including targeting senescence as therapy for AMD, if justified.

1.1. Pathobiology of AMD

The pathobiology of AMD is complex and our knowledge of it has evolved with the emergence of new imaging modalities allowing evaluation of the retinal layers of patients in real time (Fleckenstein et al., 2021). AMD can largely be sub-classified into early dry AMD, intermediate AMD, geographic atrophy (GA), and non-exudative and exudative macular neovascularization (MNV) (Fleckenstein et al., 2021). The early stages of AMD, referred to as ‘early’ and ‘intermediate’ AMD, involve the accumulation of medium-or large-sized lipid- and protein-rich extracellular deposits to the RPE. Vascular changes at the level of the choriocapillaris also emerge at this stage and include choriocapillary dropout (Mullins et al., 2011). Capillary degeneration, along with photoreceptor and in particular RPE atrophy are hallmarks of GA, resulting in significant compromise to central vision. Finally, in some

patients, the invasion of vessels originating from the choroid into the outer retina results in MNV, and extensive vision loss.

Our initial understanding of the pathology of AMD was primarily based on examination of ocular tissues from donors, often collected with variable post-mortem times and sample processing methods, which impacted the quality of the tissue. Improved techniques and stricter tissue processing protocols have not only confirmed early findings but also identified new distinct pathological changes in the macula, including but not limited to sub-retinal drusenoid deposits/reticular pseudodrusen and outer retinal tubulations (Chen et al., 2020; Rudolf et al., 2008). Of importance, the overall clinicopathological picture of AMD supports the involvement of an assortment of cells in the different stages of the disease including the photoreceptors, microglial cells, RPE, and choroidal endothelial cells, all potentially vulnerable to aging associated events such as cellular senescence (Fig. 1) (Malek and Lad, 2014; Tuttle et al., 2021).

1.2. Aging and cell senescence

The risk of developing AMD increases markedly with age. Likewise, pathological molecular and cellular conditions that can induce senescence also increase exponentially after middle age. Even though *aging* is difficult to precisely define, it corresponds to an overall decline in many physiological functions with age. In 2013, nine hallmarks of aging were proposed, including stem cell exhaustion, altered intercellular communication, genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, and increased cellular senescence (Lopez-Otin et al., 2013). These hallmarks often overlap, and of note, several are associated with cellular senescence potentially triggered by persistent DNA damage, mutations and/or oxidative stress (Campisi et al., 2019). In particular, oxidative stress, mitochondrial dysfunction, and impaired proteostasis are known to contribute to AMD development and progression (Ferrington et al., 2021; Fleckenstein et al., 2021; Paraoan et al., 2020) (Fig. 1).

Cellular senescence was originally described to entail an irreversible loss in the cells' ability to divide concomitant with resistance to cell death-inducing stimuli (Hayflick and Moorhead, 1961). Consequently, these cells accumulate with age. More recently, cellular senescence phenotypes have been observed in post-mitotic neurons and brain tissue (Jurk et al., 2012; Musi et al., 2018; von Zglinicki et al., 2021). Senescent cells characteristically secrete many inflammatory factors, as part of the complex senescence-associated secretory phenotype (SASP) (Coppe et al., 2008), and can contribute to many pathologies, including pulmonary fibrosis (Chilosi et al., 2013; Wiley et al., 2019), atherosclerosis (Childs et al., 2016), osteoarthritis (Jeon et al., 2017), type 2 diabetes (Aguayo-Mazzucato et al., 2019; Sone and Kagawa, 2005) and eye diseases including diabetic retinopathy (Crespo-Garcia et al., 2021; Oubaha et al., 2016), and glaucoma (Blasiak et al., 2017; Skowronska-Krawczyk et al., 2015). Senescent cells drive these pathologies largely by secreting pro-inflammatory cytokines, danger-associated molecular patterns (DAMPS) and chemotactic chemokines (Basisty et al., 2020). The SASP is dynamic and in some cases beneficial, such as during wound healing (Demaria et al., 2014). Here, we summarize the characteristics of senescent cells (Fig. 2).

Two major tumor suppressive pathways, governed by $p16^{INK4a}$/Rb and $p14^{ARF}$ ($P19^{Arf}$ in mice)/p53/$p21^{CIP1}$, initiate and maintain the senescent growth arrest. Many stressors enhance the expression of $p16^{INK4a}$, which inhibits a cyclin-dependent kinase (CDK) and phosphorylation of the retinoblastoma (Rb) tumor suppressor protein, thus preventing activation of the pro-proliferative transcription factor E2F. On the other hand, $p21^{CIP1}$ expression is regulated mainly by p53, which inhibits different CDK targets. The two pathways can act together synergistically to activate Rb proteins and arrest progression from G1 to the S phase of the cell cycle (Sherr, 1996; Wu et al., 2001). Increased expression of $p16^{INK4a}$ and/or $p21^{CIP1}$ is commonly observed in senescent cells.

Genotoxic stress or oncogene activation can cause a senescence response, including the secretion of SASP factors; the SASP component includes pro-inflammatory cytokines such as interleukins (IL; e.g., IL-6, IL-8, IL-1α), chemokines (e.g., CXCL1 and CXCL10), and matrix metalloproteases (MMPs; e.g., MMP1, MMP3, MMP9) (Basisty et al., 2020; Coppe et al., 2008). These molecules are frequently associated with age-associated pathologies, including chronic inflammation. However, proteomic analyses of cultured human cells induced to senescence by different agents show that each SASP is unique, consisting of hundreds of largely distinct proteins (Basisty et al., 2020). Additionally, SASP components vary depending on species (e.g., human vs mouse) and cell type (e.g., epithelial vs stromal) (Coppe et al., 2008). On the other hand, certain core SASP factors, including growth differentiation factor-15 (GDF15), stanniocalcin-1 (STC1), Serpin Family E Member 1 (SERPINE-1), and MMP1, are expressed by many types of senescent cells (Basisty et al., 2020).

Oxidative stress, the overproduction of reactive oxygen species (ROS), can damage macromolecules, including nuclear and mitochondrial DNA. The primary sources of ROS are the mitochondrial electron transport chain and NADPH oxidases, enzymes in the plasma membrane and membranes of detoxifying organelles such as phagosomes. In all cases, ROS serve as either damaging or signaling molecules, and these divergent processes interact (Brennan et al., 2009; Dickinson and Chang, 2011; Finkel, 2003). For example, as ROS increases, it can sulfenylate cysteines on dynamin-related protein 1, the protein that initiates mitochondrial fission. This sulfenylation disrupts mitochondrial dynamics, resulting in cellular senescence in some cell types (Nishimura et al., 2018; Yu et al., 2020) potentially including RPE cells. Indeed, several studies show that mitochondrial dysfunction contributes to AMD pathology (Datta et al., 2017; Ferrington et al., 2016; La Cunza et al., 2021). Furthermore, a senescence response caused by mitochondrial dysfunction in the absence of genotoxic stress induces a distinct SASP lacking certain proinflammatory factors regulated by interleukin-1 receptor signaling. This mitochondrial dysfunction-associated senescence (MiDAS) (Wiley et al., 2016) may a good candidate to investigate further in AMD.

Finally, with regards to the retina and retinal diseases, early studies often used the terms aging and senescence interchangeably. The discovery of senescent characteristics has created a need to further review the literature in a more holistic way in an attempt to tease out the results of aging versus senescence with more clarity.

2. Requirements and challenges for assessing senescence in in vivo and ex vivo models

Modeling of AMD using both in vitro and in vivo platforms remains a vital tool in exploring the contributions of pathways to disease development and progression. Each present with challenges and limitations.

2.1. Limitations to animal models of AMD as platforms to investigate senescence

The multi-faceted nature of AMD has made recapitulating phenotypic features of the disease challenging, with an overall perception that animal models of human AMD remain to be developed. Yet, a number of in vivo models have been characterized incorporating either known genetic and environmental risk factors for the disease (Malek et al., 2005; Storti et al., 2019; Toomey et al., 2015) or through the discovery of pathways compromised simply during the aging process (Choudhary et al., 2020; Hu et al., 2013; Yao et al., 2022). These models present with different phenotypic features of AMD, most frequently basal laminar deposits below the RPE, and RPE phenotypic and degenerative changes. Importantly, these models were developed taking age into consideration and as such may serve as platforms to investigate potential senescence. The observation of choroidal changes similar to that observed in dry human AMD have not been reported in animal models.

