MOLECULAR MECHANISMS OF RETINAL CELL DEGENERATION AND REGENERATION

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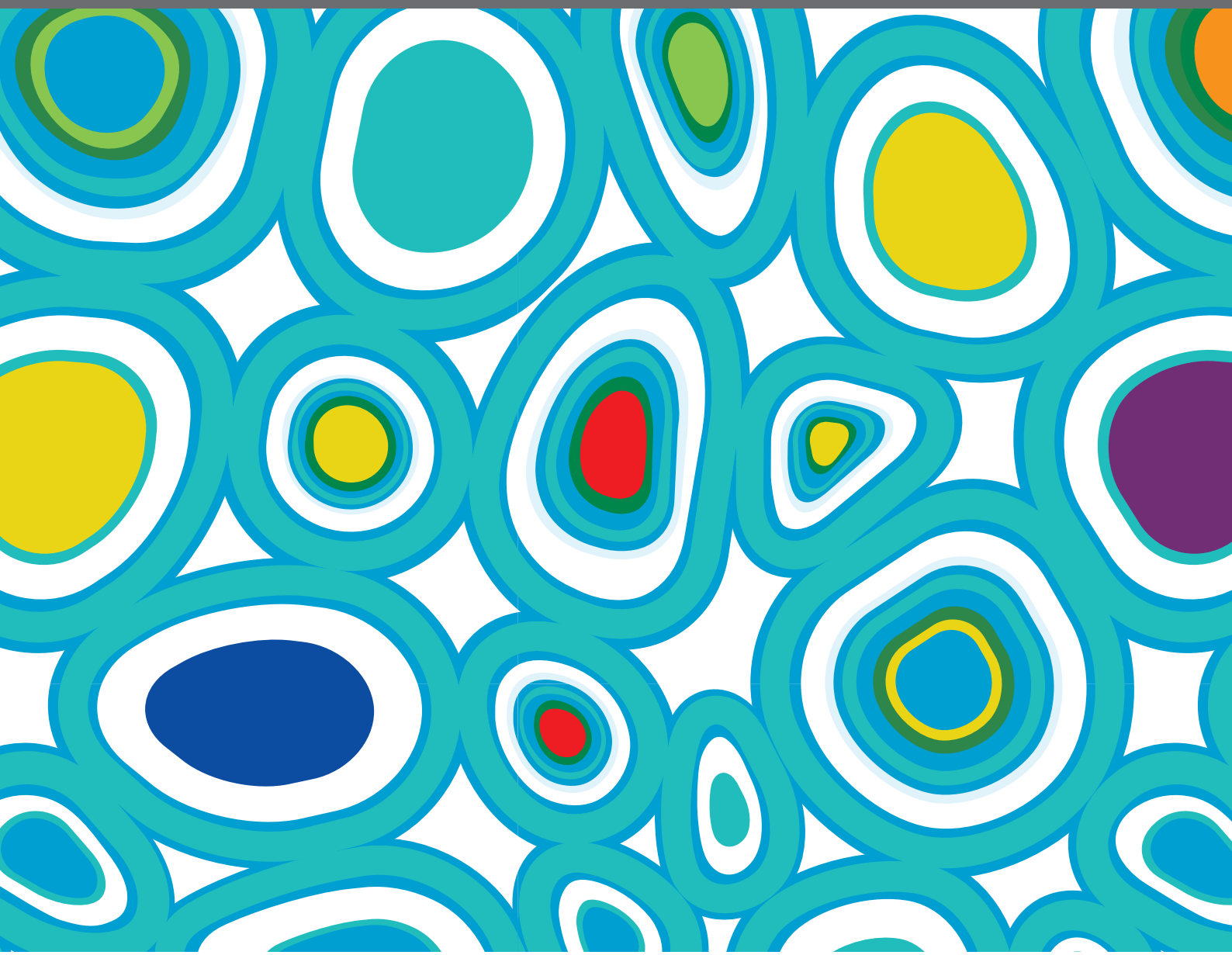

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MOLECULAR MECHANISMS OF RETINAL CELL DEGENERATION AND REGENERATION

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EDITORIAL
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Editorial: Molecular Mechanisms of Retinal Cell Degeneration and Regeneration

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Editorial on the Research Topic

Molecular Mechanisms of Retinal Cell Degeneration and Regeneration

Retinal degeneration is characterized by the deterioration of highly differentiated cells within the neurosensory retina, such as photoreceptors (PR), retinal pigment epithelium (RPE), or the choroid. Retinal remodeling culminating with cell death and topological changes in the retina is the final common pathway in many retinal degenerative diseases (RDDs) that cause irreversible blindness. Identifying the molecular and cellular mechanisms responsible for these processes is crucial for early diagnosis and prevention of these pathological outcomes to thwart blindness remains the main goal in vision research and is quite challenging. This special issue of *Frontiers in Cell and Developmental Biology*, as a result of contributions from leading groups in this field has elegantly covered the different aspects of both clinical and basic research involved in the process of retinal degeneration associated with complex or Mendelian traits highlighting new mechanistic avenues.

Retinal ganglion cell (RGC) degeneration in glaucomatous optic neuropathy poses a significant challenge in treating and managing glaucoma. With increasing evidence for vascular dysfunction in glaucoma, Wareham and Calkins highlight the potential role of neurovascular dysfunction and raise pertinent questions related to its causal role in glaucoma. The review discusses the mechanisms of vascular dysfunction in neuronal degeneration due to breakdown in autoregulation and neurovascular coupling in the neuronal, vascular, and glial cells that comprise the neurovascular unit. The authors hypothesize roles of these cells in the breakdown, possibly via the gap junction proteins that may affect cell-cell communication, cause leaky blood-retinal-barriers, predispose RGCs to apoptosis, and contribute to glaucoma progression. The modulation of vascular regulation and neurovascular interactions can represent an interesting area of neuroprotective modality for glaucoma in the future.

The retina is one of the highest oxygen-consuming tissues in the human body, making it highly susceptible to oxidative damage by reactive oxygen species (Schmidt et al., 2003). Aerobic glycolysis accounts for up to 90% of the glucose needs of the PR. The review by Rajala describes the role of aerobic glycolysis in the survival and maintenance of the PR cells with a focus on the metabolic and non-metabolic functions of pyruvate kinase isoform M2 (PKM2) expressed in the retina. Besides,

Sinha et al. have effectively highlighted the role and mechanisms by which riboflavins and their derivatives maintain retinal oxidative homeostasis. The review furthers our knowledge on the importance of this underappreciated vitamin to the retina beyond other antioxidants and vitamins such as A, D, and E. Notably, oxidative stress and choroidal vascular dysfunction are hypothesized to be critically involved in age-related macular degeneration (AMD) pathogenesis.

Tong and Wang highlight the mechanisms by which different oxidative stressors induce oxidative damage and may be responsible for AMD. The review discusses the mechanisms of RPE senescence and different modes of cell death in RPE cells induced by hydrogen peroxide (H_2O_2), 4-Hydroxynonenal (4-HNE), N-retinylidene-N-retinyl-ethanolamine (A2E), Alu RNA, amyloid β (Aβ), and sodium iodate ($NaIO_3$). The authors point toward a need for future investigations to understand the nature of RPE cell degeneration and death in AMD, which may be beneficial for developing therapeutic strategies to treat AMD patients. Besides, the review article by Hadziahmetovic and Malek further elaborates on responses and underlying mechanisms of cells vulnerable to AMD, such as the PR, RPE, microglia, and choroidal epithelial cells to oxidative damage, dysregulated lipid peroxidation, inflammatory cytokines and choroidal vascular dysfunction in the pathogenesis of AMD. Anti-angiogenic vascular endothelial growth factor inhibitors are available for treating wet AMD. However, the common dry AMD is still untreatable. The review discusses the currently available and in-trial treatment options for wet and dry AMD.

Identification of causal variants in a complex polygenic disease like AMD is challenging. Genome-wide association study (GWAS) is a useful tool for analyzing complex diseases, as they provide glimpses of the molecular pathways that lie beneath the disease landscape. Nguyen et al. describe an integrated approach of using GWAS and expression quantitative trait loci studies to prioritize functional variants that may be more likely to have a causal role and how such approaches have been successful in identifying causal genes in AMD. Likewise, Dhirachaikulpanich et al. have, for the first time, using an integrated transcriptomic approach, reported differentially expressed (DE) transcripts associated with mixed AMD in post-mortem macular (764 genes) and non-macular (445 genes) human RPE/choroid microarray and RNASeq datasets (GSE135092 and GSE29801). Interestingly, protein-protein interaction network identified two central hub genes, *HDAC1* and *CDK1*, involved in the control of cell proliferation/differentiation processes. The study highlights a useful approach to integrate publicly available datasets to increase the power of detecting functional transcripts and pathways that may provide novel insights into disease mechanisms and form the basis of future investigations.

The RPE is responsible for the proper functioning of the photoreceptors and maintenance of the blood-retinal barrier. RPE dysfunction can result in progressive loss of PR survival and subsequent loss of vision. Damaged RPE cells lose pigment, proliferate, migrate and differentiate into different cell types but may not transdifferentiate into neural retinal cells. Instead, they may form fibroblast-like cells, which, in the wet form of AMD, are involved in developing a choroidal neovascular membrane. These phenotypic changes in RPE cells are commonly referred to as 'epithelial-mesenchymal transition' (EMT) (Zhou et al., 2020). The interesting review by Zhou et al. describes the clinical and pre-clinical evidence of EMT in the RPE cells and their role in AMD, proliferative vitreoretinopathy and certain inherited forms of retinal degenerations plausibly via tight junctions and adherens junctions proteins and the unfolded protein response (UPR) pathway which is involved in the repair and removal of damaged or misfolded proteins. The authors propose a role of UPR pathway and provide evidence for the role of misfolded proteins in RPE dysfunction that may have pathological consequences.

Mutations in *BEST1* (Bestrophin 1) are responsible for different inherited retinopathies, including the autosomal dominant Best vitelliform macular dystrophy or Best disease (BD). A study by Bonilha et al. describes the retinal findings in two donors harboring heterozygous variants in the *BEST1* gene: c.886A>C (p.Asn296His) (in donor 1) or c.602T>C (p.Ile201Thr) (in donor 2) to suggest that bestrophin-1 localization is mutation-dependent and support the concept that different variants in the *BEST1* gene can result in substantially different phenotypes. In another inherited retinal disease like Bardet-Biedl syndrome (BBS), which is caused by mutations in the *BBS* gene, Song et al. using a $bbs2^{-/-}$ deficient cone dominant zebrafish model has elegantly demonstrated how the loss of *bbs2* leads to impaired visual function in larval zebrafish and progressive photoreceptor cone degeneration in adults; and how acute injury stimulates Müller glia proliferation and modest regeneration of cones. As the authors suggest, the zebrafish *bbs2* deficient model may represent an ideal tool to investigate mechanisms involved in retinal degeneration diseases to promote regeneration.

Considering the increasing rate of visual impairment and blindness worldwide (from estimated 45 million blind in 1996, to projected 76 million in 2020), it is imperative to look for feasible therapeutic approaches to prevent them (Ackland et al., 2017). The recent success of gene therapy (Luxturna™) for Retinitis Pigmentosa and Leber's Congenital Amaurosis caused by retinoid isomerohydrolase (RPE65) mutations have raised the hopes of similar ongoing trials in inherited forms of retinal diseases. The concept of deriving retinal cells from human embryonic stem cells and induced pluripotent stem cells or endogenous sources can offer a promising and exciting prospect for stem cell therapy in retinal degenerative diseases. The current status and challenges to make advancements toward therapeutics have been reviewed by Ikelle et al.

Overall, the 12 contributions that make up this special issue provide a broad overview of the molecular mechanisms involved in retinal cell degeneration and certain inherited forms of retinal diseases, recent advances and potential regeneration strategies that should be the major goal of investigators in this field. The quality of the reviews and research articles are excellent. Retinal degeneration is a vast field, and although many questions remain to be answered, exciting discoveries will continue to be made in this area in years to come.

AUTHOR CONTRIBUTIONS

GPL and AAK wrote the editorial. MRB read and edited the editorial. All authors contributed to the article and approved the submitted version.

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REVIEW
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Aerobic Glycolysis in the Retina: Functional Roles of Pyruvate Kinase Isoforms

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One hundred years ago, Otto Heinrich Warburg observed that postmitotic retinal cells are the highest oxygen-consuming cells in the body. He compared these cells to actively growing mitotic tumor cells since both cells reprogram glucose for anabolic processes, which include lipid, protein, and RNA/DNA synthesis, and for antioxidant metabolism. To achieve this metabolic reprogramming, cancer cells preferentially express a less active dimeric form, the M2 isoform of pyruvate kinase (PKM2), which shuttles glucose toward the accumulation of glycolytic intermediates that redirect cell activities into anabolic processes. Similar to cancer cells, retinal photoreceptors predominantly express the M2 isoform of PKM2. This isoform performs both metabolic and non-metabolic functions in photoreceptor cells. This review focuses on the metabolic and non-metabolic roles of pyruvate kinases in photoreceptor cell functions.

Keywords: pyruvate kinase M2, pyruvate kinase M1, posttranslational modifications, photoreceptor cells, glycolysis, Warburg effect, anabolic processes

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INTRODUCTION

Almost 100 years ago, German physiologist Otto Heinrich Warburg noticed that retina consumes the highest amount of oxygen in the body; he compared this oxygen consumption to that of a rapidly dividing cancer cell (Warburg, 1956a,b). The Warburg effect is described as a reprogramming of cancer cell's metabolism such that the cell uses more glucose than does a normal cell and redirects the glucose for use in anabolic processes, such as lipid, RNA/DNA synthesis, and NADPH generation, which results in incomplete glucose oxidation in the presence of oxygen (Warburg, 1956a). This phenomenon is also called aerobic glycolysis. The difference between a cancer cell and retinal cells, especially the highly oxygen-consuming photoreceptor cell, is mitotic versus post-mitotic. The Warburg effect is observed in fetal cells, rapidly proliferating tumor cells, and retinal cells (Warburg, 1956a,b; Winkler, 1981; Fiske and Vander Heiden, 2012; Casson et al., 2013; Ng et al., 2014; Rajala and Gardner, 2016). The question becomes why a non-dividing photoreceptor cell needs a Warburg effect. Two types of photon-absorbing preceptors are present in the retina: rods and cones. Rods are mainly used in dim light, whereas cones are needed for daylight color vison (Soucy et al., 1998). The photoreceptor is surrounded by a plasma membrane in which disc membranes are loaded with a light-absorbing G-protein coupled receptor, rhodopsin. Every day, by the onset of light, 10% of outer segment tips are engulfed by the neighboring retinal pigment epithelial (RPE) cells (LaVail, 1976), and some of the digested products

are recycled back to photoreceptor and other cells of the retina (Bok, 1985). The RPE engulfment of photoreceptors is called phagocytosis. Also, the RPE continuously provides essential nutrients, especially glucose through glucose transporters, and oxygen to photoreceptor cells for survival and maintenance (Kanow et al., 2017). Daily phagocytosis leaves a gap between the photoreceptors and the RPE. New membrane synthesis (disc biogenesis) has been very efficient to maintain the length of the photoreceptor cells for proper RPE-photoreceptor interaction (Punzo et al., 2012). It was suggested that aerobic glycolysis might be essential for photoreceptor membrane biosynthesis (Punzo et al., 2012).

In the photoreceptor cells, the dark current needs a large amount of ATP through the tricarboxylic acid (TCA) cycle. Photooxidation products generated through rhodopsin activation generate reactive oxygen species (ROS), which are toxic to the photoreceptor cell (Punzo et al., 2012; Adler et al., 2014). The pentose phosphate (PPP) pathway or hexose monophosphate (HMP) shunt, as the principal components of cellular anabolism, generate the NADPH used to reduce oxidized glutathione to reduced glutathione, which neutralizes the toxic effects of ROS (**Figure 1**). The PPP-generated NADPH is necessary for lipid biosynthesis (Punzo et al., 2012). During the photobleaching of rhodopsin, 11-*cis*-retinal is isomerized to all-*trans*-retinal; NADPH is an absolute requirement for the reduction of all-*trans*-retinal by the enzyme retinol dehydrogenase 8 (RDH8) (**Figure 1**).

Checkpoints in the glycolytic pathway and shunting the glucose to the PPP to generate NADPH and ribose are evolutionarily established (Jiang et al., 2014). Glucose redirection for anabolic processes is necessary for photoreceptors, as they require copious amounts of NADPH for disc membrane biogenesis, antioxidant metabolism, and reduction of all-*trans*-retinal as a means to detoxify this retinoid.

Although it was a century ago that Warburg noticed that tumor cells and retinal cells redirect glucose for anabolic processes, the mechanism behind the fuel redirection from oxidative phosphorylation to anabolic processes was not known until 2011. Investigators for the first time identified that higher pyruvate kinase enzyme activity results in the release of ROS during yeast respiration (Gruning et al., 2011). These investigators also observed that low enzyme activity of pyruvate kinase results in the accumulation of a substrate, phosphoenolpyruvate (PEP), that activates a negative feedback inhibitor of the PPP, triosephosphate isomerase (TPI1), which in turn activates the PPP. This activation results in the generation of NADPH for antioxidant metabolism and increased lipid synthesis (Gruning et al., 2011). These studies led other scientists to examine more closely the pyruvate kinases and their roles in energy metabolism. This study for the first time demonstrated that pyruvate kinase triggers a metabolic feedback loop that controls redox metabolism in respiring cells (Gruning et al., 2011).