There are a limited number of spontaneously developed animal models that have been used to investigate different aspects of senescence including the senescence-accelerated OXYS rats, though their ocular senescence markers remain to be investigated (Kozhevnikova et al., 2018), and the senescence-prone mouse strain 8 (SAMP8). Aged SAMP8 mice present with increased ocular autofluorescence, decreased scotopic retinal function, amyloid beta positive deposits below the RPE, RPE degenerative changes and increased $p16^{Ink4a}$ expression in the RPE, collectively supporting senescence mechanisms at play (Feng et al., 2016). Importantly, sub-retinal injection of an amyloid beta peptide in 5 month old C57BL/6 mice has been reported to impact retinal function, specifically triggering a decrease in the amplitudes of the a-, b- and c-waves on electroretinography, RPE pigmentary and degenerative changes, and increased expression of $p16^{Ink4a}$ in RPE cells (Liu et al., 2015). However, as noted in Section 4, these studies need to be confirmed using multiple measures of senescence because p16 expression is not solely associated with senescence but can also be a marker inflammation and general aging (Liu et al., 2019b).

The ocular pathology of non-human primates has also been described in some detail and though with advanced age some species develop lipid-rich deposits, the time investment to use them as animal models (over 25 years versus 2 years for mice) limit their broad use for therapeutic testing. However, examination of the pathology of retinal cross-sections from one 16- and one 29-year-old Rhesus monkey has been informative, demonstrating Senescence-associated beta-galactosidase (SA-βgal) staining in the RPE adjacent to small, hard drusen (Mishima et al., 1999), indicating that senescence may be a feature of retinal aging in the monkey eye. One caveat for these studies is that SA-bgal also labels older lysosomes, and can be a measure of lysosome aging, rather than senescence per se. Evidence for senescence in the best model for AMD available, namely human donor tissue from AMD

patients is fairly limited. Noteworthy has been immunolocalization of bone morphogenetic protein-4 (BMP4), capable of inducing expression p53 and $p21^{CIP1}$ in RPE cells, within the RPE and Bruch's membrane of patients with dry AMD (Zhu et al., 2009). In spite of this, there is a need to investigate senescence in current murine models that present with AMD phenotypes and more importantly human donor tissue, comprehensively, sub-classified based on clinical phenotype (Ferris et al., 2013) and ideally genotyped (at a minimum) (Fritsche et al., 2016; Pappas et al., 2021).

2.2. Limitations of existing in vitro models used to study senescence

The pros and cons of existing in vitro models to study important aspects of RPE cell biology have been recently discussed (Lakkaraju et al., 2020). Here, we will discuss the utility of these cell-based models to interrogate senescence in particular.

2.2.1 ARPE19, versus primary, versus stem cell derived RPE cells—Because the RPE is a terminally differentiated, postmitotic tissue, to accurately model non-replicative senescence in the RPE using in vitro models requires the use of non-dividing cell cultures. Many published studies on senescence in the RPE have used poorly differentiated cultures of the immortalized ARPE-19 cell line, which participates in the cell cycle and is susceptible to mitotic senescence. Therefore, conclusions regarding mechanisms or triggers that induce senescence in the RPE using immortalized RPE cell lines may have little relevance to RPE in vivo or to AMD. The minimum requirement to study senescence in RPE cell cultures is that the cells be well polarized, terminally differentiated, and express RPE differentiation markers (RPE 65, apical Na^+,K^+-ATPase, etc) to accurately reflect RPE in vivo. Well-characterized cell-based models include:

Primary RPE cultures established from freshly harvested retinas of mouse, porcine, or human donors recapitulate several features of RPE in situ. When grown on semi-permeable membrane supports (Transwell filters), these cultures form well-differentiated monolayers with tight junctions, trans-epithelial resistances (TER) greater than 300 ohm.cm^2, and degrade photoreceptor outer segments with kinetics comparable to that found in vivo. For each of these models, there are established protocols that have been validated in multiple publications (Blenkinsop et al., 2013; Gibbs and Williams, 2003; Maminishkis et al., 2006; Samuel et al., 2017; Toops et al., 2014).

Primary RPE cultures have proven to be especially useful in understanding mechanisms of AMD pathogenesis. Long-term cultures of human fetal and porcine RPE have been shown to constitutively secrete apolipoprotein E (ApoE), a major component of drusen, and this could be further exacerbated by exposing the cultures to active complement components (Johnson et al., 2011). Adult human RPE cultures secrete osteopontin, an inflammatory mediator, in response to oxidative stress (Lekwuwa et al., 2021). These studies suggest that the RPE is sufficient to induce biogenesis of drusen constituents. But what could be the mechanism? Using porcine RPE cultures, recent studies demonstrated that complement activation and lipid dysregulation – two major pathways implicated in AMD – cause mitochondrial injury in the RPE. The resulting alteration of mitochondrial redox status drives the formation of ApoE biomolecular condensates as potential drusen

precursors (La Cunza et al., 2021). Noteworthy, studies using RPE cultures established from adult human donors have identified defective autophagy, mitochondrial dysfunction, and decreased expression of nuclear hormone receptors in AMD donors compared with cells from unaffected controls (Ferrington et al., 2017; Ferrington et al., 2016; Hu et al., 2013; Zhang et al., 2020) (Fig. 3).

The limited availability of human donor tissue and number of passages that maintain expression of RPE cell markers, are the main drawbacks of primary cultures. While this is not a limitation for cultures from mice or pigs, one caveat for their use is the species-specific expression of genes associated with AMD: for instance, cholesterol ester transfer protein (*CETP*) is not expressed in mice, and humans are the only species that express three isoforms of *APOE*.

ES- and iPSC-RPE (Embryonic and induced pluripotent stem cell-derived RPE) cultures are now widely used not only to study various aspects of RPE cell biology (Hazim et al., 2017; Maruotti et al., 2015) but also in clinical trials launched by the National Eye Institute, for slowing or reversing vision loss associated with AMD. Cultures established from patient fibroblasts are especially useful to model disease conditions. Studies on iPSC-RPE generated from AMD donors with AMD-associated genetic risk alleles showed that these RPE cultures secreted high levels of complement proteins and drusen components including ApoE and identified nicotinamide as a potential modifier of these disease phenotypes (Saini et al., 2017).

Although iPSCs can constitute a freely available source to differentiate into RPE, there are a few significant caveats to keep in mind while studying senescence. First, iPSCs retain epigenetic memory that reflects the tissue of origin, developmental stage, and sex that can impact the efficiency of reprogramming (Efrat, 2020). This complex epigenetic regulation could in turn impact senescence response pathways in iPSC-RPE.

Immortalized RPE cell lines currently used include the human ARPE-19, human RPE-1, and rat RPE-J lines. RPE-1 (hTERT) is a telomerase immortalized line that exhibits virtually no features of RPE in situ. The cells are poorly differentiated, flat, and are more often used to study the development of the primary cilium, and not RPE biology (Lakkaraju et al., 2020). The rat RPE-J line was established by simian virus 40 (SV40)-mediated transformation, which resulted in hypodiploid RPE (Nabi et al., 1993). Moreover, these cells do not exhibit the correct apical localization of Na^+,K^+-ATPase and neural cell adhesion molecule (NCAM), which are required for RPE and photoreceptor function.

By far the most commonly used RPE cell line is ARPE-19, which was established by spontaneous immortalization of RPE from a 19-year-old donor (Dunn et al., 1996). Many studies on senescence have used poorly polarized or actively dividing ARPE-19 cells. These cells are fibroblastic in appearance, do not express key RPE proteins such as RPE65, and clear photoreceptor outer segments with very slow kinetics. Pertinent to studies on senescence, these poorly differentiated ARPE-19 cultures are very susceptible to oxidative stress (Glotin et al., 2008) or AMD-associated insults such as complement attack, in contrast to the highly robust primary RPE cultures (Radu et al., 2014; Tan et al., 2016). Therefore,

using actively dividing ARPE-19 cultures to study senescence could identify pathways and mechanisms that would not be relevant for RPE in vivo.

Two recent protocols for culturing ARPE-19 cells have shown an improvement in cell phenotype and kinetics of outer segment clearance (Hazim et al., 2019; Samuel et al., 2017). These protocols involve growing the cells in high glucose with pyruvate for 3-4 months or in nicotinamide for 2 weeks. Using these protocols, ARPE-19 cells express RPE65 and other markers of differentiation. However, the TER is low, indicative of a leaky barrier, likely because ARPE-19 cells do not express claudin-19, which is a key component of the RPE tight junction in vivo. Because barrier function is directly linked to the ability of the RPE monolayer to withstand stressors such as complement and oxidative damage, this could potentially underlie the increased susceptibility of these cells to insults that induce senescence in dividing cells. Another caveat is that ARPE-19 cells also exhibit chromosomal abnormalities, which could be another confounder in their use for studies of RPE senescence. Researchers using ARPE-19 cells to study senescence should be mindful of these caveats and at the very least, culture the cells using protocols established by Hazim et al. It is important to establish that the cells express RPE markers and are not actively dividing before the start of the experiment. Because a feature of senescence in postmitotic cells is cell cycle re-entry, using mitotic cells could mask important mechanisms that could drive senescence in the RPE.

2.2.2 RF6A versus primary choroidal endothelial versus stem cell derived choroidal endothelial cells—Changes in the outer vasculature have been noted in all the sub-types of AMD, though the clinicopathological features are quite different. In early, intermediate AMD and GA, there is evidence of choriocapillary dropout, while in MNV the choroidal vasculature undergoes abnormal vessel growth. As such, studying the biology of the choroidal endothelial cells is paramount, yet understudied. This is in part because in vitro model systems for choroidal endothelial are limited. The most commonly used cell culture model system has been a spontaneously transformed cell line originally derived from a crude choroid-retina complex isolated from the fetus of the rhesus macaque monkey, called RF/6A, which originally was reported to expresses endothelial markers. Recent careful characterization of this cell line has identified low level expression of endothelial markers including von Willebrand factor, platelet endothelial cell adhesion molecule-1 (PECAM1), VE-cadherin and cadherin 5 in RF/6A cells (Makin et al., 2018). Additionally, functional assays using RF/6A cells, found these cells to be insensitive to VEGF-A stimulation as well as shear stress. Finally, when challenged with tumor necrosis factor alpha (TNFα), expression of the endothelial-specific protein E-selectin was prominently less than that in other endothelial cell lines. Collectively, these findings highlight limitations of this cell line (Makin et al., 2018). Alternative cell culture systems which may be used included primary cultures isolated from human donor eyes (Peavey and Malek, 2020; Peavey et al., 2022; Stewart et al., 2011), which are convenient should there be accessibility to non-fixed donor eyes with short post-mortem times. Of relevance to AMD has been the creation of a conditionally immortalized choroidal endothelial cell line with decreased binding affinity for the AMD-associated 402H variant compared to 402Y (Loeven et al., 2018). An immortalized human choroidal endothelial cell line generated using lentiviral vectors

with endothelial-specific promoters to drive immortalization (Giacalone et al., 2019), and choroidal endothelial cells derived from mouse fibroblast iPSCs (Songstad et al., 2015), are also potential relevant cell culture models to incorporate in studies of AMD.

2.2.3 Senescence in spontaneously arising cell lines—Cells with a variety of phenotypes have been used to study age-related pathologies, including AMD. ARPE-19 cells, discussed above, is a spontaneously immortalized human RPE line that is not pre-malignant or malignant. Spontaneous immortalization is rare for primary human cells, but more common for mouse cells. In mouse cells many of the spontaneous immortalization events occur due to loss of p53 function (Harvey and Levine, 1991). This is significant because p53 can also regulate the SASP (Coppe et al., 2008; Davalos et al., 2013), Similarly human cells, particularly epithelial cells, are susceptible to losing p53 function upon spontaneous immortalization (Yaswen and Stampfer, 2002). Of interest, gene expression profiling of human embryonic stem cell derived (ESC)-RPE cells demonstrates greater similarity to primary RPE than to ARPE-19 cells (Klimanskaya et al., 2004). Finally, because gene expression profiles can differ substantially between primary and immortalized cells, caution and in vivo validation should be considered for interrogating aging, pathological and senescent phenotypes in intact tissues.

3. Aging-related changes in the outer retina

Given the impact of age-related vision loss on quality of life many laboratories have been investigating the morphological, physiological, and molecular changes in the aging eye using human ophthalmic data, human post-mortem specimens and animal models [reviewed in detail in (Campello et al., 2021)]. Common age-related ocular changes include cataract and low-light vision sensitivity, and chronic ocular diseases such as diabetic retinopathy, glaucoma and AMD, in which the neural retina, RPE and choroid are affected.

3.1. Retina

A key feature of the aging retina is a decline in visual function reflecting a decrease in photoreceptor activity. Studies in mice, rats, and humans (Ferdous et al., 2021; Gao and Hollyfield, 1992; Kovacs-Valasek et al., 2021) show an age-related reduction of scotopic a- and b-wave amplitudes and total number of photoreceptors. Interestingly, photoreceptor loss in aging is not uniform, rather pronounced in the peripheral retina as rods are more vulnerable to loss than cones (Curcio et al., 1993; Eliasieh et al., 2007). Surprisingly, these changes are not dependent on the diurnal/nocturnal lifestyle of the studied organism.

Another phenotype of aging is retinal thinning. Systematic studies (Samuel et al., 2011) have described age-related changes in cell number and density of the mouse retina and provided elegant quantification of dendritic arbors for all retinal neurons. Spectral domain-optical coherence tomography (SD-OCT) studies (Ferdous et al., 2021) have shown reduced thickness of the outer nuclear layer (ONL), which contains photoreceptor nuclei; confirmed by quantification of nuclei by histology. Interestingly, rod bipolar and horizontal cell dendrites extend into the ONL in aged animals suggesting changes in the extracellular matrix composition. Similar observations were made in examining the human aging retina

(Eliasieh et al., 2007) potentially indicating high levels of metabolic activity in aged bipolar and horizontal cells.

A third aging phenotype of the retina is reactive gliosis (Mansour et al., 2008; Ramirez et al., 2001) often associated with aberrant activation of Müller glia cells and astrocytes, and elevated levels of glial fibrillary acidic protein (GFAP) immunoreactivity. Glial activation is characterized by increased expression of VEGF, cytokines, extracellular matrix modifying molecules and several interleukins, which maintain the retinal inflammatory environment, as well as an overall decline in microglial functionality [reviewed in (Ma and Wong, 2016)].

Finally, the vasculature is also affected in the aging retina. Multiple studies using optical coherence tomography angiography (OCTA) have shown a decrease in vessel density in the aging human retina. Additionally, detailed mouse and human studies have found a significant drop in vascular density in age-related eye conditions including glaucoma (Yip et al., 2019), AMD (Toto et al., 2017; Vaghefi et al., 2020) and diabetic retinopathy (Liu et al., 2019a; Tonade et al., 2017). Of note, in larger animal models including canines, an evaluation of the retina, uvea, and lens revealed that the presence of tumors rather than age was associated with an increase in the senescent markers gH2AX and $p21^{Cip1}$ (Merz et al., 2019).

3.2. RPE

Like other postmitotic, metabolically active tissues, the RPE acquires specific functional deficits with age, which are exacerbated in AMD. One of the most important functions of the RPE is the daily phagocytosis and digestion of photoreceptor outer segment tips (Caceres and Rodriguez-Boulan, 2020). In the human eye, each RPE cell is in contact with ~40 photoreceptors, and the diurnal clearance of shed outer segments constitutes an enormous burden on the degradative machinery of the RPE. The importance of this process for photoreceptor health and vision is illustrated by the fact that mutations in specific phagocytic machinery are associated with inherited retinal degenerations: mutations in the MER tyrosine kinase (MERTK), which participates in outer segment ingestion, cause retinitis pigmentosa; whereas mutations in the actin motor protein myosin VIIa (MYO7A), which participates in phagosome transport in the RPE, result in Usher syndrome (Gal et al., 2000; Gibbs et al., 2003). However, there is to date limited evidence for defects in clearance of phagocytosed photoreceptor outer segments in the aging or AMD RPE. Studies on changes in RPE lysosomal hydrolase activities with age have yielded conflicting data, with one showing increased cathepsin D and acid phosphatase activities (Boulton et al., 1994) and another reporting a specific decrease in alpha-mannosidase activity but not in acid phosphatase (Wyszynski et al., 1989). Although decreased expression of the lysosomal membrane protein LAMP2 has been reported in AMD donor RPE compared to unaffected controls (Notomi et al., 2019), how this impacts photoreceptor outer segment degradation is yet to be investigated.