Another landmark observation made by Dr. Lewis Cantley, that pyruvate kinase M2 isoform is a phosphotyrosine binding protein (Christofk et al., 2008b), suggested that pyruvate kinase could potentially undergo tyrosine phosphorylation. These observations, along with yeast studies, inspired several investigators to look more closely at the biological roles of pyruvate kinases.

Pyruvate Kinase Isoforms

Pyruvate kinase is a known glycolytic enzyme involved in the last step of glycolysis by converting PEP to pyruvate. Four isoforms of pyruvate kinase have been identified: PKM1, PKM2, PKR, and PKL (Imamura and Tanaka, 1982). More than one isoform may be expressed in a single tissue, but individual cells may predominantly express one isoform (Imamura et al., 1972; Cardenas and Dyson, 1978; Israelsen and Vander Heiden, 2015). Tissues with high catabolic activity, such as muscle, heart, and the brain, predominantly express PKM1 (Israelsen and Vander Heiden, 2015). In the liver, PKL is the predominant isoform, while PKL expression is very low in the kidney (Israelsen and Vander Heiden, 2015). The expression of the PKR and PKL isoforms is restricted to certain tissues and cell types; PKR expression is restricted to red blood cells (Israelsen and Vander Heiden, 2015). There was a study conducted in 2016 shows the isoform expression of PKM1 and PKM2 in different mouse tissues. This study shows that heart, skeletal muscle, smooth muscle, brain predominantly express PKM1 whereas kidney, pancreatic islets, intestine, white fat, lung, lymphocytes, thymus, spleen, and ovaries predominantly express PKM2 (Dayton et al., 2016).

The PKM1 and PKM2 isoforms arise from a single gene as alternatively spliced products (Israelsen and Vander Heiden, 2015). The PKM gene is present in humans, mice, and rats, and each contains 12 exons (Noguchi et al., 1986; Takenaka et al., 1991). The lengths of exons 9 and 10 are identical; exon 9 is specific to PKM1, whereas exon 10 is specific to PKM2 (Clower et al., 2010). A properly spliced transcript has either exon 9 or exon 10 (Clower et al., 2010). To generate the PKM2 transcript, exon 9 must be repressed to include exon 10 in the final transcript (Clower et al., 2010). The repression of exon 9 is mediated by three splicing factors, the polypyrimidine tract binding protein (PTB), heterogeneous nuclear protein A1 (hnRNPA1), and heterogeneous ribonucleoprotein A2 (hnRNPA2), whereas binding of serine/arginine-rich splicing factor 3 (SRF3) to exon 10 promotes the inclusion of this exon in the transcript (Clower et al., 2010; David et al., 2010). The transcriptional regulation of PKM2 is well understood, but the transcriptional regulation of PKM1 is very limited. The lack of knowledge on PKM1 transcript production could be due to its expression to a lesser extent in differentiated tissues.

Regulation of PKM1 and PKM2

PKM1 and PKM2 differ in only 22 amino acids in their sequences, but the functions mediated by PKM1 and PKM2 are distinct. Interestingly, PKM2 has >95% identity and >98% similarity in sequence with PKM1. The crystal structure of human PKM1 and PKM2 has been solved (PDB ID: PKM2, 4JPG, PKM1, 3SRF). We carried out the molecular modeling of mouse PKM1 and PKM2 using the Raptor X template-based protein structure modeling web server (de la Monte et al., 2003; Peng and Xu, 2011a,b; Ma et al., 2013) and aligned, with Clustal Omega

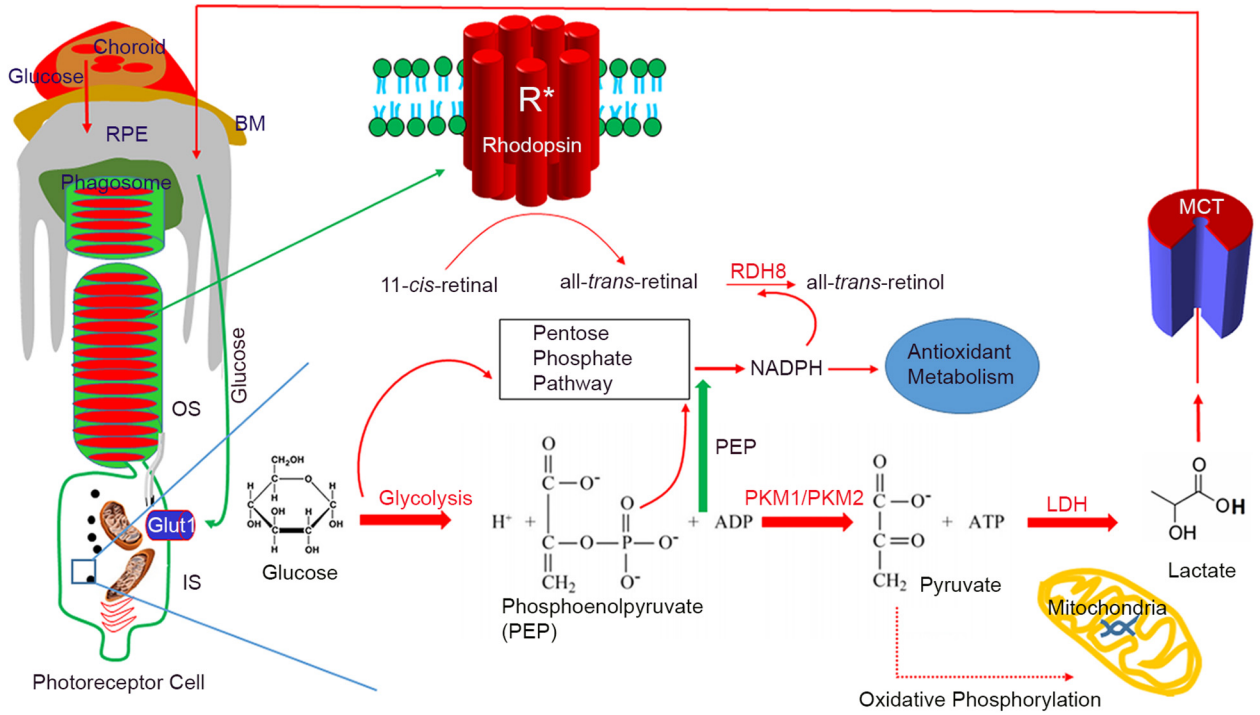

FIGURE 1 | Aerobic glycolysis in photoreceptor functions. Photoreceptors are post-mitotic cells, interdigitated with retinal pigment epithelium (RPE). Glucose enters the RPE through choroidal circulation. Bruch's membrane (BM) separates the RPE and choroid. Glucose from the RPE is transported to a photoreceptor through glucose transporter 1 (Glut1). In photoreceptor cells, the majority of glucose is redirected to anabolic processes. Every day by the onset of light, 10% of photoreceptor tips are phagocytosed by the RPE, and some of the digested lipids are recycled back to photoreceptor cells. A high rate of membrane synthesis takes place in photoreceptor cells. The redirected glucose is utilized for the anabolic processes, which include lipid synthesis, RNA/DNA synthesis, and protein synthesis. The NADPH generated through the pentose phosphate pathway (PPP) is used for lipid synthesis and reduction of all-*trans*-retinal to all-*trans*-retinol by the retinol dehydrogenase 8 (RDH8). NADPH is also needed for antioxidant metabolism. Photoreceptor cells express predominantly PKM2, while PKM1 is a minor protein. Pyruvate formed during glycolysis will be converted to lactate by lactate dehydrogenase (LDH). Lactate is transported to RPE through lactate transporters (monocarboxylate transporter), where it converts to pyruvate through LDH to fuel mitochondria. Glucose-mediated oxidative phosphorylation is minimal. PKM2 favors aerobic glycolysis and has a lower affinity for PEP, which results in the accumulation of PEP in the outer segments, which triggers the PPP. BM, Bruch's membrane; RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; RDH8, retinal dehydrogenase 8; PEP, phosphoenolpyruvate; LDH, lactate dehydrogenase; PKM1, M1 isoform of pyruvate kinase; PKM2, M2 isoform of pyruvate kinase; Glut1, glucose transporter 1; MCT, monocarboxylate transporter.

(Sievers et al., 2011), the resulting structures using the UCSF Chimera program (Pettersen et al., 2004; **Figure 2**). The majority of residues in PKM1 are identical to PKM2. A significant divergence can be noticed in the two alpha helices and a linker region with 21 of the 22 of divergent residues being localized here (aa 389–428) (**Figure 2**). The other divergent residue is (Glu 433 PKM1; Lys 433 PKM2) is located away from this region and is situated on the c-terminal side of a β-sheet downstream of these two alpha-helices. Consistent with this divergence this region shown increased estimated root-mean-square deviation of atomic position (RMSD) values compared to the full-length protein and full-length protein lacking this divergent region. Upon removal of this linker region from the full-length protein exhibited a decreased RMSD value (**Table 1**) likely indicating that this region is different between these two isoforms at a structural level. The surface plot of this divergent region in PKM1 and PKM2 further shows distinct characteristic features between both isoforms. Further studies are needed to establish the importance of this region at a functional level.

These differences between PKM1 and PKM2 seem to be localized to a part of the protein which appears key for dimerization and/or tetramerization indicating these changes might affect the substrate specificity based on the extent oligomerization (Guo et al., 2013; Israelsen and Vander Heiden, 2015). PKM1 always exists as a stable constitutive tetramer, whereas PKM2 exists in between the tetrameric and dimeric configurations, depending on the extent of posttranslational modifications and allosteric activators (Iqbal et al., 2014; Prakasam et al., 2018; Wiese and Hitosugi, 2018). Fructose 1-6-bisphosphate (FBP) (Wang et al., 2017) is one of the glycolytic intermediates of glycolysis. It binds to PKM2 and promotes the tetrameric form and increases its affinity toward its substrate, PEP. This high enzyme activity catalyzes the conversion of PEP to pyruvate and promotes oxidative phosphorylation. PKM2 undergoes tyrosine phosphorylation on tyrosine-105 (Y105) by oncogenic tyrosine kinases, glucose-mediated acetylation on lysine-305 (K305), and oxidation of cysteine-358 (C358) residue by insulin-induced ROS (Hitosugi et al., 2009; Anastasiou et al., 2011; Lv et al., 2011). These posttranslational modifications

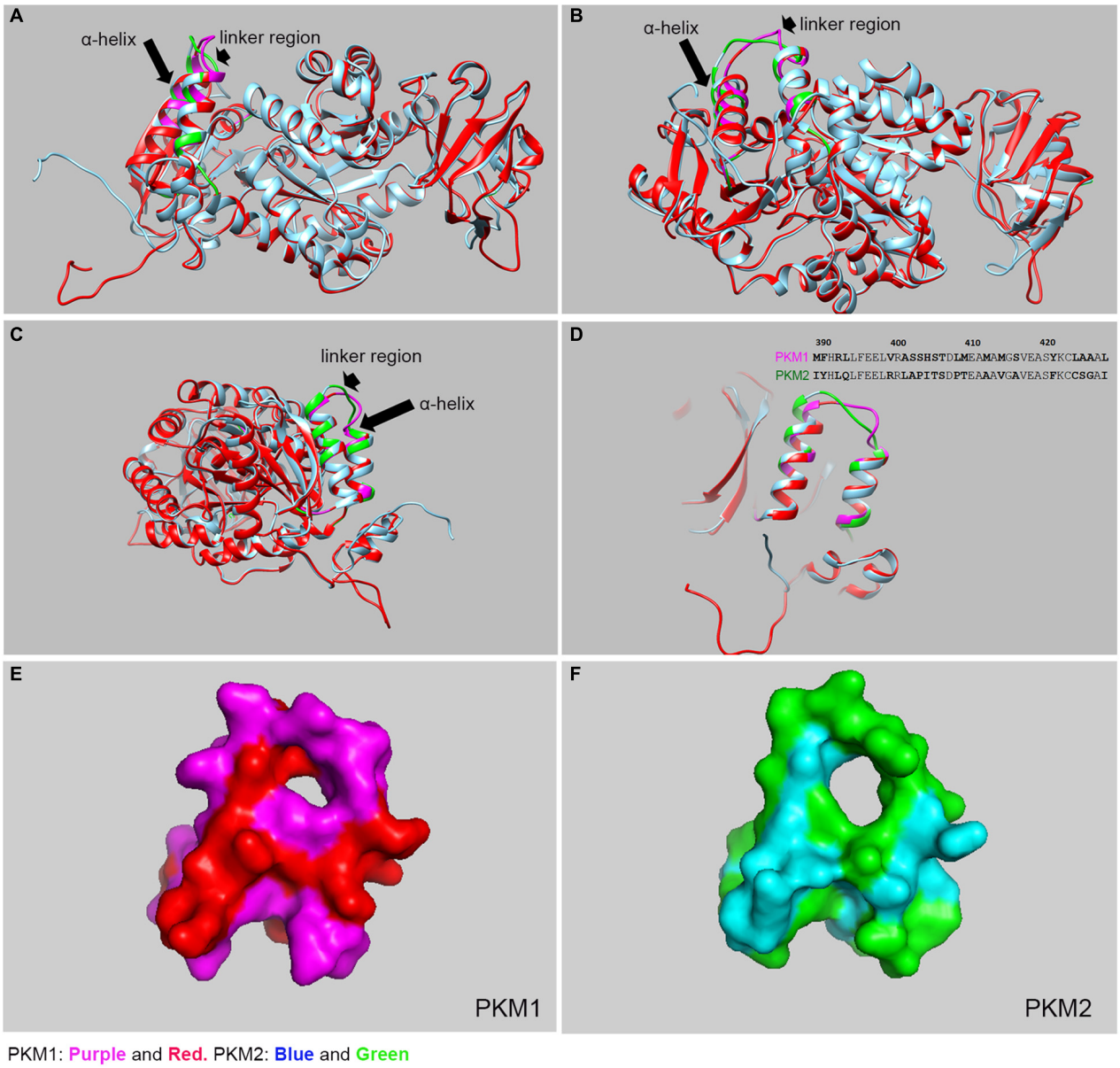

FIGURE 2 | Structural comparison between mouse PKM1 and PKM2. The crystal structure of human PKM1 and PKM2 has been solved (PDB ID: PKM2, 4JPG, PKM1, 3SRF). We carried out the molecular modeling of mouse PKM1 and PKM2 using the Raptor X template-based protein structure modeling web server and aligned, with Clustal Omega, and the resulting structures were generated using the UCSF Chimera program and PyMOL (PyMOL Molecular Graphics System, Version 4.5 Schrödinger, LLC). The structural alignment of PKM1 and PKM2 at the different views **(A–C)**. The purple color represents PKM1 divergent region/residue and the red represents PKM1 conserved region/residue. The green color represents the PKM2 divergent region/residues and the blue represents PKM2 conserved region/residue. The majority of residues in PKM1 are identical to PKM2, except a region where diverge can be noticed in the linker region (see arrowhead) and 2 alpha helices (see arrow). A significant divergence was shown to be localized to residues 389–428 which consist of 2 alpha helices and joining linker region **(D)**. *inset:* Divergent protein sequence between PKM1 and PKM2 (389–428). The surface plots of PKM1 **(E)** and PKM2 **(F)**, respectively as seen in panel **(D)**.

favor PKM2 to be in the dimeric conformation, which makes the enzyme inactive. The inactive enzyme has a low affinity for PEP that results in the activation of the PPP to promote anabolic processes.

Previous studies showed that substitution of PKM2 in tumors with PKM1 reverses the cancer phenotype (Christofk et al., 2008a). These earlier studies showed that PKM1 favors oxidative phosphorylation, whereas PKM2 promotes anabolic processes (Dong et al., 2016).

The role of PKM1 in tumor progression is controversial. More recently, PKM1 has been shown to promote the growth of multiple tumor lines (Morita et al., 2018). These studies showed that PKM1 promotes the catabolism of glucose without altering the biosynthetic pathways. The tumor progression

TABLE 1 | RMSD generated using Pymol standard algorithm.

Region	Amino acids	RMSD (Å)
Full-length protein	1–531	0.38
Divergent region (2α-helices)	389–428	0.54
Full-length protein excluding the divergent region	1–388, 429–531	0.33

Values depicted in the table are after five rounds of outlier exclusion. Complete analysis is shown in ***Supplementary Material****.*

mediated by PKM1 occurs through PKM1-mediated activation of autophagy/mitophagy. For this tumor phenotype, PKM1, but not PKM2, supports malignant cell proliferation (Morita et al., 2018). Pyruvate kinase M2 activation has been shown to protect against the progression of diabetic glomerular pathology and mitochondrial dysfunction (Qi et al., 2017).