Decreased LAMP2 expression could however interfere with autophagy, an evolutionarily conserved mechanism to clear damaged proteins and organelles. Efficient autophagy is essential for postmitotic tissues like the RPE where debris cannot be dispersed among daughter cells after cell division. RPE from AMD donors exhibit defects in autophagosome

biogenesis (decreased levels of lipidated LC3B) and autophagic flux (accumulation of long-lived proteins such as p62/SQSTM1) (Golestaneh et al., 2017; La Cunza et al., 2021). Declining autophagy and the resulting accumulation of undegraded debris can place additional stress on the aging RPE (Fig. 3).

Another feature of aging and AMD is mitochondrial dysfunction. RPE from AMD donors show increased mitochondrial fragmentation and mitochondrial DNA damage, and decreased oxidative phosphorylation (Ferrington et al., 2017; Golestaneh et al., 2017; La Cunza et al., 2021). Because the RPE is highly reliant on oxidative phosphorylation (OXPHOS) as it spares glucose for the photoreceptors, mitochondrial dysfunction with age could induce the RPE to switch to glycolysis as an energy source, and ultimately starve the photoreceptors (Kanow et al., 2017).

3.3 Choroid

The choroid is a complex tissue located between the sclera and neural retina and its underlying RPE. Playing a central role in providing oxygen and nutrients to the overlying retina, it is heavily vascularized and can be broadly divided into the *Haller's layer*, composed of large blood vessels, Sattler's layer, composed of medium diameter blood vessels, and the choriocapillaris, a network of fenestrated capillaries. It is home to a high concentration of melanin, which may in part protect the choroidal micro and macro-vessels from light toxicity. It is also rich in fibroblasts, resident immune cells, extracellular matrix molecules including collagen and elastic connective tissue. The integrity of the choroid is paramount to vision, as any damage has the potential to lead to degenerative changes in the retina due to lack of vascular support or abnormal neovascularization or edema. Indeed, OCT evaluation of choroidal thickness in non-AMD individuals aged 21 to 86 years, has revealed thinning in the fovea with age by approximately 3 μm/year (Wakatsuki et al., 2015). Studies on blood flow and choroidal vascularity, in which luminal and stromal components of the choroid have been measured have also found a decline with increase in age (Emeterio Nateras et al., 2014; Nivison-Smith et al., 2020). The reported changes in melanocytes with age are less clear and dependent on method of analysis. When measured with fluorescence, a decrease in melanocytes was observed with age (Weiter et al., 1986), while biochemical measurements showed no changes with age (Hayasaka, 1989). Finally, fewer adrenergic fibers with less varicosities, a decrease in nerve fibers to the submacular region, and a decrease in hyaluronic acid in the choroid, have been measured with age in studies with relatively small cohort sizes (Jablonski et al., 2007; Nuzzi et al., 1996; Tate et al., 1993). These studies remain to be corroborated in larger populations.

4. Senescence

Replicative senescence, is an adaptive mechanistic response to prolonged stress, associated with dividing cells and cells challenged with a cancerous signal (e.g. *ras-induced senescence*). However, an increasing number of studies show *non-replicative senescence* can occur in post-mitotic cells including neurons. Senescent cells are rare at young ages, but are generally only a minor population (a few percent) even at old ages (He and Sharpless, 2017). Although several markers of senescent cells have been reported, no

single marker can confirm the presence and state of senescence in vivo. SA-βgal activity is a widely used marker and reflects the increase in lysosomal activities that generally accompany senescence-inducing stresses (Itahana et al., 2007). Therefore, SA-βgal staining is often used as a first step to identify senescent cells in culture and/or tissues. As a second step, expression of $p16^{INK4a}$ and/or $p21^{CIP1}$ is also commonly used to identify senescent cells (Itahana et al., 2007; Lopez-Dominguez et al., 2021). However, upregulation of $p16^{INK4a}$ and $p21^{CIP1}$ is not always caused by senescence. For example, a subpopulation of macrophages can express $p16^{Ink4a}$ and SA-βgal activity (Hall et al., 2016).

γ-H2AX and 53BP1 foci, markers of DNA double strand breaks, are also useful to identify senescent cells. Both often increase in primary cells from aged mice and humans, and skin of aged primates (Herbig et al., 2006). GATA Binding Protein 4 (GATA4) is a transcription factor that regulates SASP factors. It is usually degraded by autophagy, but, upon senescence, it is stabilized and activates nuclear factor kappa B (NF-κB), resulting in SASP secretion (Kang et al., 2015). High mobility group box 1 (HMGB1) typically resides in the nucleus but is expelled from the nucleus of senescent cells and secreted, where it functions as a damage-associated molecular pattern (DAMP) molecule (Davalos et al., 2013). Finally, bioactive lipids including prostaglandins, leukotrienes and monounsaturated fatty acids, were recently shown to increase in senescent cells (Cormenier et al., 2018; Wiley et al., 2021). Thus, multiple markers are best used to detect senescent cells (Gorgoulis et al., 2019). Collectively, these markers are appropriate endpoints and should be considered when evaluating senescence in the ocular space.

4.1 Evidence for senescence in the retina

Though there are no comprehensive reports on senescence in retinas of AMD donor tissues, several studies have investigated the presence of senescent markers in the retina of other ocular diseases including glaucoma and diabetic retinopathy, that may provide some insight relevant to AMD. In glaucoma, SA-βgal positive cells have been detected in the trabecular meshwork (Liton et al., 2005) and in retinal ganglion cells in the glaucomatous retina, concomitant with SASP expression (Skowronska-Krawczyk et al., 2015). In follow-up studies, the use of p16-3MR transgenic mice or administering a senolytic drug to remove senescent cells, induced by high intraocular pressure (IOP), has provided support for potential therapy (Rocha et al., 2020). Importantly, existing clinical data has shown that senolytic exposure as a treatment for other health complications, is not associated with decreased visual acuity, elevated intraocular pressure, or senolytic-related adverse ocular effects (El-Nimri et al., 2020). These data support the hypothesis that, when controlled for dosage and frequency, senolytic drugs might be applicable to treat ocular diseases including glaucoma.

In a mouse model of retinopathy of prematurity (ROP), cells devoid of oxygen become senescent and secrete SASP molecules, induce aberrant vasculogenesis (Oubaha et al., 2016), trigger the unfolded protein response (UPR) with consequent activation of classical senescence associated factors including p53 and $p16^{Ink4a}$ (Crespo-Garcia et al., 2021). Of note, use of metformin decreased the expression of these markers and could thus be a potential therapeutic for further investigation. That said, in the context of AMD, recent

retrospective studies looking at the association between diabetic medication use and AMD risk, have not reached a consensus on the impact of metformin on AMD. A number of studies have found a decreased risk for AMD with metformin use (Blitzer et al., 2021; Brown et al., 2019) while others have reported no association and/or an increased hazard for AMD (Eton et al., 2022; Gokhale et al., 2022; Vergroesen et al., 2022). The study designs and cohorts used in these series of retrospectives varied, which may account for the different findings. Another therapeutic approach suggested to target senescence is intravitreal administration of small molecule inhibitors of senescence as well as removal of *p16*^{Ink4a}-expressing cells, in retinal diseases associated with abnormal vascularization (Crespo-Garcia et al., 2021).

Certain markers associated with senescence have also been detected in aged retinas with microaneurysms (Lopez-Luppo et al., 2017). Systematic analysis of the retinal layers for the senescence phenotype found that neurons, but not glial cells, and blood vessels express senescence-associated markers. In an Alzheimer's disease transgenic mouse characterized by amyloid beta immunoreactivity in the photoreceptor layer, rod degeneration concomitant with increased p16^{ink4a} and p21 protein expression in the outer segments has been observed in 9-month-old mice, supporting a link between photoreceptor degeneration and senescence marker expression (Zhang et al., 2021). Interestingly, the Lopez-Luppo *et al* study found that cones rather than rods expressed the senescence marker *p16*^{Ink4a} in retinas with microaneurysms. The finding that senescent cells are resistant to apoptosis, yet secrete SASP affecting surrounding cells, may explain why in several ocular diseases rod cell death precedes death of cones. These hypotheses and more detailed studies of senescence in the retina are critically needed, in order to move forward with a better understanding of the links between aging and senescence in retinal diseases including AMD.