Non-metabolic Functions of PKM2

PKM2 is known to regulate glycolytic activity and anabolic processes (Zhang et al., 2019). Besides, PKM2 has an intrinsic protein kinase activity and phosphorylates proteins on tyrosine, threonine, and serine residues (Gao et al., 2012; Israelsen and Vander Heiden, 2015). Interestingly, PKM2 uses ADP and PEP as phosphate donors, instead of ATP (Israelsen and Vander Heiden, 2015). PKM2 is also a transcriptional co-activator and mediates non-glycolytic nuclear function by phosphorylation (Luo and Semenza, 2012; Hsu and Hung, 2018). Oncogenic mediated tyrosine phosphorylation of PKM2 on Y105 results in the accumulation of glycolytic intermediates and redirects glucose for anabolic processes. Under physiological conditions, the dephosphorylation of PKM2-Y105 is mediated by protein tyrosine phosphatase 1B (Bettaieb et al., 2013; Prakasam et al., 2018). PKM2 undergoes phosphorylation on S37 by EGFR-activated extracellular signal-regulated kinase (ERK) 1/2. IGF-1R activated Akt phosphorylates PKM2 on S202, and proviral insertion in murine lymphoma 2 (PIM2) facilitates the nuclear translocation of PKM2 with the help of nuclear importin α5 (Prakasam et al., 2018). The nuclear-translocated PKM2 facilitates the transcriptional activation of β-catenin and signal transducer and activator of transcription 5 (STAT5), and enables the expression of several genes, including cyclin D1, lactate dehydrogenase (LDH), PKM2, glucose transporter 1 (GLUT1), and cMyc, to redirect the glucose metabolism that is essential for cancer progression (Prakasam et al., 2018). It was also shown that nuclear PKM2 regulates the Warburg effect (Yang and Lu, 2013).

Pyruvate Kinase Isoforms in the Retina

The retina is a post-mitotic neuronal tissue consisting of seven layers of cells, including rod and cone photoreceptor cells. These cells are metabolic, and their energy expenditure is almost equivalent to that of a cancer cell. Glycolysis is indispensable for photoreceptor cell survival; ablation of this pathway resulted in retinal degeneration (Chinchore et al., 2017), whereas the upregulation of this pathway is neuroprotective (Zhang et al., 2016). Aerobic glycolysis is essential for normal rod function and prevents cone degeneration in retinitis pigmentosa (Petit et al., 2018). Shunting glucose to aerobic glycolysis regenerates cone outer segment synthesis (Wang et al., 2016, 2019). Furthermore, the rod-derived cone viability factor has been shown to promote cone photoreceptor survival by stimulating aerobic glycolysis (Ait-Ali et al., 2015). It has also been shown that stimulation of adenosine monophosphate-activated protein kinase (AMPK) by metformin protects photoreceptor and RPE in three mouse models of retinal degeneration (Xu et al., 2018). In cancer cells, metformin increases aerobic glycolysis and reduced glucose metabolism through the citric acid cycle (Andrzejewski et al., 2014) and might similarly protect the photoreceptors. Both rod and cone photoreceptor cells are close contacts with retinal pigment epithelial (RPE) and Müller cells. Choroidal circulation brings glucose to the retina. The glucose enters the RPE cell via glucose transporters and is then delivered to rod photoreceptor cells (Punzo et al., 2012; Hurley et al., 2015). Altered mitochondrial metabolism in the RPE is the prime cause of RPE dysfunction and age-related macular degeneration (AMD) (Morohoshi et al., 2012; Ferrington et al., 2017; Riazi-Esfahani et al., 2017; Fisher and Ferrington, 2018). RPE cells do not use glucose for metabolism. Through aerobic glycolysis, glucose is converted to lactate. Through lactate transporters (Adijanto and Philp, 2012), lactate is transported to RPE cells, where it can be converted to pyruvate by the action of LDH and then converted to pyruvate, fueling the mitochondria for oxidative phosphorylation (**Figure 1**). In this regard, the RPE cell is dependent on oxidative phosphorylation, whereas the photoreceptor cell is more dependent on glycolysis (Kanow et al., 2017). The lactate made in the photoreceptor cells is also transported to Müller cells, where it is converted to pyruvate, and fuels the mitochondria for oxidative phosphorylation. Thus, a metabolic ecosystem exists between RPE, photoreceptor, and Müller cells (Kanow et al., 2017).

The retina expresses both PKM1 and PKM2. However, the expression is cell-specific. PKM1 is predominantly expressed in the inner plexiform layer and ganglion cell layer, and weakly expressed in the rod inner segments (Rajala et al., 2016). PKM2 is predominantly expressed in the inner segments and outer plexiform layer of the photoreceptors and weakly expressed in the inner plexiform and ganglion cell layers (Lindsay et al., 2014; Rajala et al., 2016).

To understand the gene networking that controls neuronal cell reprogramming in response to injury, a team of researchers has carried out single-cell RNASeq analysis from the chick, mice, and zebrafish (Hoang et al., 2019). Their transcriptomic and epigenetic analysis show that during injury several genes regulate the neuronal regeneration in the vertebrate retina (Hoang et al., 2019). These authors have deposited the data and created an interactive search at https://proteinpaint.stjude.org/F/2019.retina.scRNA.html. From this publicly available database, we searched the pyruvate kinase (*Pkm*) gene expression and found that it is expressed differently in various cell types of the retina (**Figure 3**). Consistent with our previous studies that pyruvate kinase expressed in both rod, cone and the inner retinal layer of the retina (**Figure 3**). This analysis does not differentiate the individual isoforms of either PKM1 or PKM2, but give in general the expression profile of *Pkm* gene. In

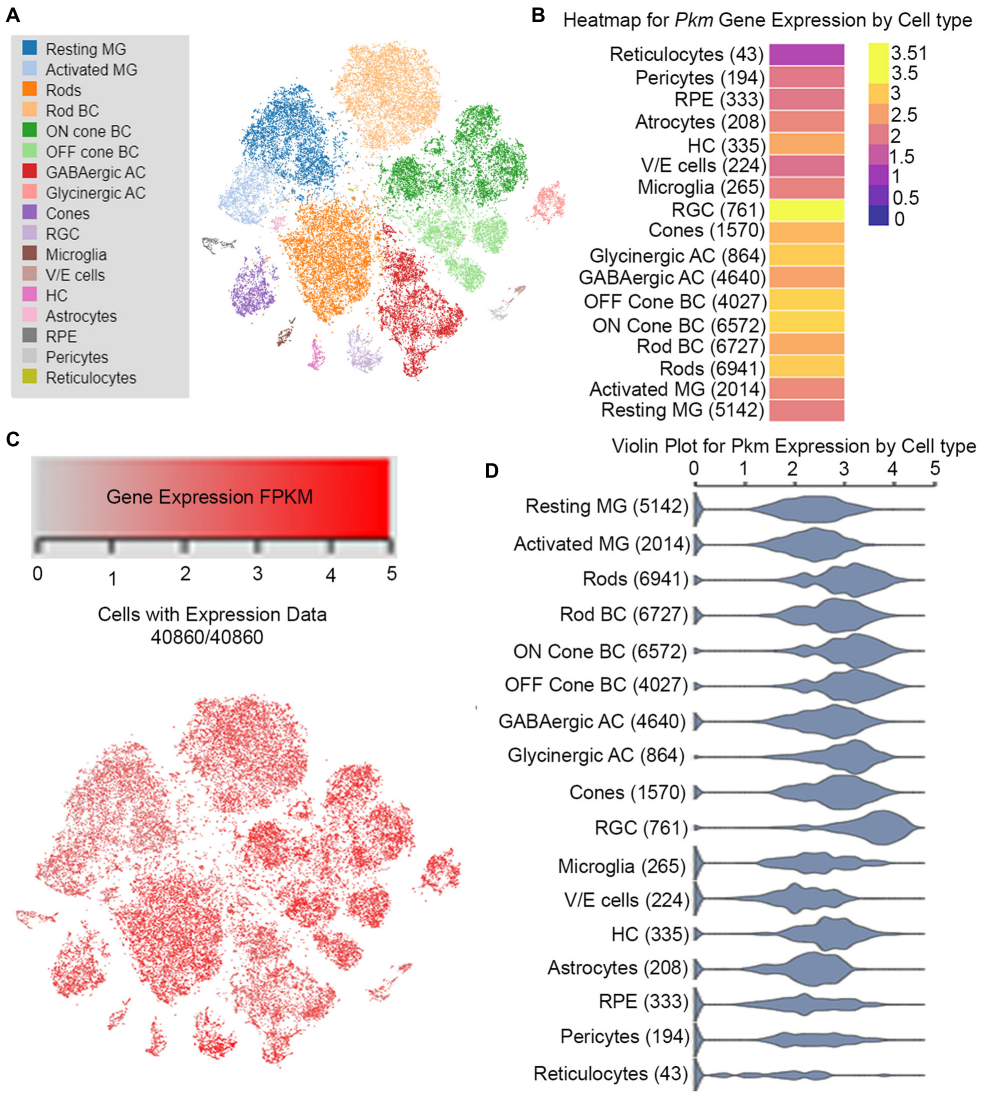

FIGURE 3 | Cell-specific *Pkm* expression in adult mouse retina on single-cell RNA-seq analysis. From this publicly available database https://proteinpaint.stjude.org/F/2019.retina.scRNA.html, we searched the *Pkm* gene expression in various cell types of the retina from single-cell RNA-seq analysis **(A)**. t-distributed stochastic neighbor embedding (t-SNE) plots of gene expression distribution in adult mouse retina **(B)**. Each dot represents a single cell. Levels of gene expression of *Pkm* in different cell types **(C)**. FPKM: Fragments/Kilobase of transcript per Million mapped reads. Violin plots showing expression levels *Pkm* gene in different cell types of the mouse retina **(D)**.

photoreceptors, PKM2 undergoes tyrosine 105 phosphorylation in a light-dependent manner (Rajala et al., 2016), similar to cancer cells (Hitosugi et al., 2009). This phosphorylation has been shown to inhibit pyruvate kinase activity and supports the notion that reduced PKM2 activity promotes anabolic activity. Several investigators have deleted the PKM2 gene specifically in rod photoreceptor cells or performed pan-retinal deletion of PKM2 with short hairpin RNAs (shRNA) (Chinchore et al., 2017). A profound retinal degeneration phenotype was reported when PKM2 was knocked down with shRNA (Chinchore et al., 2017). Conditional deletion of PKM2 in rod photoreceptor cells resulted in a very slow retinal degeneration. However, by 5 months, knockout mice experienced reduced rod function (Rajala et al., 2018a). It is well known that exon 10 (PKM2) suppresses the expression of exon 9; this is consistent with the transcription repression reports that deletion of PKM2 in photoreceptor cells produces upregulation of PKM1 in these cells (Chinchore et al., 2017; Wubben et al., 2017; Rajala et al., 2018a). In rods, PKM1is a minor protein compared with PKM2 (Rajala et al., 2016). The structural and functional changes observed in PKM2-deleted rods could be due to the upregulation of PKM1. Interestingly, combined knockdown of PKM1 and PKM2 has been shown to shorten rod outer segments (Chinchore et al., 2017). This phenotype could be reversed by supplementing PKM2 cDNA, but not PKM1 cDNA (Chinchore et al., 2017). In the presence of endogenous PKM2, forceful expression of

PKM1 has been shown to reduce the length of rod outer segments (Chinchore et al., 2017).

Since PKM1 knockout mice are not available, the functional role of PKM1 in the retina is currently unknown. Mouse rods lacking PKM2 showed upregulation of PKM1, yet failed to complement the reduced rod function (Rajala et al., 2018a). In the retina, PKM2 is present around 150 pmol and PKM1 is present around 26 pmol (Rajala et al., 2018a). In the absence of PKM2, there was a three-fold compensatory increase of PKM1 with 65 pmol of PKM1. PKM2 is a highly abundant protein in rods, and its concentration is very close to that of rhodopsin (Lyubarsky et al., 2004). It was previously reported that a single allele of rhodopsin gene gives 30 million rhodopsin molecules per rod; around 550–650 pmol rhodopsin per 6.4 million rods was estimated in wild-type mice with two alleles (Lyubarsky et al., 2004). In cancer cells, switching PKM2 with PKM1 reverses the cancer phenotype (Christofk et al., 2008a). One study found no evidence for a shift in PKM1 to PKM2 expression during tumorigenesis (Bluemlein et al., 2011).

Metabolic glucose flux experiments showed that loss of PKM2 resulted in the accumulation of glycolytic intermediates, with a decrease in pyruvate and lactate levels (Rajala et al., 2018a). In this regard, photoreceptors do not behave like cancer cells. The conversation of PEP to pyruvate is significantly slower in rod-specific PKM2 knockout mice. Even though the PKM1 is upregulated in rods lacking PKM2, there is less pyruvate kinase activity and accumulation of glycolytic intermediates. The accumulation of glycolytic intermediates in the absence of PKM2 suggests that PKM1 has a higher Km for PEP *in vivo* or that there is a lower level of pyruvate kinase activity. PKM1 upregulation in the outer retinas of PKM2 knockout mice showed increased expression of genes involved in glucose metabolism, which led to chronic degenerative changes in the outer retinas of PKM2-deleted mice (Wubben et al., 2017). These studies led to the hypothesis that reprogramming metabolism may be a novel therapeutic avenue for photoreceptor neuroprotection during stress conditions. Interestingly, the deletion of PKM2 in rods resulted in the accumulation of glycolytic intermediates, which also resulted in increased levels of NADPH (Rajala et al., 2018a). PKM2 is also expressed in cones. Mouse cones lacking PKM2 undergo early onset of cone degeneration (Rajala et al., 2018b).

PKM2 Regulates the Photoreceptor-Specific Protein Expression

In mice, rods lacking PKM2 show decreased expression of cGMP-phosphodiesterase 6β (Pde6β) and GTPase-activating protein, regulators of G-protein signaling 9 (RGS9) (Rajala et al., 2018a). Increased levels of cGMP bind to the cGMP-gated channel and facilitate the influx of ions (dark current) that depolarize the photoreceptor (Kaupp and Seifert, 2002; Yau and Hardie, 2009). Pde6β hydrolyzes cGMP and regulates the cGMP-gated channel at the plasma membrane of the rod outer segments (Kaupp and Seifert, 2002). Decreasing the cGMP levels in the cell blocks the inflow of dark-current and the cell becomes hyperpolarized (Kaupp and Seifert, 2002). Changes in the levels of cGMP alter the phototransduction kinetics (Takemoto and Cunnick, 1990).

Pde6β is specific to photoreceptor cells. However, a 2013 study reported that aggressive breast tumors and breast cancer cell lines overexpress Pde6β (Dong et al., 2013). Interestingly, these tumors also expressed increased PKM2 (Israelsen et al., 2013). Consistent with the overexpression of PKM2 and Pde6β in tumors, photoreceptor cells lacking PKM2 express reduced levels of Pde6β (Rajala et al., 2018a). The reduced expression of Pde6β is due to a non-metabolic role of PKM2 as a transcriptional co-activator of Pde6β. Consistent with this notion, PKM2 increases Pde6β promoter activity *in vitro* (Rajala et al., 2018a). PKM2 can also indirectly regulate PD6β, as it requires Hsp90 and its co-chaperone AIPL for maturation (Aguila and Cheetham, 2016). It was reported previously that Hsp90 inhibition blocked Hsp90-AILP interaction results in PDE degradation (Xu et al., 2017). Further, mutations in the gene encoding AIPL1 cause Leber congenital amaurosis (LCA) (Dharmaraj et al., 2004). Hsp90 promotes cell glycolysis, proliferation, and inhibition of apoptosis by regulating PKM2 abundance via threonine-328 phosphorylation in hepatocellular carcinoma (Xu et al., 2017). Further, PKM2 directly interacts with Hsp90, suggesting that the PKM2 interaction with Hsp90 might regulate cellular levels of Pde6β. Further studies are needed to delineate the PKM2-Hsp90-Pde6β axis.

Mouse rods lacking PKM2 show accumulation of glycolytic intermediates and decreased levels of pyruvate and lactate, suggesting that less pyruvate kinase enzyme activity is available to generate pyruvate in the presence of increased expression of PKM1 (Rajala et al., 2018a). These studies raise an important open question in photoreceptor biology. That is, there is a question of how photoreceptors survive in the absence of glycolysis, as they are highly metabolic in the dark and require copious amounts of ATP. The possible sources of ATP to preserve photoreceptors until mice reach 5 months of age are also unknown.

The major source of pyruvate production is glycolysis. An alternative pathway to produce pyruvate has been reported (Vander Heiden et al., 2010). In this PEP pathway, the substrate of pyruvate kinase acts as a phosphate donor in the phosphorylation of the glycolytic enzyme phosphoglycerate mutase (PGAM1) in PKM2-expressing cells (Vander Heiden et al., 2010). PEP is transferred onto the catalytic histidine (His^{11}) residue on human PGAM1. This reaction has been shown to occur with physiological concentrations of PEP and produced pyruvate in the absence of PKM2 activity. Pyruvate can also be produced through amino acid metabolism (Chang and Goldberg, 1978; Weber, 2001).

Upregulated PKM1 failed to complement PKM2 function in rods. Thus, there must be another source of acetate to fuel the TCA cycle to generate ATP. There is no potential source of ATP generation in PKM1-upregulated, PKM2-deleted rods. However, the source could be 3-carbon compounds, such as pyruvate and lactate, from Müller cells. Müller cells receive lactate from photoreceptor cells to fuel their mitochondria by converting the lactate to pyruvate through LDH (Kanow et al., 2017). PKM2 is not expressed in Müller cells under physiological conditions

(Lindsay et al., 2014). However, when we analyzed a publicly available database of the single Müller cell transcriptome of the rhodopsin KO mouse showed that at the time points of rod- and cone-degeneration, PKM2 expression in the Müller cells is significantly higher than that in non-degenerating wild type mice (Roesch et al., 2012). Thus, it is evident that retinal degeneration induces the expression of PKM2 in Müller cells.