4.2 Evidence for senescence in the RPE

Landmark studies in neurons conducted by Rita Levi-Montalcini in 1960 showed that as neurons mature, they undergo terminal differentiation and become resistant to apoptosis. In post-mitotic neurons, expression of cell cycle markers is accompanied by mitochondrial and endoplasmic reticulum stress, thought to be a prelude to cell death. However, because these post-mitotic cells are difficult to replace, it is likely that senescence may play a pro-survival role to preserve valuable cells. In postmitotic tissues like the RPE, the evidence for senescence is limited, and whether it plays a protective or detrimental role remains unclear (Sapieha and Mallette, 2018). Increased SA-βgal staining has been observed in aging human and non-human primate RPE. However, whether this is true senescence or increased immunoreactivity of long-lived lysosomes in postmitotic cells remains to be established. This distinction is especially important because SA-βgal activity has been detected in brain tissue independent of age or senescence.

As discussed earlier, the vast majority of published studies on senescence in the RPE have used the immortalized ARPE-19 cell line, which actively participates in the cell cycle and is susceptible to replicative senescence (Aryan et al., 2016). Therefore, conclusions regarding mechanisms or triggers that induce senescence in the RPE using poorly differentiated RPE cell lines might have little relevance to RPE cells in vivo or to AMD. Specifically,

undifferentiated RPE cultures grown on plastic have been noted to show an age-related increase in the expression of p53, $p16^{INK4a}$ and $p21^{CIP1}$. Similarly, sub-confluent human fetal RPE cells when exposed to oxidants begin to express senescent biomarkers (Sreekumar et al., 2022). To what extent these studies are representative of the aging RPE cells in vivo must be further evaluated using additional culture models and/or in vivo models. One example of in vitro – in vivo confirmation studies worth noting involved determining the effect of amyloid beta, a molecular component of drusen (Dentchev et al., 2003; Johnson et al., 2002) in RPE cells. Cultured undifferentiated RPE cells when exposed to amyloid-beta display characteristics of senescence including SA-βgal activity, increased $p16^{INK4a}$ expression, along with an increase in expression of pro-inflammatory molecules, such as IL-8, IL-33, MMP9 and VEGF (Cao et al., 2013; Liu et al., 2012; Yoshida et al., 2005). Amyloid-beta exposure also decreased tight junction-related proteins, such as ZO-1 and occlusion (Cao et al., 2013). Though this study involved the use of undifferentiated RPE cells, interestingly, in vivo studies involving subretinal injection of amyloid-beta also triggered an increase in the expression of $p16^{Ink4a}$ in the RPE, upregulated IL-6 and IL-8, compromised the integrity of basal infoldings and increased the formation of autophagic vacuoles in the RPE (Liu et al., 2015). Because the expression of p16, IL6, and IL-8 is also increased in inflammatory conditions, whether or not amyloid beta exposure in vivo stimulates additional markers of senescence and how this is relevant to AMD remains to be determined.

A few studies have used the more physiologically relevant RPE cultures, harvested from human donor eyes, to study senescence (Chaum et al., 2015; Sreekumar et al., 2016; Yamada et al., 2020). Polarized RPE grown on transwells treated with strong oxidants lead to mitochondrial damage and increased expression of $p16^{INK4a}$. This is significant since in AMD, mitochondria display focal loss of cristae, decreased electron density of the matrix, and more advanced mitochondrial alterations, reflecting mitochondrial dysfunction (Feher et al., 2006; Ferrington et al., 2016; Ferrington et al., 2021). In general, the senescent MiDAS phenotype, that has been observed upon mitochondrial dysfunction, has a distinct SASP profile that differs from the classical SASP profile (Wiley and Campisi, 2016; Wiley et al., 2016). Future studies will need to determine whether or not RPE cells in AMD patients have a senescent MiDAS phenotype.

It is important to note, that in terminally differentiated neurons and cardiomyocytes, undigested lipids and proteins accumulate in the form of lipofuscin granules, which constitute a surrogate marker for aging. Lipofuscin also accumulates in RPE lysosomes with age; however, RPE lipofuscin is formed as a by-product of the visual cycle and almost entirely composed of vitamin A metabolites called bisretinoids (Sparrow, 2016). Whether RPE lipofuscin is a marker of aging or senescence, it is clear that it derails critical homeostatic functions and compromises RPE health. Bisretinoids in RPE lysosomes lead to a secondary accumulation of cholesterol, which activates acid sphingomyelinase. The resulting increase in ceramide interferes with microtubule based trafficking, leading to autophagic defects and makes the RPE susceptible to complement-mediated mitochondrial fragmentation (Kaur et al., 2018; Tan et al., 2016; Toops et al., 2015). Bisretinoids can also undergo photo-oxidation, leading to the production of free radicals and DNA damage

(Ueda et al., 2016). These pathways could directly or indirectly drive RPE dysfunction and dedifferentiation (Fig. 3).

Finally, an important in vivo study recently demonstrated the relationship between *Serpinf1*, necessary for RPE PEDF production and cell senescence, revealing that the absence of *Serpinf1* in mice, resulted in increased SA-βgal activity in RPE cells and induced the expression of senescence-associated genes (Rebustini et al., 2022). Additional in vivo studies investigating the distribution of putative senescent cells in in vivo models that present with AMD-like phenotypes as well as in human AMD donor tissues would complement studies such as that of Rebustini et al. in supporting an active role for senescence in the development and progression of AMD. Should additional data emerge supporting senescent RPE cells in AMD, an important question will be if these cells remain functional. Multinucleated RPE cells, post-mitotic cells, retain levels of phagocytic activity comparable to proliferative single nucleated cells (Chen et al., 2016). Further research is needed to determine how senescent RPE cells modify the microenvironment of the retina and lead to AMD.

4.3 Evidence for senescence in the choroid

As mentioned earlier accessibility to bona fide choroidal endothelial cells has impeded research, with researchers often using the RF/6A cell line as a launching point to examine cell senescence in vitro. In one such study, replicative senescence in vitro was attempted using RF/6A cells, in which cells were passaged frequently for greater than 20 passages (Cabrera et al., 2016). Beta galactosidase staining was found to increase along with the expression of $p21^{Cip1}$, an inhibitor of cyclin-dependent kinase expression, which collectively was used to demonstrate the state of senescence in these high passage endothelial cells. When compared to low passage cell, high passage cells were found to be significantly less flexible and considerably stiffer, correlating with higher cytoskeletal Rho activity and greater susceptibility to complement injury, potentially supporting senescence-associated choroidal endothelial cell stiffening as a contributor to choriocapillary atrophy, observed in early dry AMD. On the other hand, there are a number of in vivo studies that provide evidence for senescent cells in the posterior pole using the experimental laser-induced choroidal neovascularization mouse model, which on flat mount and in cross-sections stain positively with SA-βgal (Chae et al., 2021) and point to the involvement of myeloid cells (Schlecht et al., 2021).

5. Anti-aging therapeutics and AMD

Anti-aging therapy can be thought of in two ways. The first is to prevent the aging process, the other is to reverse it. Many large cohort studies are looking at prevention, including the Age-Related Eye Disease Study (AREDS), in which the long term effect of antioxidants and zinc significantly reduce the risk of AMD progression and associated vision loss (Age-Related Eye Disease Study 2 Research et al., 2014; Age-Related Eye Disease Study Research, 2001; Chew et al., 2013; Seddon et al., 2016). Slowing the progression of AMD from intermediate to advanced stages could save eyesight, and even decrease mortality (Clemons et al., 2004). However, once a normal cell enters senescence, it appears unlikely

to reverse. Recently, it was shown that many age-related pathologies can be improved by eliminating senescent cells, indicating that senescence-targeted therapy is a promising candidate for the treatment of specific age-related eye diseases associated with abnormal blood vessel growth (Crespo-Garcia et al., 2021; Rocha et al., 2020). Another consideration, for senescence targeted therapy is the density of senescent cells. Should a high density of senescent cells be observed, an important question to address prior to therapy is whether or not eliminating these cells would destabilized the existing tissue and potentially be more detrimental.