The PKM2 isoform is expressed in both rod and cone photoreceptors (Rajala et al., 2016), but cone photoreceptors lacking PKM2 undergoes rapid cone degeneration compared to rods lacking PKM2 (Rajala et al., 2018b). The rod degeneration is slow in mice lacking PKM2, however, they eventually degenerate, suggesting that other compensatory mechanisms might contribute to the survival of photoreceptor cells. The open question becomes how PKM2 knockout mouse retinas survive and the source of energy production in the retinas remains an open question. One possibility could be the utilization of amino acids for the conversion pyruvate. Consistent with this possibility, cysteine catabolism and serine biosynthesis pathways support the production of pyruvate during pyruvate kinase knockdown in pancreatic cancer cells (Yu et al., 2019). This possibility cannot be ruled out in mouse retinas lacking PKM2. In ischemia/reperfusion injury, pyruvate dehydrogenase kinase (PDK) inhibitor has recently been shown to inhibit retinal cell death and improves energy metabolism in rat retinas (Sato et al., 2020). PDK phosphorylates pyruvate dehydrogenase (PDH) and inhibits the conversion cytosolic pyruvate to mitochondrial acetyl-CoA, the substrate for the TCA cycle (Sutendra et al., 2013, 2014). PDK undergoes phosphorylation by tyrosine kinase receptor signaling and the phosphorylated form of PDK inhibits PDH, which results in the inhibition of mitochondrial oxidative phosphorylation (Sutendra et al., 2013, 2014). PDK inhibitors are commonly used to treat cancers to facilitate the conversion of pyruvate to acetyl CoA, which enters into the TCA cycle and produces ATP (Sutendra et al., 2013). A possibility cannot be ruled out that PKM2 deletion may inhibit the phosphorylation of PDK that might promote the oxidative phosphorylation, which may keep the retinas stay alive for some time. If this idea holds, modulating PKM2 levels may promote photoreceptor survival in retinas that are predetermined to degenerate. Especially, in the degenerating retinas (for example, mouse models of *retinitis pigmentosa*), reducing the anabolic activity and redirect the pyruvate to fuel mitochondria for oxidative phosphorylation may prevent retinal cell death and promote retinal cell survival.

Another possibility is the β-oxidation of fatty acid-generated acetate in the mitochondria. A 2016 study reported that photoreceptors utilize both fatty acids and glucose for ATP production, and defects in the fatty acid transport pathway were shown to cause AMD (Joyal et al., 2016). If this pathway is active, then there would be an inflow of fatty acids from other cells at a higher level for membrane and outer segment renewal processes. We know from the literature that upon phagocytosis of rod outer segments in the RPE, the n3 and n6 polyunsaturated fatty acids (PUFA) are recycled back to photoreceptor inner segments for rod outer segment membrane phospholipid synthesis (Bazan et al., 1986; Stinson et al., 1991). These studies suggest that there could be a mechanism by which large quantities of fatty acids are delivered to inner segments from the RPE for oxidative production of ATP and membrane synthesis.

Based on this published work, we can hypothesize that some of the fatty acids recycled to inner segments from the RPE come from the choroidal circulation, and the inner segments may receive more fatty acids than are needed for membrane synthesis. The remaining fatty acids could be oxidized in the mitochondria to generate acetate to fuel the TCA cycle. Nevertheless, it has been shown that the n3 PUFAs is an indispensable constituent of outer segment membranes (Benolken et al., 1973; Wheeler et al., 1975). If these n3 PUFAs are utilized for acetate generation, their levels in the outer segments would be significantly decreased in diets that are deficient in PUFA precursors. This does not happen *in vivo* (Anderson and Maude, 1972), suggesting that there might be two pools of fatty acids in the inner segments: the PUFAs needed for membrane synthesis and another pool that could be used in the mitochondria for β-oxidation, especially shorter chain saturated and mono-unsaturated fatty acids that are directed to the mitochondria for β-oxidation.

We must keep in mind that we are dealing with a chronic, steady-state adaptive response to ablation of a gene, rather than an acute response to events that may have occurred over a matter of days. Thus, we are measuring responses caused by the long-term loss of the normal source of pyruvate for the TCA cycle's production of ATP. In the retina, NADPH serves three important functions: reducing all-*trans*-retinal to all-*trans*-retinol, reducing oxidized glutathione, and providing reducing equivalents for lipid and protein synthesis. The key to understanding in PKM2 knockout mice retinas and how these mice survived and the source of energy production in these retinas remain open questions.

CONCLUSION

Studies in the retina show that aerobic glycolysis is essential for retinal cell survival. Although photoreceptors are post-mitotic, PKM2 is the predominant isoform in these cells. In tumor cells, the substitution of PKM2 with PKM1 reversed the tumor phenotype. In photoreceptor cells, the deletion of PKM2 upregulates PKM1, yet it fails to clear the accumulation of glycolytic intermediates. Studies of retinal photoreceptor cells show that PKM2 is required for rod and cone photoreceptor function and survival and that PKM2 regulates photoreceptor cells both metabolically and non-metabolically.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00266/full#supplementary-material

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REVIEW
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The Neurovascular Unit in Glaucomatous Neurodegeneration

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Glaucoma is a neurodegenerative disease of the visual system and leading cause of blindness worldwide. The disease is associated with sensitivity to intraocular pressure (IOP), which over a large range of magnitudes stresses retinal ganglion cell (RGC) axons as they pass through the optic nerve head in forming the optic projection to the brain. Despite clinical efforts to lower IOP, which is the only modifiable risk factor for glaucoma, RGC degeneration and ensuing loss of vision often persist. A major contributor to failure of hypotensive regimens is the multifactorial nature of how IOP-dependent stress influences RGC physiology and structure. This stress is conveyed to the RGC axon through interactions with structural, glial, and vascular components in the nerve head and retina. These interactions promote pro-degenerative pathways involving biomechanical, metabolic, oxidative, inflammatory, immunological and vascular challenges to the microenvironment of the ganglion cell and its axon. Here, we focus on the contribution of vascular dysfunction and breakdown of neurovascular coupling in glaucoma. The vascular networks of the retina and optic nerve head have evolved complex mechanisms that help to maintain a continuous blood flow and supply of metabolites despite fluctuations in ocular perfusion pressure. In healthy tissue, autoregulation and neurovascular coupling enable blood flow to stay tightly controlled. In glaucoma patients evidence suggests these pathways are dysfunctional, thus highlighting a potential role for pathways involved in vascular dysfunction in progression and as targets for novel therapeutic intervention.

Keywords: neurodegeneration, glaucoma, neurovascular unit, vasculature, neurovascular coupling, gap junctions

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INTRODUCTION

Glaucoma is an age-related disease of the visual system and a leading cause of irreversible blindness worldwide (WHO, 2020). Clinical classification schemes of the several forms of glaucoma hinge upon a key anatomic feature of the anterior segment, the iridocorneal angle, which is defined by the angle formed where the iris and cornea meet. In open-angle glaucoma, the angle is sufficiently wide to allow normal outflow of aqueous humor from the anterior chamber to the drainage canals in the trabecular meshwork at the base of the cornea. In the most common form of the disease, primary open-angle glaucoma (POAG), the angle is open but there is progressive resistance within the outflow pathways that can lead to an increase in intraocular pressure (IOP). The disease causes degeneration of the optic nerve through sensitivity to IOP which remains the only modifiable risk factor. Over a range of magnitudes, IOP stresses retinal ganglion cell (RGC) axons as they

pass unmyelinated through the optic nerve head (ONH) and form the myelinated segment of the nerve and visual projection to the brain. In the anterograde direction from the ONH, axon degeneration involves transport dysfunction and eventual disassembly with subsequent pruning of synaptic termination sites in central projection sites in the brain (Calkins, 2012). In the retrograde direction back toward the retina, RGC dendritic arbors shrink and lose complexity as excitatory synapses are eliminated, though the cell body and unmyelinated axon segment persist until later in progression (Buckingham et al., 2008; Calkins, 2012). In late stages of disease progression, RGCs degenerate completely and retinal nerve fiber layer (RNFL) thickness is significantly decreased.

That the ONH is a critical juncture for pathogenic processes that underlie neurodegeneration in glaucoma is underscored by its unique structure and physiology (Sigal and Ethier, 2009; Burgoyne, 2011; Tamm et al., 2017; Lawlor et al., 2018). Through its architecture, complex IOP-dependent forces at the ONH translate to biomechanical stress at the lamina cribrosa and ultimately, to RGC axons as they pass through (Yan et al., 1994; Burgoyne et al., 2005; Downs, 2015). The ONH is also an important site for both systemic and local vascular dysfunction that likely contributes to progression. Glaucoma involves significant comorbidity with multiple vascular conditions, including migraine, arterial hypertension and hypotension, low ophthalmic artery blood pressure, and diabetes mellitus (Dienstbier et al., 1950; Hayreh, 1969, 2001). Vascular dysfunction and insufficiency at the ONH as well as in the retina can lead to ischemia that contributes to RGC degeneration (Hayreh et al., 1970; Flammer, 1984). In normal tension glaucoma, which occurs without overt elevations in IOP, vascular dysfunction may be a primary driver of disease progression through increased oxidative stress at the level of the retina and ONH (Trivli et al., 2019). Mild and repetitive hypoxic events due to small fluctuations in IOP may lead to an unstable oxygen supply, generating chronic, low-grade ischemia-reperfusion injury that differs from sustained hypoxic insults resulting from acute elevations in IOP (Flammer, 2001; Nita and Grzybowski, 2016). In both cases, however, the main consequence is progressive oxidative stress that challenges the metabolic resources RGCs require in transmitting the retinal image to the brain.

Despite the association between systemic vascular dysfunction and glaucoma, controversy remains concerning the extent of involvement of neurovascular dysfunction in RGC degeneration during glaucoma (Hayreh, 2001). Much of the data addressing vascular changes in eyes of patients have been collected using techniques that have limitations; for example, limitations in the technology available to accurately measure blood flow in the retina and ONH (discussed below). Difficulties arise when discerning whether vascular abnormalities precede glaucomatous degeneration as most studies of the vasculature in patients are carried out in those already clinically diagnosed with glaucoma (for a recent review see Ahmad, 2016). Nevertheless, there is mounting evidence to support a role for vascular dysfunction in some cases of glaucoma. Critical questions still remain, including (1) whether vascular changes precede other insults thereby increasing RGC susceptibility, (2) if vascular dysfunction follows neuronal degeneration from a breakdown in neurovascular coupling, and (3) whether particular vascular pathways are dysfunctional and, if so, if they are targets for therapeutic intervention. In the sections that follow, we will review known facts that address these questions and others that have bearing on the vascular contribution to glaucomatous neurodegeneration.

VASCULAR DYSFUNCTION IN GLAUCOMA

The high metabolic nature of the retina necessitates a continual supply of metabolites and removal of oxidative waste (Buttery et al., 1991; Wong-Riley, 2010; Country, 2017). The retina has a conflicting requirement of blood supply and minimal interference with light. The evolution of two vascular supplies meet this conflict: the choroid supplies photoreceptors that comprise one-third of the retina, and intra-retinal vessels supply the remaining two-thirds of the retina (Kur et al., 2012). The inner layers of the retina that require a proximal blood supply include the outer plexiform layer, the inner plexiform, and the ganglion cell layer (Dowling, 1987). The vascular system supplying the ONH is more complex than in the retina (Harris et al., 2005). Blood flow to the ONH is primarily supplied by the posterior capillary artery circulation via the peripapillary choroid and short posterior ciliary arteries, except for the surface nerve fiber layer which is supplied from the central retinal artery circulation (Onda et al., 1995) (**Figure 1A**). Blood flow regulation at these sites involves multiple metabolic and vasoactive pathways (**Figure 1B**).

In all stages of glaucoma, early through to late progression, functional and morphological changes appear in the microvasculature of both the retina and ONH, independently of IOP (Newman et al., 2018). Ocular blood flow measurements have improved over recent years but remain technically challenging. Compared with healthy controls, ocular blood flow is disturbed in glaucoma patients and is a recognized factor that contributes to progressive visual field loss (Galassi et al., 2003; Grieshaber and Flammer, 2005). Ocular blood flow is more reduced in patients who have IOP in a normotensive range, compared with patients who experience ocular hypertension (Kaiser et al., 1997). Ocular hypotensive medications have the potential to improve ocular blood flow in the eye, but studies to date have had difficulties disentangling the effects of lowered IOP and improved circulation in the eye (Januleviciene et al., 2012).

In addition to blood flow, advances in fundus imaging have enabled vessel diameters close to the optic disc to be routinely performed on patients. Retinal microvascular caliber is therefore one of the most commonly reported biomarkers, with high reproducibility using semi-automated quantification methods (Li et al., 2013; Newman et al., 2018). However, there are limitations in the quantification of more specific vessel artifacts that require assessment by trained observers, such as focal arteriolar narrowing (Wong, 2004). Vessel caliber measurements indicate that arteriole vessel narrowing is associated with optic nerve damage and severity of optic neuropathy (Jonas and Naumann,

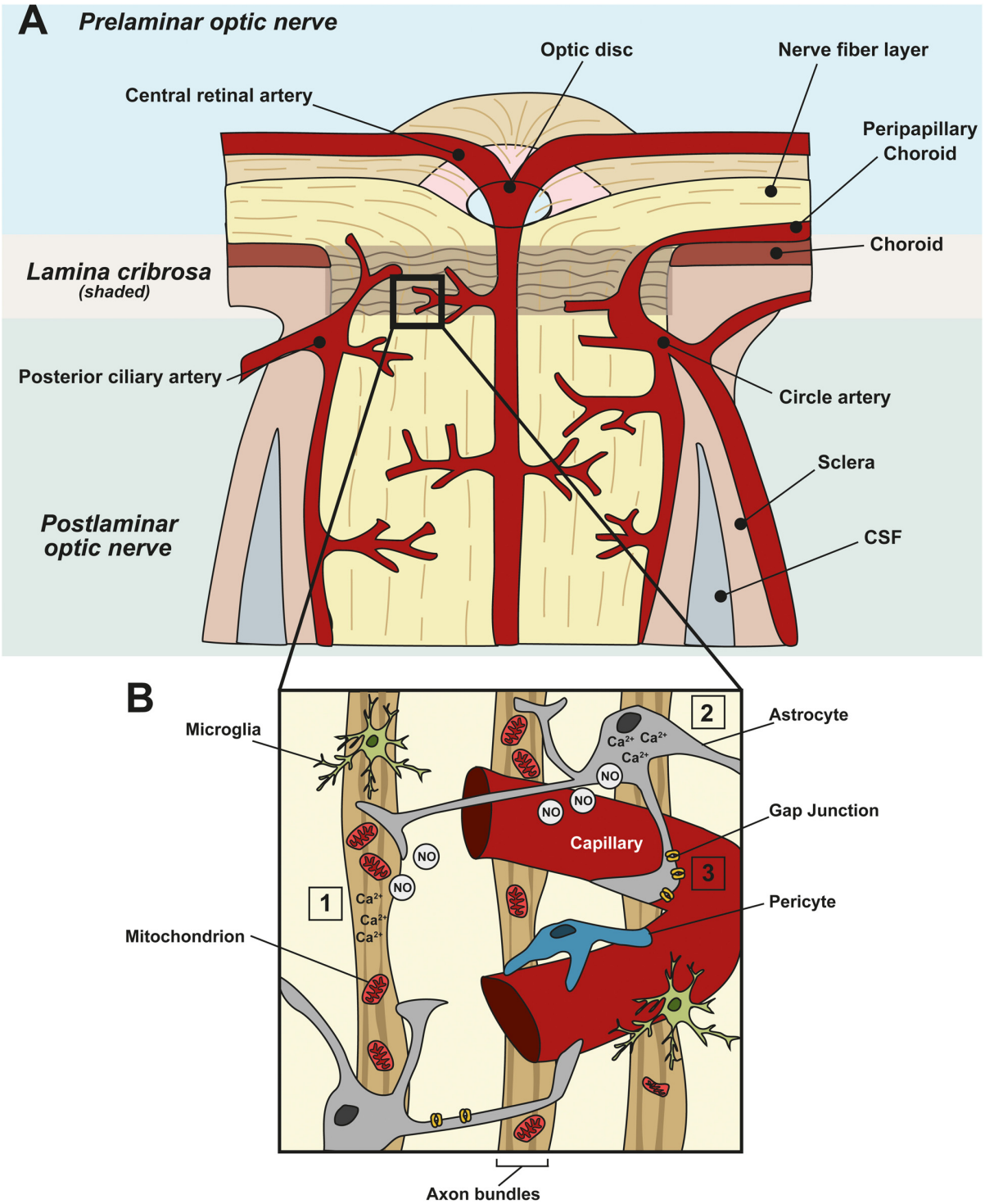

FIGURE 1 | Neurovascular coupling in the ONH and vascular dysfunction in glaucoma. **(A)** Schematic showing the blood supply at the ONH. Prelaminar, lamina cribrosa, and postlaminar sections are indicated. The primary blood supply to the ONH and retina comes from the choroid, the central retinal artery, the posterior ciliary artery and the circle artery. **(B)** Enlargement of the boxed area in **(A)** showing a capillary and its associated cells in the ONH. Astrocytes, pericytes, and endothelial cells of blood vessels constitute the neurovascular unit (NVU), to link local neuronal activity to vascular changes. In healthy tissue, when there is a spike in neuronal activity (1), or metabolic demand, it leads to an increased intracellular concentration of Ca^{2+} in neurons and (2) astrocytes. This, in turn, leads to the generation of nitric oxide (NO), a vasoactive gaseous messenger, which can diffuse to nearby blood vessels, altering blood flow. In glaucoma, apoptotic neurons and reactive astrocytes lead to the breakdown of this coupling. (3) In addition, in glaucoma, ischemia, and perfusion instability damages astrocyte–astrocyte gap junctions, leading to miscommunication between astrocytes and neurons.