5.1. Pros and cons of senescence targeting drugs

Various molecules have been investigated to stop or at least slow aging. At least three major pathways regulate the SASP: the DNA damage response (DDR) pathway, the p38MAPK pathway, and the mTOR pathway. All these pathways converge on the NF-κB transcription factory, which drives the inflammatory phenotype. Persistent SASP expression produces a chronic pro-inflammatory microenvironment and can cause neighboring cells in tissues to function inappropriately. Some of these pathways could be inhibited by senomorphics: small molecules that can selectively inhibit certain aspects of the SASP and thus reduce some of the deleterious effects of senescent cells. Drug repurposing studies have shown that drugs such as rapamycin, an mTOR inhibitor, metformin, an AMPK activator, and ruxolitinib, a JAK inhibitor, block pathways that regulate the SASP and protect against age-related pathologies. However, when the treatment stops, the deleterious molecules are secreted again and pathologies triggered by senescent cells can recur (Crespo-Garcia et al., 2021). Additional consideration for therapy is potential off-target effects that may result in untoward effects. For example, one study has shown that cones are functionally dependent on the mTOR signaling pathway, and that stimulation of this pathway can delay cone death in a mouse model of retinitis pigmentosa (Punzo et al., 2009).

Eliminating senescent cells (senolysis) may be a more promising approach to diminish their adverse effects. Caveat being if the density of senescent cell is not such that eliminating them would compromise the integrity of the tissue. Transgenic mouse models, in which senescent cells can be eliminated throughout the body have uncovered a surprising number of age-related pathologies that are due, at least in part, to the presence of senescent cells (Gorgoulis et al., 2019). In addition, a number of small molecules can have senolytic effects, including tyrosine kinase inhibitors coupled with a flavonoid (dasatinib and quercetin) (Zhu et al., 2015), a Bcl inhibitor (ABT263), (Chang et al., 2016), and a glutaminase inhibitor (Johmura et al., 2021).

Currently, a Bcl-xL inhibitor UBX1325, is being tested in a phase 2a human clinical trial as a senolytic to treat refractory diabetic macular edema and neovascular age-related macular degeneration (NCT04537884). As discussed above, diabetic retinopathy is associated with senescence and eliminating senescent cells can improve the avascular area (Crespo-Garcia et al., 2021; Oubaha et al., 2016). One potential problem with senolytics is tissue atrophy due to the removal of specific, albeit small, populations of cells. Cells with low proliferative capacity, such as retinal cells, including photoreceptor cells and RGCs, are mostly post-mitotic and could be targeted by the drugs. Moreover, given this population of senescent

cells is generally small (He and Sharpless, 2017), losing a small population in exchange for the possibility of maintaining tissue function may be acceptable for many age-related diseases. However, the continuous removal of p16-positive senescent cells *in vivo* can result in systemic perivascular fibrosis (Grosse et al., 2020). Further research is needed to determine the appropriate duration and interval of senolytic treatments to effectively clear senescent cells in the context of AMD.

5.2. Tools available to study senescence in vivo, ex vivo, and in animal models

Several mouse models have been developed to study senescence, either by inducing senescence conditionally, reprograming epigenetic changes with age, or eliminating senescent cells. One informative model created a cell type-selective mouse to generate senescent cells by crossing a floxed Ercc1 knockout ($Ercc1^{-/fl}$), selectively deficient in transcription-coupled DNA repair, with Vav-$iCre^{+/-}$ (Yousefzadeh et al., 2021) to localize DNA damage to hematopoietic cells. This model demonstrated accelerated aging in immune cells, notably B cells, T lymphocytes, natural killer T cells, macrophages and monocytes, which all expressed significantly higher levels of $p16^{Ink4a}$ and $p21^{Cip1}$, similar to that of wild-type aged mice. Aging effects in other tissues, such as kidney, pancreas and intervertebral discs, were also accelerated cell non-autonomously in this model. Another strategy deployed to study age-related epigenetic changes specifically in retinal ganglion cells (RGCs) expressed Yamanaka factors (excluding MYC) to alter the DNA methylation signature with age. This reprograming reversed vision loss following glaucomatous damage (Lu et al., 2020). While cellular senescence was not assessed in this model, elevated IOP, sufficient to cause glaucoma and progressive RGC degeneration, was associated with elevated $p16^{INK4a}$ expression (Skowronska-Krawczyk et al., 2015), and early elimination of senescent cells, either by $p16^{Ink4a}$-dependent expression of a viral thymidine kinase gene and treatment with ganciclovir (Demaria et al., 2014) or by senolytic treatment (desatinib), which restores RGCs and evoked visual potentials (Rocha et al., 2020). Of relevance to AMD, rapid expression of SA-βgal in RPE cells has been reported in the laser induced experimental mouse model of CNV, the outer retinal degeneration model created by injecting sodium iodate to ablate the RPE, and in 9-week-old mice treated with doxorubicin to induce senescence (Chae et al., 2021; Sreekumar et al., 2022). Interestingly, retinal degeneration in mice subjected to doxorubicin is more evident than RPE degeneration and the atrophy of the photoreceptor outer nuclear layer is alleviated by treatment with nutlin-3a, a murine double minute 2 (MDM2, p53 target gene and E3 ubiquitin ligase) antagonist. By taking advantage of existing tools and by continuing to generate new conditional animal models, it should be possible to better understand whether or not specific cell types are particularly deleterious when senescent, and to develop therapeutics to more specifically target the most deleterious cell types.

5.3. Therapeutic targeting of ocular tissues in AMD

Which tissue compartment should be targeted in AMD is a critical point of discussion when considering therapies. The retina, RPE, and choroid are all involved in the pathogenesis of AMD and as such drugs that may improve their cellular health and function should be considered. So far, in AMD patients, the presence of senescent cells in the retina, a complex multicellular tissue, housing the light sensitive photoreceptors as well as microglial

cells, both compromised in disease, has not been reported. Targeting the compromised RPE cells is attractive, as the RPE regulates homeostasis of ions and pH between photoreceptors and metabolic waste products from the retina, and transports nutrients to the retina. RPE cells also converts retinol to 11-cis-retinal, regulate photoreceptor outer segments, and secrete neurotrophic factors to maintain photoreceptor integrity. Furthermore, the RPE forms the blood-retina barrier. All these specialized functions depend on the expression and polar distribution of receptors, transporters, channels and enzymes that are markers of a differentiated RPE. Understanding the role of senescent RPE cells during the progression of degenerative diseases such as AMD are central prior to targeting them with senolytic treatments. Finally,

degenerative changes to the choroid play an important role in the different clinical sub-types of AMD. Loss of choroidal endothelial cells or choriocapillary dropout is a classic hallmark of early dry AMD. Morphometric analysis of the choriocapillary density and vascular lumen to stroma ratio in the outer choroid of donor tissue from patients with geographic atrophy reveal an even larger loss of the choriocapillaris (Sohn et al., 2019). This finding combined with the known vascular changes in wet AMD, support choroidal changes are an underlying pathological event in all clinical sub-types of AMD and therefore a valid tissue site for testing therapeutics. A common denominator in targeting any of these tissues is the need for further studies demonstrating the extent to which senescence is a factor in AMD and at which cellular levels does it occur.

5.4. Evidence needed to support senescence does in fact play a role in AMD and where should the field go from here

Currently, the data on senescence markers in the posterior pole in AMD patients or models are not definitive enough to conclude the extent to which senescence plays a role in the etiology of the disease. However, the potential that senescence may contribute to the pathogenesis of AMD, necessitates pursuing this line of research, in a more comprehensive way. The most convincing data to support a role for senescence would begin with detailed phenotypic profiling of a large cohort of donor tissue from AMD patients versus non-AMD, classified based on their clinical sub-type, sex, and genotype. This is the necessary minimum given the multi-factorial nature of AMD. The next stages would include molecular profiling including single cell transcriptomics and cell cyle profiling to help clarify which populations of AMD vulnerable cells express senescent markers, vital information should therapeutics need to be considered.