1989; Jonas et al., 1989; Lee et al., 1998; Papastathopoulos and Jonas, 1999). A longitudinal study of glaucoma patients linked early blood vessel narrowing with disease progression; over a 10-year follow-up period, patients with narrowed retinal arteriole caliber were associated with a greater risk of developing glaucoma (Kawasaki et al., 2013). In this particular study, RNFL measurements were not carried out, so a correlation between microvascular changes and RNFL thickness could not be determined. Nonetheless, generalized narrowing of retinal vessel caliber is a phenomenon associated with glaucomatous optic neuropathy and RNFL thinning that occurs independently of elevated IOP; ocular hypertensive patients without glaucoma pathology did not exhibit vessel narrowing (Rankin and Drance, 1996; Mitchell et al., 2005; Amerasinghe et al., 2008). This finding is corroborated in the pediatric population (De Haseth et al., 2007), lending support to the notion that vascular changes in glaucoma are independent of IOP and may be associated with other pathological features.

In addition to vessel narrowing, further along in disease progression, OCT-angiography in glaucoma patients shows reduced vessel density in retinal capillary layers (Yip et al., 2019; see also Quigley et al., 1984). In the very early stages of glaucoma, macula vessel dropout is common and there is a significant association between ONH vessel density with peripapillary RNFL thickness (Suh et al., 2016; Yarmohammadi et al., 2016a,b; Hou et al., 2019). Elevated IOP combined with decreased perfusion pressure is correlated with reduced retinal vessel density, which may lead to a reduction in blood flow to retinal tissues (Baek et al., 2019). These changes observed in humans also reflect in animal models of the disease. In a rat model of elevated IOP, there is reduced capillary volume, perimeter, diameter and density in the optic nerve head (Moreno et al., 2014). In the DBA/2J mouse model of glaucoma, choroid and retinal blood flow reduce as age and IOP increase (Lavery et al., 2012).

Of particular importance to the health of RGC axons is the microvascular perfusion at the ONH. In glaucoma there is a general compromise of the vasculature in the ONH and surrounding regions (Liu and Neufeld, 2000; Jia et al., 2012; Liu et al., 2015; Scripsema et al., 2016; Akil et al., 2017; Nascimento et al., 2019). Thus glaucoma is often associated with an unmet need for metabolites and O_2 due to insufficient blood flow, or 'ischemia' (Osborne et al., 2004; Kaur et al., 2008; Schmidt et al., 2008). In fact, the posterior lamina is implicated as the primary site of disruption in glaucoma, and emerging studies show a significant decrease in vessel density and blood flow in POAG in the deeper layers of the ONH compared with controls (Nascimento et al., 2019). Microvascular density correlates with RGC axon volume across all areas at the ONH, but the correlation is greater at the posterior lamina cribrosa, further emphasizing the importance of changes in vascular parameters at this site (Kang et al., 2018). Narrowing of retinal blood vessels is also characteristic of advanced glaucomatous optic nerve damage, indicating that vascular changes occur in the retina in addition to the site of injury at the ONH (Jonas et al., 1989; Rankin and Drance, 1996). ONH blood flow velocity is reduced to a greater extent in glaucoma patients with visual field progression compared to those with non-progression (Yamazaki and Drance, 1997). Furthermore, eyes with progressive visual field defects in NTG patients had lower blood vessel velocities (Kaiser et al., 1997; Yamazaki and Drance, 1997).

Changes in vascular morphology, i.e., narrowing of vessels and complete vessel dropout, are indicative of deleterious changes in blood vessel tone and blood flow regulation at the level of the neurovascular unit (NVU). There is a higher incidence of these changes in glaucoma patients at all stages of disease progression. Interestingly, focal arterial narrowing and other microvascular changes are also associated with other non-glaucomatous optic neuropathies (Jonas et al., 1991; Rader et al., 1994), suggesting that changes in vessels occur across a wide range of IOP values and may well arise from dysfunctional RGCs, leading to impaired blood flow and vessel narrowing. When RGCs are dysfunctional, for example, RGCs that are experiencing higher levels of ROS, or cells that are undergoing cell death, they are not as metabolically active and due to lower nutritional demand, blood flow decreases. Therefore blood flow dysregulation in glaucoma likely exacerbates the progressive loss of RGCs. The next sections of this review will highlight critical pathways in blood flow regulation in the retina and ONH.

BLOOD FLOW REGULATION IN THE EYE

The vessels of the retina and ONH have evolved mechanisms that enable blood flow to meet the dynamic metabolic demands of the tissue (Kur et al., 2012). Such mechanisms include tight autoregulation and neurovascular coupling. Autoregulation enables vascular beds in the retina and ONH of healthy eyes to maintain a continuous blood flow and supply of metabolites despite fluctuations in ocular perfusion pressure (OPP, Alm and Bill, 1973). To determine autoregulation capacity in patients, measurements of blood flow differences are carried out before and after the OPP is artificially increased or decreased. The normalized blood flow change represents the autoregulation capacity at any given OPP level tested. Changes in blood flow in response to OPP changes plotted on a graph constitute a classic autoregulation curve (**Figure 2A**). The curve includes a plateau region across a range of OPP where the blood flow is fully compensated by autoregulatory mechanisms. When the OPP fluctuations exceed the autoregulation range defined by this plateau, vasomotor adjustments are incomplete and blood flow will gradually decrease or increase passively as OPP changes. Autoregulation is achieved through changes in blood vessel tone and through neurovascular interaction. Typically, when blood vessels experience changes in blood pressure, they alter the resistance and the tone of their vessel walls as part of the 'myogenic response' in order to maintain a continuous flow through the tissue (Hayreh, 2001). Arterioles will contract or relax in response to an increase or decrease in intravascular pressure, respectively (Boltz et al., 2013; Prada et al., 2016).

Two key vasoactive molecules mediate blood vessel tone and blood flow: nitric oxide (NO) and endothelin-1 (ET-1; Nyborg and Nielsen, 1990). NO is a potent vasodilator released by smooth muscle cells and endothelial cells which acts via pericytes to dilate capillaries (Hayreh, 1997). ET-1 is a potent

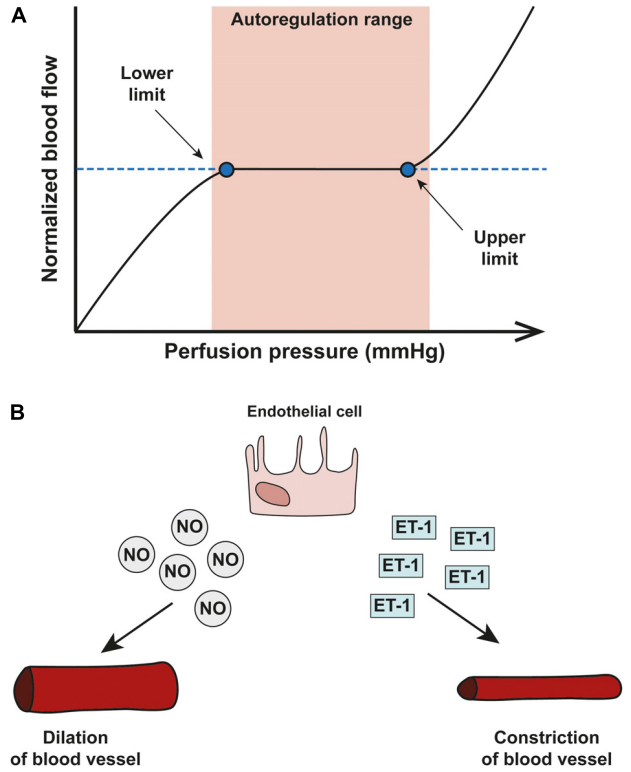

FIGURE 2 | Blood flow autoregulation in the eye. **(A)** A schematic showing an autoregulation curve that describes the relationship between normalized blood flow (*y*-axis) and perfusion pressure (*x*-axis). Autoregulation can only operate within a critical range of OPP and once OPP surpasses the optimal range (shown in pink), autoregulatory systems start to break down. **(B)** The two important vasoactive substances released by endothelial cells are nitric oxide (NO) and endothelin-1 (ET-1) and autoregulation of the vascular system in the eye relies on a delicate balance between the two; NO is a potent vasodilator released by smooth muscle cells and endothelial cells which acts via pericytes to dilate capillaries. Opposite in function is ET-1, a potent vasoconstrictor.

vasoconstrictor released from endothelial cells that acts on three primary receptors: ET_A, ET_{B1}, and ET_{B2} receptors. ET_A receptors are present in vascular smooth muscle and mediate the vasoconstrictive properties of ET-1 (Orgul et al., 1999; Resch et al., 2009a; Schmidl et al., 2011). ET_{B1} receptors are present on endothelial cells and facilitate vasodilation (Schmidl et al., 2011). ET_{B2} receptors on the other hand mediate constriction of vessels (Pollock et al., 1995).

Aside from its role in regulating blood flow, ET-1 mediates a multitude of responses in the retina through targeting ET-1 receptors on several cell types. In rodents, intravitreal and peribulbar administration of ET-1 activates receptors on RGCs altering the rate of RGC axonal transport and promotes apoptotic cell death of RGCs (Stokely et al., 2002; Yorio et al., 2002; Chauhan et al., 2004; Lau et al., 2006; Taniguchi et al., 2006; Krishnamoorthy et al., 2008). ET-1 receptor activation on astrocytes promotes their proliferation (Lau et al., 2006). Moreover, ET-1 reduces expression of RGC mitochondrial oxidase enzymes, implicating a role for ET-1 in RGC bioenergetics (Chaphalkar et al., 2020). The effect of ET-1 on RGCs and other cell types directly is beyond the scope of this review, but reviewed well in Shoshani et al. (2012).

In vascular autoregulation, a delicate balance between concentrations of NO and ET-1 mediates appropriate vessel response to maintain blood flow (**Figure 2B**; Orgul et al., 1999; Venkataraman et al., 2010). In glaucoma, mounting evidence suggests dysfunction in the pathways of vasoactive mediators.

BLOOD FLOW REGULATION IS IMPAIRED IN GLAUCOMA

Patients with glaucomatous optic neuropathy have abnormal autoregulatory responses due to the dysfunction of the cells involved in these processes (Pournaras et al., 2004; Galambos et al., 2006; Feke and Pasquale, 2008; Prada et al., 2016). Cellular stress derived from pressure changes at the ONH, combined with impaired autoregulatory responses which triggering ischemia may accelerate glaucomatous RGC degeneration (Trible et al., 1993).

Elevated serum levels of ET-1 and other biochemical markers of endothelial function in the aqueous humor of glaucoma patients suggest that endothelial dysfunction is associated with disease pathology (Sugiyama et al., 1995; Noske et al., 1997; Resch et al., 2009a; Ghanem et al., 2011; Cellini et al., 2012; Li et al., 2016). There is a statistically significant correlation between microvascular endothelial function and severity of POAG in the Malay population (Bukhari et al., 2016). In patients, blocking both ET_A and ET_B receptors results in increased blood flow through the retina, choroid and ONH. In DBA/2J mouse studies, delivery of bodentan, a dual ET_A and ET_B receptor blocker, significantly protects against glaucomatous damage at the ONH (Resch et al., 2009b; Howell et al., 2011). In addition, administration of ET-1 in proximity to the optic nerve head leads to ischemia, and the appearance of clinical indications of glaucoma including increased cupping of the optic disc, which leads to subsequent RGC loss (Orgul et al., 1996; Chauhan et al., 2004; Cioffi et al., 2004). Mice with endothelium-specific overexpression of ET-1 exhibit both retinal vascular dysfunction and progressive loss of RGCs over 10–12 months (Mi et al., 2012). Importantly, a recent study in mice directly links IOP elevation to vascular endothelial dysfunction, which bolsters findings of endothelial dysfunction in glaucoma patients where elevated IOP is apparent. In the study, elevated IOP blunts retinal arteriole reactivity in response to the endothelium-dependent vasodilator acetylcholine, but not to the endothelium-independent nitric oxide donor, nitroprusside. Also, retinal arteriole responses to changes in perfusion pressure are compromised in eyes with elevated pressure, suggesting that autoregulation is impaired (Gericke et al., 2019). In the DBA/2J mouse model of inherited glaucoma, several molecular changes in the ONH are detectable before damage to optic nerve axons have been elucidated, and these include endothelin induction in microglia (Howell et al., 2011).

As well as perturbations in the endothelin-1 pathway, there is also longstanding evidence that impaired NO signaling is

implicated in glaucoma (Haefliger and Anderson, 1997; Polak et al., 2007; Wareham et al., 2018). NO is a gaseous signaling molecule, however, high NO concentrations can be neurotoxic and induce oxidative stress through the formation of reactive nitrogen species (Pacher et al., 2007). In a rat model of glaucoma, RGC degeneration is linked with increased nNOS expression and RGC loss was prevented by NOS inhibition (Neufeld et al., 2002). NO is not always deleterious to ocular function, and a delicate balance in NO production is therefore necessary to support a healthy cellular environment. Production of NO by NO-synthase (NOS) enzymes in the ONH is essential for controlling the vascular tone of the region (Haefliger et al., 1992, 1993, 1999). When NO production is blocked systemically by inhibition of NOS in glaucoma patients, both choroidal and ONH blood flow do not decrease to the same extent as in healthy patient controls, suggesting that elevated basal NO in glaucoma patients may be a compensatory mechanism to ensure optimal ocular blood flow (Polak et al., 2007). On the other hand reports of decreased levels of NO were found in the aqueous humor of POAG patients (Doganay et al., 2002), as well as a reduction in the levels of cGMP, a signaling molecule downstream of NO production (Galassi et al., 2004). In animals, impaired NO signaling has also been linked with glaucomatous characteristics. A mouse line deficient in the alpha subunit of the guanylate cyclase ($GC1^{-/-}$), an enzyme activated by NO and responsible for the production of cGMP), develop POAG over time, characterized by RGC axon loss, modest increases in IOP and impaired retinal vascular function (Buys et al., 2013). RGC loss is linked to deficiencies in the NO-cGMP signaling pathway in two animal models of glaucoma and treatment with tadalafil, a phosphodiesterase inhibitor prevents RGC degeneration, independently of IOP (Wareham et al., 2018).

Dysfunction in the NO signaling pathway, either through up-regulation, or down-regulation, is a likely contributor to abnormal ocular blood flow; an increase or decrease in NO shifts the balance between vasoconstrictive and vasodilatory mediators. Poor ocular perfusion is directly detrimental to RGC health, leading to ischemia, oxidative stress, and lack of metabolic support. In addition, a reduction in ocular perfusion may also increase the sensitivity of the cells to other glaucoma-related stressors conveyed at the ONH that further exacerbate disease progression.

NEUROVASCULAR COUPLING IN GLAUCOMA

Neuronal activity and blood flow are tightly coupled in the central nervous system in a phenomenon known as 'functional hyperemia' – a spike in neuronal activity evokes increased blood flow to the area (Roy and Sherrington, 1890). After initial observations in the brain, the general consensus was that homeostatic regulation of blood flow was dependent on local metabolite concentration in a negative feedback loop. In this mechanism, increased neuronal activity leads to increased energy demand, for example, the additional ATP consumption that is required to reset ion gradients after an action potential (Attwell et al., 2010). A reduction in ATP is perceived as an increased need for metabolites in tissues and thus induces dilation of blood vessels. However, the vascular supply to tissues after neuronal activity far supersedes the metabolic requirements of the tissue, so a feedback mechanism working alone has been discredited (Powers et al., 1996). More recent work has shown that glial cells play a major role in neurovascular coupling (NVC) via a feedforward mechanism (Vaucher et al., 1997; Attwell et al., 2010; Petzold and Murthy, 2011). In this process, neuronal activity leads to neuronal signaling to nearby blood vessels or astrocytes, which leads to the release of vasoactive agents thereby increasing blood supply (Attwell et al., 2010). The latest consensus is that both feedforward and feedback mechanisms are at play; the initial feed-forward mechanisms that over-supply neurons with nutrients may be balanced by a feedback mechanism that is metabolism-dependent and responsive to the accumulation of vasoactive metabolic by-products (Iadecola, 2017). The objective of these mechanisms medicated by the NVU is to meet the metabolic needs of the neurons. A triumvirate of cell types comprise the NVU (**Figure 3A**): vascular cells (vascular smooth muscle cells, pericytes and endothelial cells), glial cells (astrocytes, microglia, and oligodendrocytes), and neurons (Iadecola, 2004; Attwell et al., 2010; Hamilton et al., 2010; Winkler et al., 2011).

The NVC response has been elegantly demonstrated in the ONH and retina with experiments investigating hemodynamic responses to flicker-light stimulation (Riva et al., 1986, 1997; Garhofer et al., 2003, 2005; Gugleta et al., 2012, 2013a). In glaucoma patients, this response is dysfunctional; flicker-light induced retinal vasodilation responses are diminished (**Figure 3B**, Garhofer et al., 2004). This impaired response is likely due to reduced neuronal activity and altered glial cell function in the disease (Kornzweig et al., 1968; Hernandez et al., 2008). However, it could also be directly related to fluctuations in IOP, or vascular dysfunction in the tissue. In an experiment where short term IOP elevations of up to 43 mmHg were inflicted in healthy subjects, flicker-light stimulations in the retina were maintained, suggesting that diminished responses in glaucoma patients are not necessarily directly due to changes in IOP alone (Garhofer et al., 2005). Thus, the dysfunctional NVC response observed in glaucoma patients probably arises from dysfunction at the cellular level provoked by other, non-IOP-related stressors.

PERICYTES AND THE NVU

The involvement of pericytes in the regulation of blood flow in the retina and the relation to glaucomatous disease has been largely unexplored. Pericytes are embedded in the basement membranes of microvessels, and extend their processes along capillaries, pre-capillary arterioles and post-capillary venules (Sweeney et al., 2016). Pericytes express several types of muscle contractile proteins (Herman and D'amore, 1985) and are involved in propagating vasomotor signals along the length of capillaries (Peppiatt et al., 2006; Puro, 2007). Pericytes are responsive to vasoactive molecules described earlier, e.g., NO, and other circulating metabolites, such as ATP (Haefliger and Anderson, 1997; Kawamura et al., 2003). Pericytes function

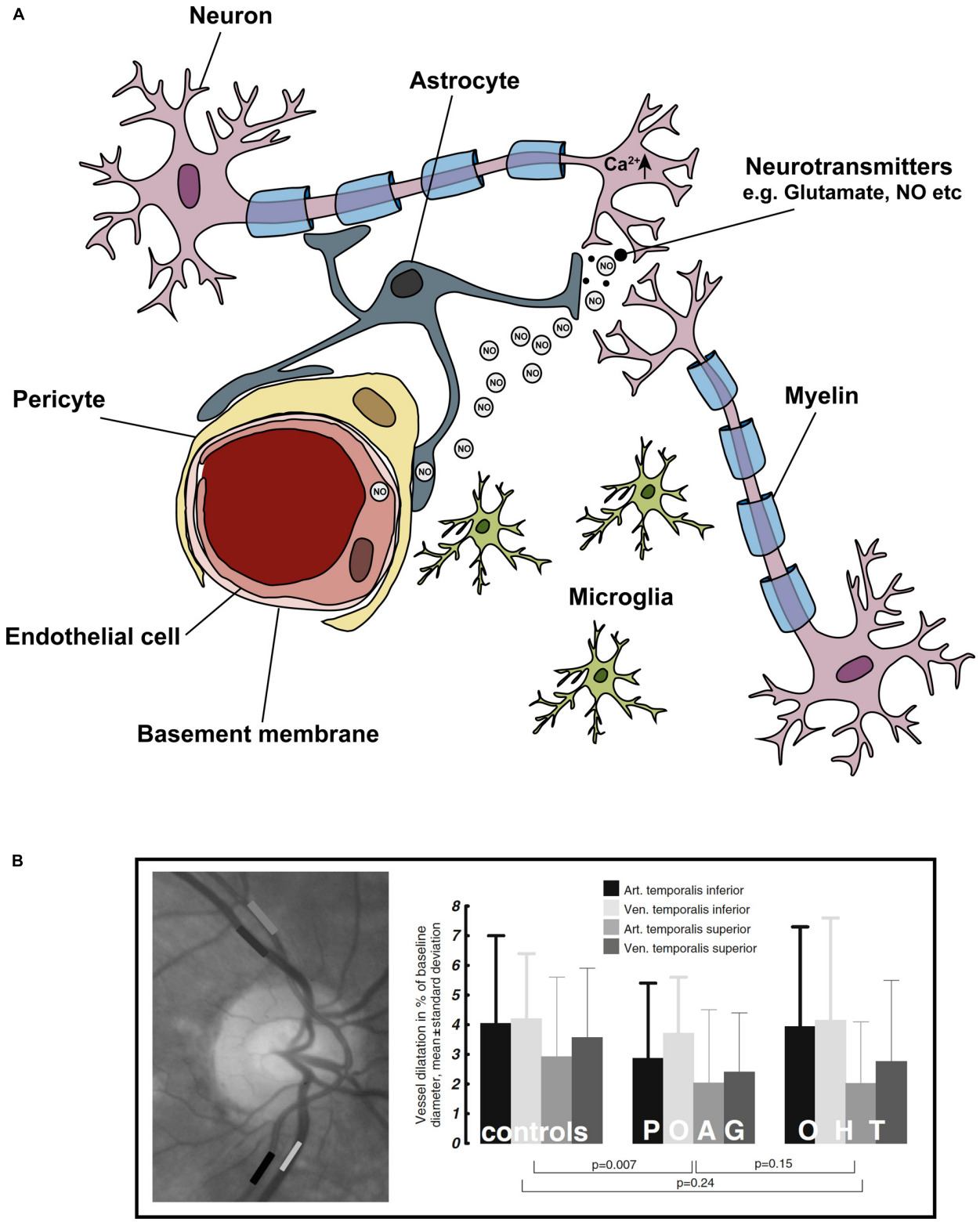

FIGURE 3 | Cells of the 'neurovascular unit' and the light flicker response. Neurovascular coupling describes the coupling of neuronal activity to vascular responses. **(A)** Shows the cells comprising the NVU, these include neurons (in the eye specifically – RGCs), astrocytes, microglia, pericytes, and endothelial cells. In general, a spike in neuronal activity leads to an increase in intracellular Ca^{2+}, which generates NO. NO diffuses to local blood vessel endothelial cells, activating K^{+} channels, which leads to downstream vasodilation and increased blood flow. The light flicker response demonstrates the tight coupling of neuronal activity (in response to light)

(Continued)

FIGURE 3 | Continued
and change in vessel diameters in the retina. In glaucoma, this light flicker response is diminished. **(B)** To measure the light flicker response in the retina, a fundus video is used (image left) where the temporal inferior artery and vein, and temporal superior artery and vein are clearly visible. Areas of analysis are shown in grayscale boxes. Graphical representation the light flicker response in control, ocular hypertension (OHT) and glaucoma patients shows diminished vessel response with disease. Figure adapted from Gugleta et al. (2013b).

similarly to smooth muscle and endothelial cells, possessing a number of ion channels and transporters that help to mediate changes in capillary diameter. Like smooth muscle, alterations in pericyte tone and contractile ability change with intracellular Ca^{2+} levels (Sakagami et al., 1999, 2001; Wu et al., 2003). The overall tone of pericyte-containing microvessels is the result of a balance between Ca^{2+}-mediated contractility and by NO-mediated relaxation (Kutcher et al., 2007).

As an integral cell of the NVU, it is unlikely that pericytes are immune to cellular changes or dysregulation. In an experiment mimicking glaucoma in mice, elevated IOP stimulated the expression of β-III-tubulin, a neuronal cell marker, in both pericytes and endothelial cells, suggesting that vascular cells respond to changes in IOP via alterations in protein expression (Prokosch et al., 2019). It is not yet understood how these expression changes relate to regulation of blood flow or in NVC responses in the retina, however, the results suggest that pericytes respond to IOP-related stress through changes in gene expression. Furthermore, pericytes themselves are vulnerable in ischemic conditions; retinal ischemia reduces the ability of pericytes to relax after constriction, leading to a further decrease in blood flow (Hall et al., 2014; Sweeney et al., 2016; Alarcon-Martinez et al., 2019). Glaucoma has been described as a vasospastic disease, whereby retinal ischemia-reperfusion injury repeatedly occurs, rather than a single ischemic event (Flammer et al., 2001). Reduced retinal blood flow in glaucoma may lead to pericyte dysfunction, which may further impact reperfusion of retinal tissue. Glaucoma is associated with aging, and changes in pericytes that occur with age may also contribute to, and encourage, degeneration of RGCs. In the aged rat retina, there is breakdown of the normal vascular architecture and reduced pericyte-endothelial cell contact (Hughes et al., 2006). Pericytes express a number of gap junction proteins that facilitate cell–cell communication with other cells of the NVU, and expression changes of these gap junctions in glaucoma may also play a role in vascular dysfunction (discussed below). With their central role in retinal perfusion, the role of pericytes in ocular perfusion and blood flow in glaucoma is crucial in our understanding of vascular dysfunction in the disease and is an area that warrants further investigation.

VASCULAR CONNECTIVITY IN GLAUCOMA

Astrocytes are the predominant glial cell type of the unmyelinated ONH, and their close proximity to blood vessels hints at their importance in mediating blood flow (Balaratnasingam et al., 2014). Astrocytes react to neurotransmitters released during neuronal activity (such as glutamate) by increasing their intracellular Ca^{2+} levels, prompting the release of vasoactive substances, such as K^+ (Porter and Mccarthy, 1996; Filosa et al., 2006). Astrocytes are key players in the neurovascular coupling response and in healthy eyes, they are quiescent and mediate normal neurovascular responses (Bachoo et al., 2004), however, in response to changes in the ONH environment, such as changes in IOP, or ischemia, astrocytes become reactive, promoting the degradation of RGCs and their axons (Varela and Hernandez, 1997; Hernandez et al., 2008). Since changes in glial reactivity are significantly implicated in various stages of glaucoma, it is plausible that all cells of the NVU in some way have a role to play in the breakdown of NVC in the retina and ONH that contributes to disease progression. Multiple pathways integral to the NVU are differentially regulated in glaucoma, and evidence for their role in glaucoma progression is outlined below.

In NVC, NO surfaces as an important player when considering direct neuronal signaling to blood vessels. An increase in neuronal activity causes the synaptic release of glutamate, activating NMDA receptors on neurons, leading to increased intracellular levels of Ca^{2+}. An elevated level of Ca^{2+} triggers a cascade of events leading to the activation of nNOS, which generates intracellular NO. NO can directly activate BKCa channels (Bolotina et al., 1994), or indirect activation can occur through NO-derived cGMP (Stumpff et al., 1997). Activation of BKCa channels leads to K^+ efflux and cell hyperpolarization. Cell hyperpolarization causes voltage-operated (L-type) Ca^{2+} channels to close, reducing Ca^{2+} influx, leading to vasorelaxation of vascular smooth muscle cells. This chain of events leads to vasodilation and increased blood flow (Cavet et al., 2014; Prada et al., 2016). Dysfunctional NO signaling is implicated in glaucoma pathogenesis and endothelial dysfunction. Inhibitors of NOS attenuate light-induced vasodilation in the retina and the ONH (Kondo et al., 1997) and increased levels of NO are observed in the ONH in response to changes in neuronal activity in flicker-stimulation (Buerk and Riva, 2002). Reactive astrocytes have been shown to produce excess amounts of NO through the activation of inducible-NOS (iNOS) (Neufeld et al., 1997; Liu and Neufeld, 2000), increasing free radicals and causing damage to local axons. Interestingly, despite high levels of NO in the ONH, blood flow is still often impaired. NO signaling represents a double-edged sword paradigm; NO in excess is detrimental to cells, causing great damage to cellular components and their dysfunction; this likely impacts vascular function. However, lower levels of NO, however, can fine-tune vascular responses. The role of NO in the NVC response may therefore lie in modulation, rather than mediation of the response; in the rat retina, high concentrations of NO leads to vasodilation of nearby blood vessels, whereas lower concentrations causes vasoconstriction (Metea and Newman, 2006). Impaired NO signaling in astrocytes

may indeed contribute to atypical neurovascular responses, causing reductions in ocular blood flow that ultimately lead to ischemia and subsequent RGC degeneration.

In the retina and optic nerve, gap junction channels between cells mediate intercellular communication, such as the communication between astrocytes and blood vessels. This communication can occur from glial cell to glial cell, or through glial cells communication to other cell types. The concept of neuron-glial connectivity, or 'gliotransmission,' was initially introduced to account for the active transfer of neuroactive molecules, from glia to neurons (Bezzi and Volterra, 2001). Initial evidence that connexin channels played a role in neuron-glial interactions was demonstrated using co-culture models and *ex vivo* brain slices (Nedergaard, 1994; Froes et al., 1999; Giaume and Theis, 2010). Increases in Ca^{2+} generated in astrocytes triggered Ca^{2+} responses in co-cultured neurons; an effect abolished by connexin channel blockers, suggesting that astrocytic-neuronal connectivity is mediated, in part by gap junctions. These junctions are integral to cell-to-cell transfer of electrical conductance (Na^+, K^+, and Ca^{2+}) and small molecules such as ATP/ADP, glutamate, and glucose, and second messengers (e.g., NO, cGMP, and cAMP; Bloomfield and Volgyi, 2009; Giaume et al., 2020). The five major neuronal classes in the vertebrate retina form diverse coupling networks by gap junctions formed by connexin proteins (Sohl and Willecke, 2003; Sohl et al., 2005). Gap junction channels have been implicated in numerous cellular processes including in maintaining ionic balance, synaptic plasticity, metabolic substrate trafficking, and cellular survival (Andrade-Rozental et al., 2000; Wright et al., 2001; Bloomfield and Volgyi, 2009; Spray et al., 2013). Gap junction-mediated gliotransmission is a vastly growing field, and we refer the reader to a recent review (Giaume et al., 2020).

In the retinal NVU, gap junctions are integral in the maintenance of the blood–retinal-barrier (**Figure 4**), and in glaucoma, changes in gap junction expression may compromise the blood–retinal-barrier, exacerbating neuronal degeneration (**Figure 4B**). Ischemia and ocular perfusion instability in glaucoma damages astrocyte–astrocyte gap junctions; under experimental elevated-IOP conditions, there is decreased gap junction communication between ONH astrocytes (Malone et al., 2007). This may lead to loss of continuity and communication between astrocytes and other cells of the NVU, including pericytes and neurons, which can disrupt ionic and metabolic homeostasis in the tissue and eventually alter blood flow (Hernandez et al., 2008). Indeed, expression of Cx43 in pericytes is important in the development, maturation, and maintenance of the blood–brain-barrier and also in retinal blood flow (Trost et al., 2016; Giaume et al., 2020), but changes in connexin expression in pericytes as they relate to glaucoma progression have not been investigated to date. In healthy rabbits, uncoupling the gap junctions between astrocytes impairs ONH blood flow regulation (Shibata et al., 2011). Ischemia also differentially regulates the hemichannels of gap junctions (Thompson et al., 2006). In human glaucomatous eyes, Cx43 expression is upregulated at the level of the lamina cribrosa and in the peripapillary and mid-peripheral retina in association with glial activation (Kerr et al., 2012). *In vitro* studies show that an increase in hydrostatic pressure leads to loss of gap junction communication and redistribution in human astrocytes (Malone et al., 2007). Conversely, in other models of retinal ischemia, blockade of Cx43 reduced overall cell death and injury in the retina and ONH (Kerr et al., 2012).

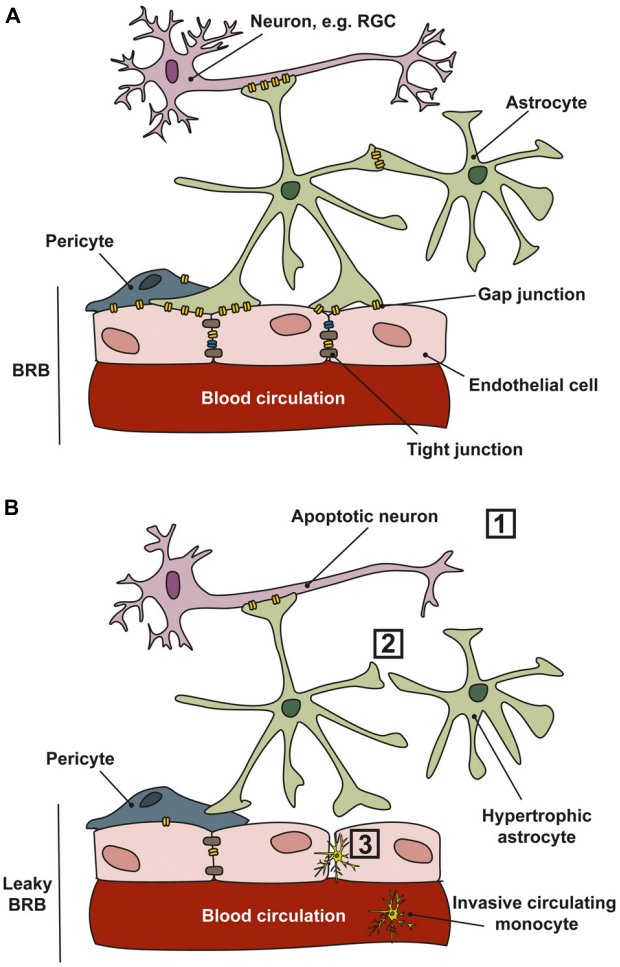

FIGURE 4 | Cell communication in the NVU. **(A)** In healthy tissues, retinal ganglion cell (RGC) neurons, astrocytes and endothelial cells communicate through gap junctions which permit the movement of electrical conductance (Na^+, K^+, and Ca^{2+}) and small molecules such as ATP/ADP, glutamate, and glucose, and second messengers. Gap junctions exist in the retina and ONH between neurons, glia and between vascular cells. **(B)** In glaucoma, several changes occur that affect communication between the cells of the NVU. (1) RGCs are vulnerable to stressors that lead to apoptosis; (2) cell–cell communication is lost as astrocytes and other glial cells become reactive and gap junction expression is reduced; (3) a reduction in gap junction expression, and loss of tight junctions between vascular cells leads to a leaky blood–retinal-barrier (BRB), allowing the infiltration of circulating monocytes into the retina and ONH.