With regards to treatment there are a number of important questions to contemplate when considering the use of senolytic drugs: 1. Which cells should be targeted, the whole retina, microglial cells, photoreceptors, RPE cells, choroidal endothelial cells? 2. Are the senescent cells still active and functional?; 3. If senescent cells are removed, is it possible for the missing neurons to regenerate to avoid tissue atrophy?; 4. If senescent cells that are still functional, are removed, how will it affect vision?; 5. What if most of the cells of a given type are senescent, should we treat the tissue with senolytic drugs and risk losing other cell types? Finally, until studies on a molecular level are done, whether all cells assume the same

type of senescence remains unknown; maybe some options could be specific to a given cell type.

6. Conclusions

The field of senescence is relatively young and new discoveries on its underlying mechanisms are published daily. Case in point, even though the fate of senescent cells involves resistance to death and secretion of SASPs, the phenotypic features, consequences, and triggers of senescence are cell and tissue specific, in part due to the diversity in the aging rate of organs throughout the body (Nie et al., 2022); adding to the complexity of this cellular process. It is therefore of great interest to understand the extent to which the retina and RPE undergo senescence in a manner similar to dividing cells, the degree to which the process differs, and/or if there are novel mechanisms underlying ocular senescence.

The discovery of senolytic factors has revolutionized the field of age-related conditions. Some of these compounds are safe for use in patients as they were previously FDA-approved drugs for other conditions. In addition, pre-clinical studies are promising (Boccardi and Mecocci, 2021). As an alternative, the use of senostatic/senomorphic drugs could be considered (Boccardi and Mecocci, 2021). These molecules quench the most deleterious segments of the SASP without removing the living cell. This type of approach most likely will require sustained treatment but might be the best option for now.

Since retinal neurons and RPE are largely non-dividing cells, removal will directly affect cell-cell connectivity with potential deleterious effects on activity. Although the technology is not there yet, one can imagine that replacement therapy or transdifferentiation approaches could be used to fill the space of missing cells. In particular, studies performed in lower vertebrates (Lahne et al., 2020) such as zebrafish are exciting, as it has been shown that Müller cells can undergo re-differentiation to the cell type that has been lost. Unfortunately, recent detailed studies have described an evolutionary change in the Muller glia's ability to transdifferentiate *in situ* in response to damage (Hoang et al., 2020) and future work is needed to establish the feasibility of this approach in mammals (Eastlake et al., 2021) Current approaches including overexpression of cell-lineage specific transcription factors to transdifferentiate Muller cells into the specific neuron may prove to be more applicable (Todd et al., 2021) and further ideas are needed to fill this need in treating age-related eye conditions.

Finally, the degree to which senescence in the posterior pole may contribute to development and progression of AMD is of high interest. Hopefully, exploring the questions discussed along with studies in progress, including single cell transcriptomics and cell cycle profiling of donor retinal tissue in correlation with clinical stage of AMD, will provide much needed clarity and insight into the role of senescence in disease pathogenesis and provide support for senescent – associated ocular therapies, if indicated.

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Abbreviations

AMD	age-related macular degeneration
RPE	retinal pigment epithelium
GA	geographic atrophy
SASP	senescence-associated secretory phenotype
DAMPS	danger-associated molecular patterns
CDK	cyclin-dependent kinase
Rb	retinoblastoma
MMPs	matrix metalloproteases
ROS	reactive oxygen species
MiDAS	mitochondrial dysfunction-associated senescence
SAMP8	senescence-prone mouse strain 8
BMP4	bone morphogenetic protein-4
iPSC-RPE	induced pluripotent stem cell derived RPE
TER	transepithelial resistance
ApoE	apolipoprotein E
hTERT	human telomerase reverse transcriptase
SD-OCT	Spectral domain-optical coherence tomography
ONL	outer nuclear layer
OCTA	optical coherence tomography angiography
MERTK	MER tyrosine kinase
MYO7A	actin motor protein myosin VIIa

OXPHOS	oxidative phosphorylation
SA-βgal	Senescence-associated beta-galactosidase
GATA4	GATA Binding Protein 4
HMGB1	High mobility group box 1
IOP	intraocular pressure
RGC	retinal ganglion cells
TBK1	TANK-binding protein 1
UPR	unfolded protein response
NET	neutrophil extracellular traps
IL	interleukin
CETP	cholesterol ester transfer protein
SV40	simian virus 40
NCAM	neural cell adhesion molecule
PECAM1	platelet endothelial cell adhesion molecule-1
VEGF	vascular endothelial growth factor
GDF15	growth differentiation factor-15
STC1	stanniocalcin-1
SERPINE-1	Serpin Family E Member 1
TNFα	tumor necrosis factor alpha
ESC	embryonic stem cell
GFAP	glial fibrillary acidic protein
LAMP	lysosomal membrane protein
OXPHOS	oxidative phosphorylation
NF-κB	nuclear factor kappa B
DAMP	damage-associated molecular pattern
DDR	DNA damage response
MDM2	murine double minute 2

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Highlights

- Advanced age is a major risk factor for developing age-related macular degeneration (AMD), a complex blinding disease.
- Age-related changes are seen throughout the posterior pole, at the level of the retina, retinal pigment epithelium, and choroid, impacting vision and contributing to disease development.
- The lack of therapies for AMD, necessitate further discovery of pathways that are affected in aging.
- Recent observation of a senescence-like phenotype in post-mitotic, terminally differentiated cells in aged mice, has led to the hypothesis that senescence may play a role in AMD.
- The extent to which senescent cells accumulate in the aged eye and the degree to which these cells may trigger AMD development needs to be investigated comprehensively in order to consider the use of senolytics and senomorphics for therapy.

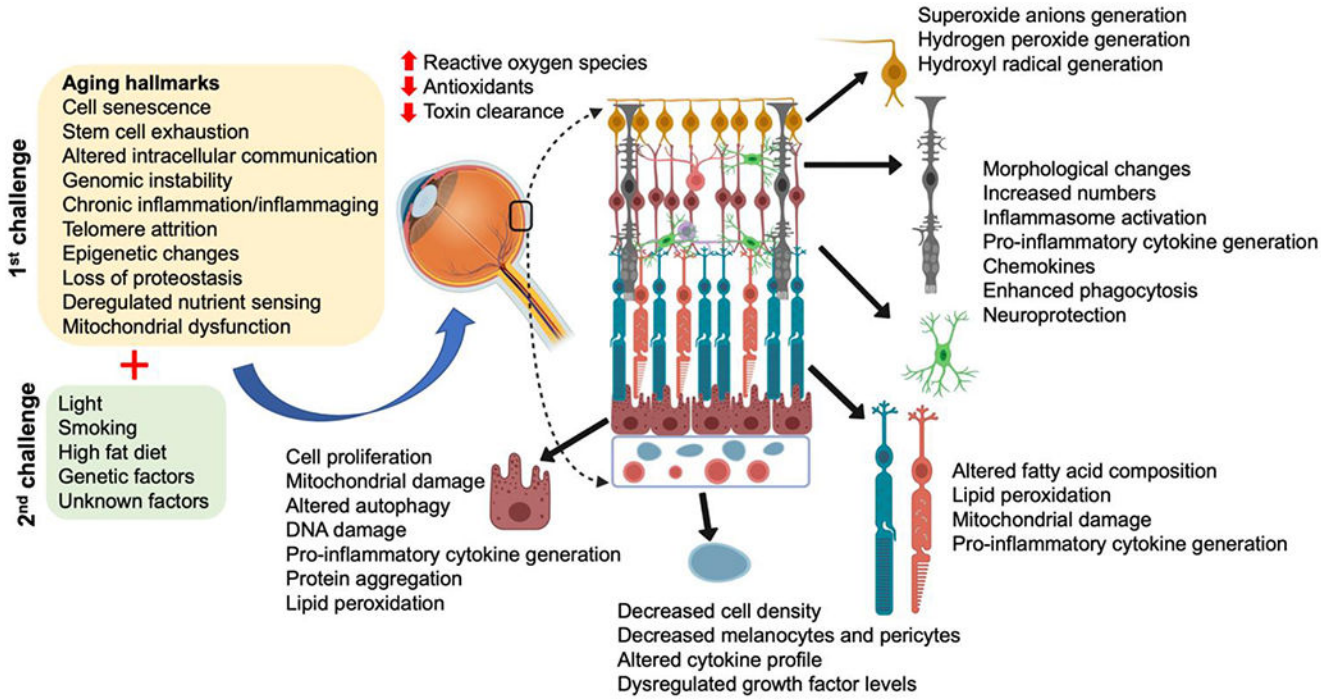

Figure 1.