In the optic nerve and ONH, the processes of astrocytes are interconnected via gap junction proteins Cx30 and Cx43, allowing intercellular communication that contributes to maintaining a homeostatic cellular environment (Quigley, 1977; Rose and Ransom, 1997; Rash et al., 2001). Expression of Cx43 on astrocytes increases during chronic stress (Frisch

et al., 2003; Giaume and Theis, 2010; Kerr et al., 2011). *In vitro*, elevated hydrostatic pressure causes astrocytes to alter localization and phosphorylation state of Cx43 (Malone et al., 2007). Increased phosphorylation of Cx43 leads to gap junction uncoupling (Warn-Cramer et al., 1998), whereas decreased phosphorylation is correlated with a decrease in gap junction communication (Godwin et al., 1993) or an increased in gap junction conductance (Moreno et al., 1994). These studies do not provide direct *in vivo* evidence for increased connectivity between astrocytes under glaucoma stress but suggest that glaucoma-related stresses can alter the connectivity of astrocyte gap junction proteins and their activity.

The role of gap junctions in the vasculature of the retina and ONH is less well established. Direct electrical communication of vascular cells via gap junctions has been shown to mediate the vasomotor tone and propagation of vasomotor response in the retina (Ivanova et al., 2017). Most recently, expression of another gap junction, Cx45, was shown to form electrical synapses on RGC axons in the optic nerve (Smedowski et al., 2020). In other degenerative eye diseases, such as diabetic retinopathy, down-regulation of gap junctions leads to the breakdown of the blood–retinal-barrier (Oku et al., 2001; Bobbie et al., 2010; Tien et al., 2016). There is evidence elsewhere in the CNS for gap junction association with tight junctions; in the porcine blood–brain-barrier, Cx43- and Cx40-containing gap junctions are required for the endothelial barrier (Nagasawa et al., 2006). In mice, connexins Cx43 and Cx40 are expressed throughout the retina on glia and retinal vasculature, whilst Cx37 is expressed along endothelial cells throughout the retinal vascular tree (Ivanova et al., 2019). In particular, Cx43 is expressed at tight junctions and between astroglia and endothelial cells, suggesting that these gap junctions have an integral role in maintenance of the blood–retinal-barrier (Ivanova et al., 2019). Another gap junction, not yet linked to vascular communication in the retina, is Cx36 which is found throughout the inner retina, but not the optic nerve. The expression of Cx36 been shown to increase with elevated IOP in a mouse model of glaucoma (Akopian et al., 2017). Blockade of Cx36 prevents RGC degeneration, suggesting a role of Cx36 in promoting apoptosis through inter-neuronal communication of death-signals, however, a role for Cx36 in the function of the blood–retinal-barrier is yet to be explored.

CONCLUSION AND FUTURE DIRECTIONS

The observation that systemic and ocular vascular dysfunctions are correlated with the incidence of glaucoma raises an important question; does vascular dysfunction precede glaucomatous optic neuropathy, increasing the sensitivity of RGCs to pressure at the ONH, or is it merely a secondary consequence of other pathological changes in the disease, e.g., increased inflammation? Treatments so far have focused on the anterior chamber with current therapies aimed at lowering IOP, the only modifiable risk factor for the disease. Such drugs address IOP by modulating the amount of aqueous humor produced by the ciliary body, or by improving outflow through the trabecular meshwork (Weinreb et al., 2014). These treatments have variable success rates, with many patients requiring additional invasive surgery. A good proportion of patients continue to progress despite adhering to these treatment regimens – visual field loss is inevitable. In most cases treatments that target IOP serve only to delay progression of the disease, they do not prevent degeneration of RGCs and their axons.

The fact that glaucomatous optic neuropathy occurs at all levels of IOP, and that patients progress regardless of interventions to regulate IOP suggests that there are other factors that contribute to the degeneration of RGCs and their axons in the visual projection. As we have outlined, these factors include increased vascular dysfunction, and ocular hemodynamics are critical players in the progression of glaucoma (Flammer, 1994). Systemic vascular diseases such as hypertension and hypotension are correlated with glaucoma (Tielsch et al., 1995), and changes in the vasculature of glaucoma patients, for example, disc hemorrhages are also evident in both early and late stages of the disease. Ischemia at the ONH leads to increases in oxidative stress and inflammation, as well as a decrease in the supply of essential nutrients and metabolites. Such stressors not only lead to degeneration of RGCs directly, but also render RGCs more sensitive to mechanical stresses conveyed at the ONH. An important question is how all of these glaucomatous stressors interplay to cause sensitivity of RGCs. A novel hypothesis that may marry together vascular dysfunction with inflammation and biomechanical stress of tissue in the retina and ONH is the idea that the eye possesses an ocular glymphatic system (Wostyn et al., 2017). Such a glymphatic system would have a similar role in the eye as it does in the brain, primarily as an exit for toxic waste products. It would be interesting to investigate whether there is paravascular communication between the surroundings of the retinal vascular system and the surroundings of the central retinal vessels in the optic nerve, and how vascular factors may alter glymphatic flow.

As technologies for *in vivo* imaging of vasculature in the retina and ONH of glaucoma patients improve, evidence is mounting in support of vascular abnormalities coinciding with optic neuropathy in glaucoma. A key question moving forward in glaucoma research is how can we target vascular function in the design and development of new treatments? Vascular dysfunction in glaucoma likely arises from impaired functioning of cells in the NVU, and a loss of connectivity between neurons, glia, and endothelial cells. Thus, future research into the pathways involved in this intercellular communication, as outlined in this review, is key to our understanding of the role of NVC in glaucomatous disease and efforts to delineate the temporal changes in NVC and RGC death require urgent investigation. Vascular dysfunction occurs at all levels of IOP and can affect RGC health directly, however, we do not fully understand the role of these pathways in RGC survival. Further work in these areas will lead to therapies that are aimed at mediating proper vascular regulation and therapies that promote neurovascular

interactions. Both of these are attractive novel areas to explore in the search for neuroprotective therapies in glaucoma.

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LW and DC collected the analysis and wrote the manuscript.

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Expression of R345W-Fibulin-3 Induces Epithelial-Mesenchymal Transition in Retinal Pigment Epithelial Cells

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Purpose: To investigate the role of protein misfolding in retinal pigment epithelial (RPE) cell dysfunction, the effects of R345W-Fibulin-3 expression on RPE cell phenotype were studied.

Methods: Primary RPE cells were cultured to confluence on Transwells and infected with lentivirus constructs to express wild-type (WT)- or R345W-Fibulin-3. Barrier function was assessed by evaluating zonula occludens-1 (ZO-1) distribution and trans-epithelial electrical resistance (TER). Polarized secretion of vascular endothelial growth factor (VEGF), was measured by Enzyme-linked immunosorbent assay (ELISA). Differentiation status was assessed by qPCR of genes known to be preferentially expressed in terminally differentiated RPE cells, and conversion to an epithelial–mesenchymal transition (EMT) phenotype was assessed by a migration assay.

Results: Compared to RPE cells expressing WT-Fibulin-3, ZO-1 distribution was disrupted and TER values were significantly lower in RPE cells expressing R345W-Fibulin-3. In cells expressing mutant Fibulin-3, VEGF secretion was attenuated basally but not in the apical direction, whereas Fibulin-3 secretion was reduced in both the apical and basal directions. Retinal pigment epithelial signature genes were downregulated and multiple genes associated with EMT were upregulated in the mutant group. Migration assays revealed a faster recovery rate in ARPE-19 cells overexpressing R345W-Fibulin-3 compared to WT.

Conclusions: The results suggest that expression of R345W-Fibulin-3 promotes EMT in RPE cells.

Keywords: RPE, dedifferentiation, EMT, protein misfolding, macular degeneration

INTRODUCTION

Retinal pigment epithelial (RPE) cells, photoreceptors, and the choroid form a functional unit required for healthy vision. The unique structure and polarity of the RPE monolayer is critical to maintain photoreceptors. Atrophy and dysfunction of the RPE with subsequent loss of photoreceptors plays a fundamental role in numerous retinal degenerations. RPE dysfunction manifests as a loss of barrier function, disrupted polarization, and downregulated expression of RPE signature genes and microRNA-204/211 (Wang et al., 2010; Adijanto et al., 2012). Recent evidence suggests that RPE cells lose terminal differentiation and acquire a mesenchymal cell phenotype in several retinal degenerations (Ghosh et al., 2018; Goldberg et al., 2018; Wu et al., 2019). Furthermore, misfolded proteins accumulate in RPE cells with age, but the relationship between misfolded protein accumulation and RPE epithelial–mesenchymal transition (EMT) remains unclear.

During EMT, cells lose their epithelial-specific markers and increase expression of mesenchymal drivers. Historically, it is thought that in healthy tissues, fully differentiated epithelial cells exert specific functions and maintain their phenotype after development. However, EMT can be activated under pathological circumstances, facilitating epithelial cells to obtain an enhanced migration ability and increase their production of extracellular matrix (ECM) components. Clinical evidence suggests that RPE cells undergo EMT with upregulated mesenchymal cell markers and enhanced migration ability in several degenerative retinal diseases, including inherited rod-cone degenerations, inherited macular degeneration, age-related macular degeneration (AMD), and proliferative vitreoretinopathy (Hirasawa et al., 2011; Tamiya and Kaplan, 2016; Ghosh et al., 2018; Goldberg et al., 2018; Wu et al., 2018). Optical coherence tomography (OCT) has detected intraretinal hyper-reflective foci (HRF) in various retinal diseases, which have been interpreted as migratory RPE cells, macrophages or hard exudates (Kuroda et al., 2014; Piri et al., 2015; Miura et al., 2017). Combined polarimetry with auto-fluorescence to discriminate between RPE cells, inflammatory cells, and hard exudates, revealed that a portion of HRF showed signs of RPE migration in patients with the early stages of AMD, substantiating the possibility that RPE cells have the capacity to migrate into the neuroretina (Miura et al., 2017).

Intracellular misfolded protein accumulation has been shown to induce loss of differentiation through activation of the unfolded protein response (UPR; Pallet, 2012; Lamouille et al., 2014). Similarly, stress due to intracellular amyloid-β aggregates has been shown to dissociate tight junctions (Park et al., 2014). These studies are consistent with data in other cells and systems that have revealed an interaction between the UPR and EMT (Zhong et al., 2011; Lenna and Trojanowska, 2012; Pang et al., 2016; Santamaria et al., 2019). The UPR is regulated by three endoplasmic reticulum (ER) transmembrane sensors, IRE1α, PERK, and ATF6 (Lin et al., 2007; Walter and Ron, 2011). The activation of IRE1α-XBP1 signaling promotes EMT in breast, lung, and liver tissues (Li et al., 2015; Mo et al., 2015; Cuevas et al., 2017; Liu et al., 2019). Several studies have shown that the UPR and TGF-β-induced EMT signaling pathways interact at the level of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (JNK/p38-MAPK) in an IRE1-dependent manner (Urano et al., 2000; Santibanez, 2006; Liu et al., 2019). To date, the potential role of the IRE1α-XBP1 in RPE cell dysfunction has not been explored.

Fibulin-3 is a secretory protein and contains six epidermal growth factor (EGF)-like domains and a fibulin domain (Zhang and Marmorstein, 2010). Fibulin-3 is expressed throughout the body including the eyes. A single arginine-to-tryptophan point mutation, R345W, is responsible for the inherited macular degeneration, Doyne honeycomb retinal dystrophy (Stone et al., 1999; DHRD; Garland et al., 2014; Fernandez-Godino et al., 2018), and is known to impair Fibulin-3 secretion from RPE (Marmorstein et al., 2002; Roybal et al., 2005; Hulleman and Kelly, 2015). Prior studies have also shown that R345W-Fibulin-3 is misfolded and migrates faster than the wild-type (WT) form on non-reducing gels (Marmorstein et al., 2002). Thus, overexpression of R345W-Fibulin-3 provides a useful model to study the impact of protein misfolding on RPE phenotype and dysfunction.

In this study, we investigated the role of fibulin-3 protein misfolding on RPE cell dedifferentiation and dysfunction. We infected primary RPE cells with lentivirus carrying luciferase-tagged wild-type or R345W-Fibulin-3. Our results show that expression of R345W-Fibulin-3 activates ER stress via the IRE1α/XBP1 pathway, which in turn attenuates RPE cell differentiation, indicated by disrupted tight junctions, impaired polarization, and downregulation of RPE signature gene expression. Moreover, we report that the expression of R345W-Fibulin-3 upregulates EMT markers and enhances the migration ability of RPE cells.

MATERIALS AND METHODS

Plasmids

Lentiviral constructs containing naturally secreted *Gaussia* Luciferase (GLuc) and GLuc tagged wild type or R345W Fibulin-3 were described previously (Hulleman et al., 2013). ViraPower^{TM} Lentiviral Expression systems (Thermo Fisher Scientific, Waltham, MA, United States) were used to produce Lentiviruses in 293T cells by calcium phosphate transfection.

Cell Culture

Human fetal RPE (hfRPE) cells were generously provided by Dr. Sheldon S. Miller, Dr. Kapil Bharti, and Dr. Arvydas Maminishkis (National Eye Institute, Bethesda, MD, United States) and cultured following the protocol published previously (Maminishkis et al., 2006). In brief, hfRPE cells were maintained in MEM medium (α modification) with N1 supplement, glutamine, non-essential amino acid, penicillin–streptomycin, taurine, hydrocortisone, triiodothyronine, and 5% fetal bovine serum (heat inactivated) at 37°C with 5% CO_2. Human fetal RPE cells were seeded on human ECM (#354237, Corning Life Sciences, Tewksbury, MA, United States) coated 12 mm polyester (PET) Transwell® inserts with 0.4 μm pores in 12-well plate (#3460, Corning Life Sciences, Tewksbury, MA, United States)

with 150K cells per well. Medium was changed twice a week. At the beginning of seven weeks after seeding, hfRPE cells were infected with Lentiviral GLuc-tagged WT-Fibulin-3, GLuc-tagged R345W-Fibulin-3, or GLuc tag only at MOI 10 with 6 μg/ml hexadimethrine bromide (#H9268, MilliporeSigma, Burlington, MA, United States) for 4 h a day for 5 days, resulting in a copy number of 55 ± 9 (mean ± SEM) in WT group versus 57 ± 3 (mean ± SEM) in mutant group.

ARPE-19 Tet-On cells with Lentiviral GLuc, GLuc-tagged WT- or R345W-Fibulin-3 were described previously (Hulleman et al., 2013). Inserted genes were expressed only in the presence of Doxycycline (1 μg/ml, Dox, #D9891, MilliporeSigma, Burlington, MA, United States). ARPE-19 Tet-On cells were maintained at 37°C with 5% CO_2 in DMEM (Dulbecco's Modified Eagle's Medium)/Hams F-12 50/50 Mix (#10-092-CV, Corning Life Sciences, Tewksbury, MA, United States) supplemented with 10% fetal bovine serum (FBS, #100106, BenchMark™ GeminiBio, West Sacramento, CA, United States) and penicillin–streptomycin.

Immunocytochemistry

Cells in Transwell® inserts were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were washed twice with PBS, then treated with 0.1 M glycine for 15 min and permeabilized with 0.1% Triton X-100 for three times, 2 min each. Cells were blocked with 10% normal donkey serum for 2 h at room temperature then incubated with rabbit polyclonal anti- zonula occludens-1 (ZO-1) (1:100, #61-7300, Thermo Fisher Scientific, Waltham, MA, United States) overnight at 4°C. Cells were washed three times in PBS and incubated with Alexa Fluor® 488 donkey anti-rabbit IgG (H + L) for 1 h (1:500, #711-546-152, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, United States). Nuclei were counterstained with Hoechst 33342 (1 μg/ml, #B2261, MilliporeSigma, Burlington, MA, United States). The Transwell® membranes with cells were mounted on microscope slides with Aqua-Poly/Mount medium (#18606-20, Polysciences, Warrington, PA, United States). Images were acquired using a Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Enzyme-Linked Immunosorbent Assays

Cell culture media were collected from the upper and lower chambers of Transwells after incubation for 48 h. Vascular endothelial growth factor (VEGF) enzyme-linked immunosorbent assays (ELISAs; Quantikine; R&D Systems, Minneapolis, MN, United States) were performed according to kit instructions. Optical densities were determined within 30 min with a SpectraMax 190 microplate reader (Molecular Devices, San Jose, CA, United States) at 450 nm with wavelength correction at 570 nm. All VEGF ELISA experiments were run in biological and technical triplicate.

Luciferase Assay

Cell culture media was collected weekly from apical and basal compartments of Transwell® inserts after Lentiviral infection, spun at 12,000 *g*, 15 min at 4°C, and stored at -20°C. Cells were lysed in 1× Passive Lysis Buffer (#E1941, Promega, Madison, WI, United States). *Gaussia* Luciferase activities were measured by BioLux® *Gaussia* Luciferase Assay Kit (#E3300, New England BioLabs, Ipswich, MA, United States) in triplicate according to the manufacturer's standard protocol using a Sirius Tube Luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany).