Overview of aging and AMD: The combination of aging (1st hit) and other stressors/modifiers (2nd hit) can impact the integrity and function of ocular cells in the posterior pole triggering AMD. Cells affected include the retinal ganglion cells (yellow), microglial (green) and Müller cells (dark grey), photoreceptors (aqua/orange), retinal pigment epithelial cells (burgundy) and choriocapillaris/endothelial cells (light blue). Select consequences have been listed next to each cell. Figure modified from (Hadziahmetovic and Malek, 2020).

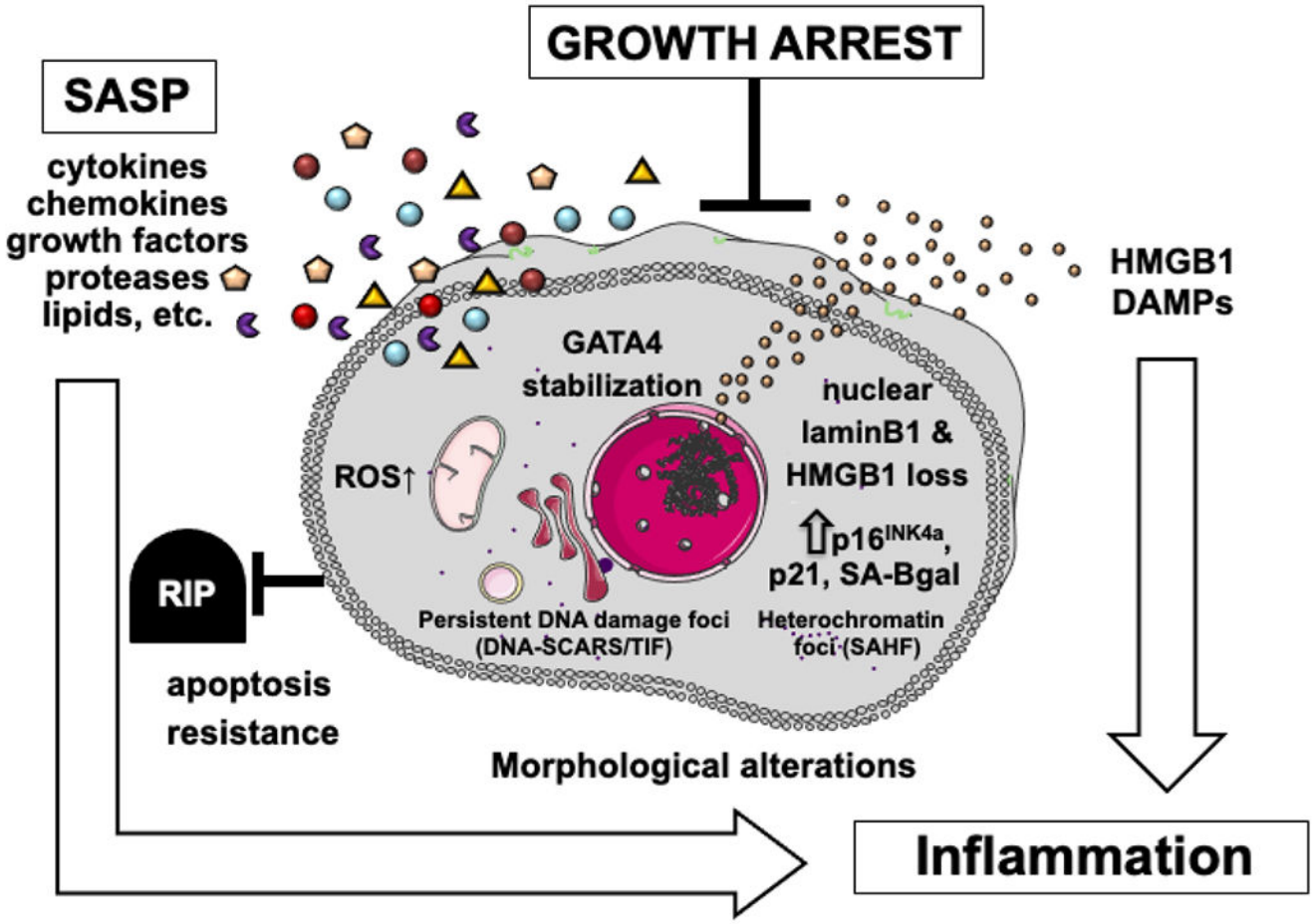

Figure 2.

Definition of senescent cells: Cellular senescence is a cell fate in which both intrinsic and extrinsic signals can cause an irreversible cell cycle arrest, accompanied by many phenotypic changes. These phenotypic changes have also been reported in non-replicative senescence. Senescent cells acquire a complex, often pro-inflammatory, secretory phenotype termed the senescence-associated secretory phenotype (SASP), which can cause chronic inflammation. SA-βgal: senescence-associated beta-galactosidase; HMGB1: high mobility group box protein 1; DAMPs: damage-associated molecular patterns; ROS: reactive oxygen species; GATA4: GATA binding protein 4; SAHF: senescence-associated heterochromatin foci; DNA-SCARS: DNA segments with chromatin alterations reinforcing senescence; TIF: telomere dysfunction-induced foci.

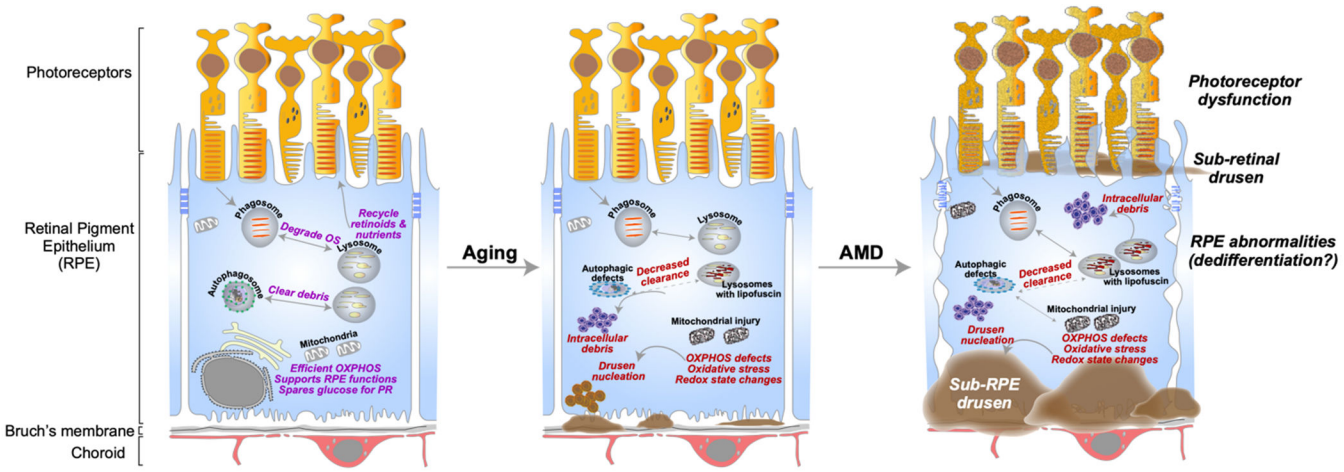

Figure 3.

RPE dysfunction and dedifferentiation in aging and AMD: Left panel: The retinal pigment epithelium (RPE) performs numerous functions critical for photoreceptor health and vision. These include the daily phagocytosis and clearance of photoreceptor outer segments (OS) and recycling retinoids and nutrients to photoreceptors. The RPE relies on oxidative phosphorylation (OXPHOS) for its high energy needs and spares glucose for the photoreceptors. Middle panel: Age-related accumulation of vitamin A metabolites in the form of lipofuscin in RPE lysosomes interferes with critical functions such as autophagy and OXPHOS. Declining mitochondrial function can lead to redox state-mediated phase separation of proteins, resulting in the nucleation of drusen-like aggregates. Right panel: Genetic and environmental risk factors in AMD (complement activation, lipid dysregulation, etc.) can act as “tipping points” to exacerbate these deficits, increase drusen formation, and eventual cause RPE atrophy and photoreceptor dysfunction. Figure adapted from (La Cunza et al., 2021).