Real-Time PCR

Total RNA was extracted from samples using the AllPrep DNA/RNA/Protein Mini Kit (#80004, Qiagen Sciences, Inc., Germantown, MD, United States) according to the manufacturer's protocol. RNA was eluted with RNase-free water. Total RNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). For reverse transcription, 0.25 μg of total RNA was used in the SuperScript™ IV First-Strand cDNA Synthesis System (#18091050, Thermo Fisher Scientific, Waltham, MA, United States) with a mixture of 50 μM oligo-dT and 50 ng/μl random hexamers as primers. Real-time primers were designed with Primer3web, version 4.4.0[1] and validated using melt curve analysis and agarose gel electrophoresis. PCR primers used in this study are shown in **Supplementary Table 1**. Significant gene expression was evaluated using the FastStart Universal SYBR Green Master (ROX; #4913850001, Roche Molecular Systems, Inc., Branchburg, NJ, United States) in triplicate in QuantStudio™ 3 Real-Time PCR Systems (Thermo Fisher Scientific, Waltham, MA, United States). The relative quantity of mRNA was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the comparative $2^{-\Delta\Delta Ct}$ method.

Cell Migration Assay

ARPE-19 cells were cultured in 96-well ImageLock Microplates (Essen Bioscience Inc., Ann Arbor, MI, United States) to confluence (n = 8), and scratches were made using a 96-pin WoundMaker™ (Essen Bioscience Inc., Ann Arbor, MI, United States). The wells were then washed with PBS to remove cell debris. Wound images were acquired automatically by the IncuCyte™ software system (Essen Bioscience Inc., Ann Arbor, MI, United States). Images were collected at 1-h intervals for the duration of the experiment (72 h). The data were then analyzed via IncuCyte S3 Software (Essen Bioscience Inc., Ann Arbor, MI, United States) using the Relative Wound Confluence integrated metric.

Western Blots

Western blots were performed as described previously (Zhao et al., 2018). The primary antibodies used in this study were rabbit anti-GLuc (1:5,000, #E8023, New England Biolabs, Ipswich, MA, United States) and mouse anti-GAPDH (1:1,000, #sc-32233, Santa Cruz Biotechnology, Inc., Dallas, TX, United States).

Trans-Epithelial Electrical Resistance

Trans-epithelial electrical resistance (TER) of hfRPE cells was measured weekly after seeding in Transwell® inserts using the

[1]http://bioinfo.ut.ee/primer3/

EVOM^2 Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL, United States). In each plate, one coated insert without cells was measured as blank and final resistance was calculated by multiplication of net resistance ($R_{Total} - R_{blank}$, Ω) with effective membrane area (cm^2). The electrodes were rinsed with 70% ethanol and sterile pre-warmed culture medium.

Transmission Electron Microscopy

Cells were washed twice with PBS and fixed in 1/2 strength Karnovsky fixative (2% paraformaldehyde, 2.5% glyceraldehyde, pH 7.3) for 1 h at room temperature and further fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 h. Samples were dehydrated in a graduated ethanol series terminating in pure acetone and embedded in LX-112 (Ladd Research, Williston, VT, United States). Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate and viewed with a JEOL JEM1400 Transmission Electron Microscope (JEOL USA Inc., Peabody, MA, United States), Penn State College of Medicine Imaging Core Facility.

Statistics

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Prism (GraphPad, Inc., La Jolla, CA, United States). One-way analysis of variance and multivariate analysis were used to determine differences within groups. When identified, a student's *t*-test was used to compare differences between groups. $p < 0.05$ was considered statistically significant.

RESULTS

Primary RPE Cells Exhibit Terminal Differentiation on Transwell Filters

Primary RPE cells were placed on Transwell filters, and several endpoints were used to test the differentiation status at four weeks. Transmission electron microscopy (TEM) imaging showed that primary RPE cells displayed appropriate structural polarity with apically located microvilli, tight junctions, and melanosomes, and basally located nuclei and basal infoldings (**Figures 1A,B**). Barrier function was assessed by quantifying TER over time. After 4 weeks, the TER plateaued at ∼1,400 Ω cm^2 (**Figure 1C**). Tight junction expression and distribution was confirmed by confocal imaging. Zonula occludens-1 immunoreactivity was abundant and continuous along the cell borders (**Figure 1D**). These data confirm that primary RPE cells grown on Transwells are polarized and differentiated.

Expression of R345W-Fibulin-3 Activates the IRE1α/XBP1 Pathway in RPE Cells

A prior study revealed that in ARPE-19 cells, expression of R345W-Fibulin-3 activates ER stress via the IRE1α/XBP1 pathway (Roybal et al., 2005). This study showed that overexpression of R345W-Fibulin-3 induces the elevated expression of Binding immunoglobulin protein (BiP) also known as glucose regulated protein-78 (GRP-78) at both the mRNA and protein levels and induces increased expression of XBP1 at mRNA level (Roybal et al., 2005). To investigate the specific ER stress pathway(s) induced by mutant Fibulin-3 in primary RPE cells, total RNA was isolated at 14 weeks post-infection ($n = 3$), and quantified by rtPCR. Consistent with the previous study, IRE1α/XBP1 pathway and transcription factor C/EBP homologous protein (CHOP) were elevated in primary RPE cells expressing mutant Fibulin-3 compared to controls (**Figure 2A**). mRNA levels of ER chaperone, BiP/GRP-78 and GRP-94 were also elevated compared to cells expressing WT-Fibulin-3 (**Figure 2B**). These results suggest that expression of mutant Fibulin-3 activates ER stress in RPE cells primarily through the IRE1α/XBP1 pathway.

Expression of R345W-Fibulin-3 in RPE Cells Disrupts Tight Junction Protein Distribution and Impairs Barrier Function

To investigate the effects of R345W-Fibulin-3 expression on RPE permeability barrier function, we first examined ZO-1 distribution using the primary RPE cell culture system described above. Immunocytochemistry (ICC) was conducted at 9 or 12 weeks post-infection. Confocal imaging revealed that ZO-1 distribution was dramatically disrupted and disorganized in the mutant group relative to the WT group (**Figure 3A**). To quantify these changes, the number of RPE nuclei associated with continuous ZO-1 was counted in nine regions of the 625 μm^2 confocal image. Relative to controls, continuous ZO-1 distribution in the mutant group was reduced by 38% ($p < 0.01$) after 9 weeks of infection and by 90% after 12 weeks ($p < 0.01$; **Figure 3B**). These data indicate that the expression of mutant Fibulin-3 in RPE cells disrupts tight junction protein distribution.

To assess the impact of disrupted tight junction protein distribution on a critical aspect of RPE cell function, TER was monitored over time. Four weeks after being seeded, the TER of all cells reached a plateau of approximately 1,400 Ω cm^2. Seven weeks after seeding, cells were infected with GLuc-tagged WT-Fibulin-3, GLuc-tagged R345W-Fibulin-3, or GLuc tag only. At 3 weeks post-infection, the TER was reduced to 800–1,000 Ω cm^2 in all three groups, but no significant differences were observed between groups. Starting at 4 weeks post-infection, the TER of cells expressing R345W-Fibulin-3 was significantly reduced relative to the GLuc-tagged WT-Fibulin-3 and GLuc tag only groups. Trans-epithelial electrical resistance in the GLuc-tagged R345W-Fib3 group was 70.7% lower at 9 weeks post-infection ($n = 8$, $p < 0.01$) and 78.4% lower at 12 weeks post-infection ($n = 8$, $p < 0.01$; **Figure 4**). These data are consistent with the altered ZO-1 distribution described above and further indicate that expression of mutant Fibulin-3 induces loss of RPE barrier function.

Expression of R345W-Fibulin-3 in Primary RPE Cells Reduces the Basal Secretion of VEGF

It is well established that polarized secretion of proteins by RPE cells is critical for RPE, photoreceptor, and choroidal

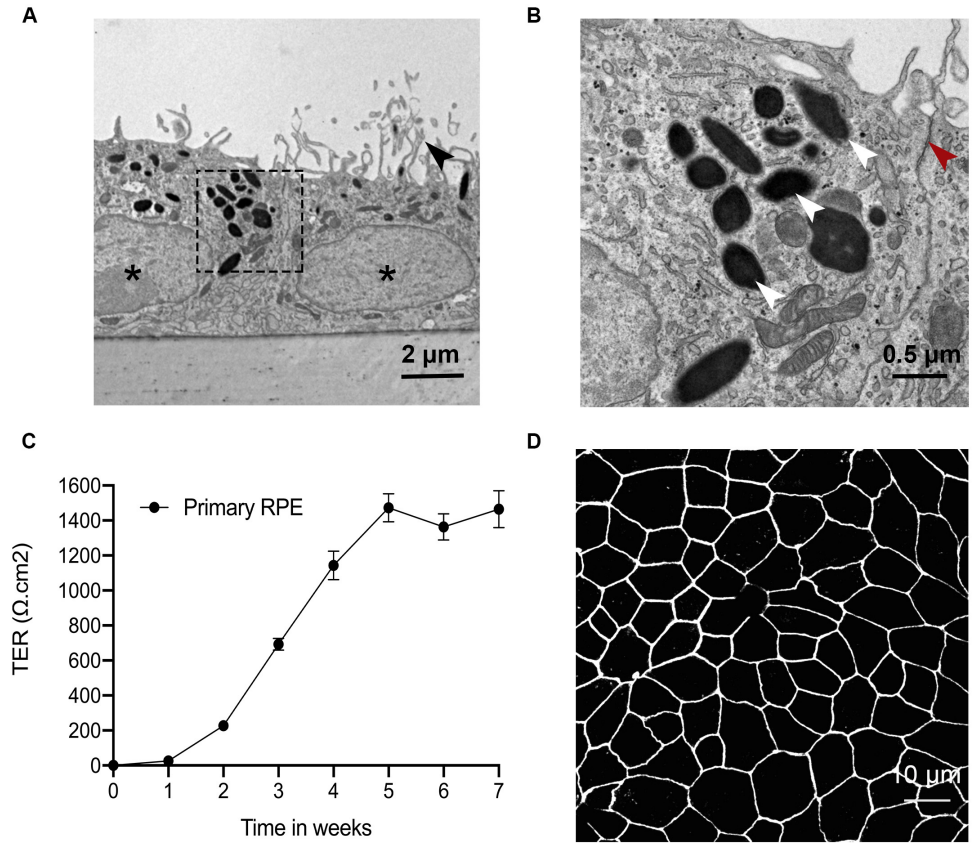

FIGURE 1 | Primary RPE cells have a terminal differentiation phenotype on Transwell filters. Primary RPE cells were cultured on Transwell filters, and their phenotype was evaluated by TEM, TER and tight junction protein ICC. **(A)** RPE cells grew in a single monolayer with apical microvilli (black arrowhead), melanosomes, basal nuclei (asterisks), and membrane infoldings. **(B)** Magnification of the indicated area in A shows RPE cells with clearly visible tight junctions (red arrowhead) and melanosomes (white arrowhead). **(C)** Barrier function was assessed by quantifying TER over time. After four weeks, the TER plateaued at ∼1,400 Ω cm^2. **(D)** ZO-1 immunoreactivity was continuous along the cell borders.

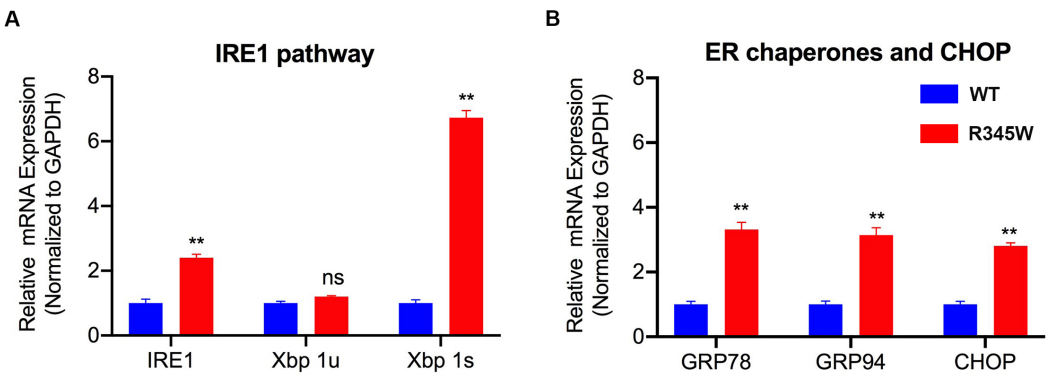

FIGURE 2 | Expression of R345W-Fibulin-3 elevates markers of ER stress in primary RPE cells. mRNA isolated from primary RPE transfected with WT or R345W-Fibulin-3 was quantified by rtPCR. **(A)** IRE1α/XBP1 mRNA were elevated in primary RPE cells expressing mutant Fibulin-3 compared to controls, while Xbp 1u was unaltered. **(B)** mRNA levels of ER chaperone, GRP-78 and GRP-94, and transcription factor CHOP were also elevated compared to cells expressing WT-Fibulin-3. (n = 3, values are mean ± SEM, $^{**}p < 0.01$).

homeostasis, and that the polarized secretion of VEGF is of particular significance (Bhutto et al., 2006; Maminishkis et al., 2006). In terminally differentiated RPE cells, VEGF is primarily secreted in the basal direction to promote the growth of the choroidal vasculature (Marneros et al., 2005). To test whether mutant Fibulin-3 induction alters the polar secretion of growth

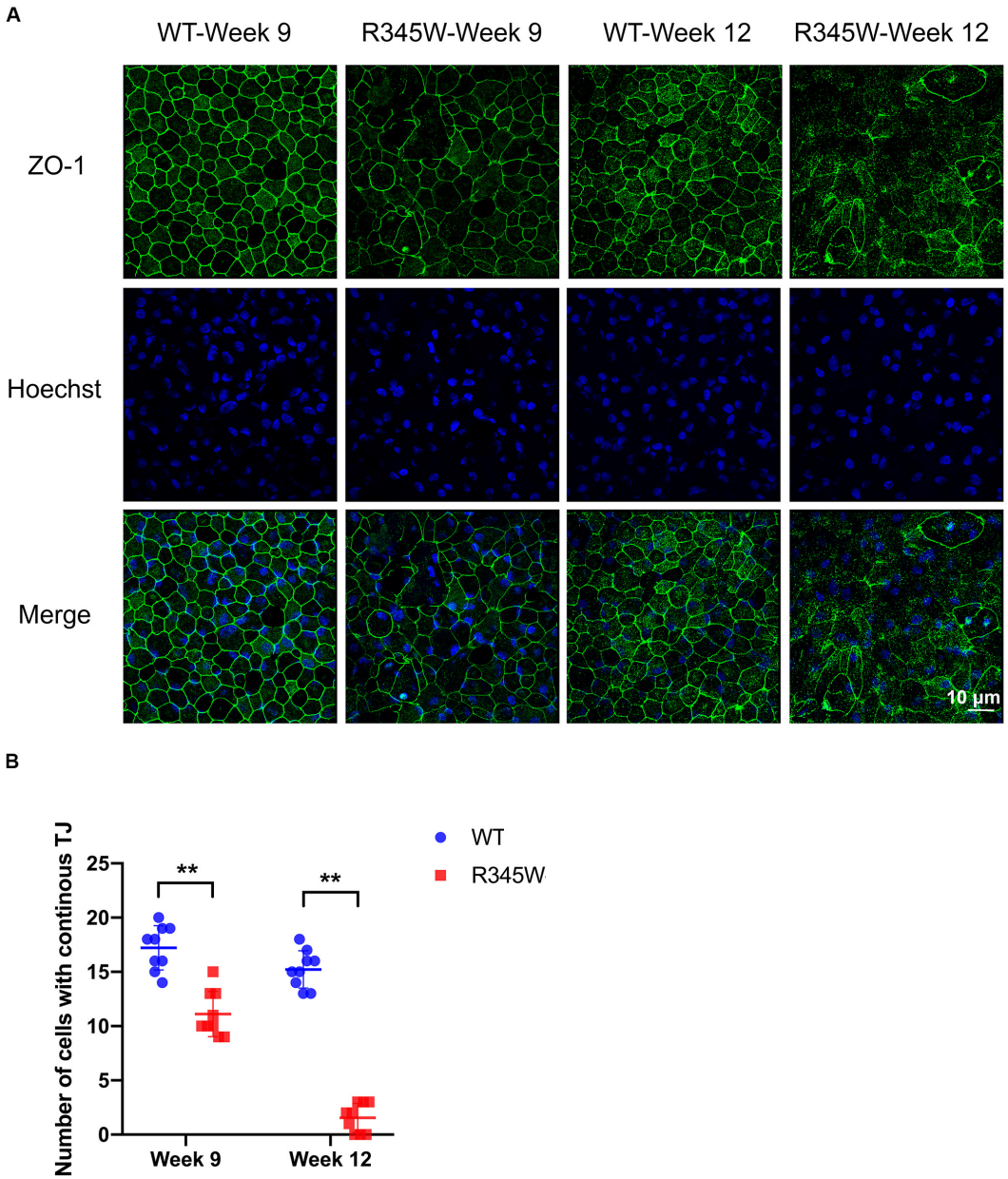

FIGURE 3 | ZO-1 distribution is disrupted in primary RPE cells expressing R345W-Fibulin-3. The expression and distribution of ZO-1 tight junction protein was assessed in primary RPE cells 9 and 12 weeks after infection with WT- or R345W-Fibulin-3 **(A)** Maximum projection confocal images of ZO-1 ICC. Green: ZO-1; Blue: Hoechst-stained nuclei. Confocal imaging reveals that ZO-1 distribution was dramatically disrupted in RPE cells expressing R345W-Fibulin-3 at 9 and 12 weeks post-infection. **(B)** Cells with continuous ZO-1 distribution were counted in nine different areas of 625 μm^2. The number of cells with continuous tight junctions was significantly lower in RPE cells expressing R345W-Fibulin-3 at 9 and 12 weeks post-infection compared to WT infected controls (n = 9, values are mean ± SEM, $^{**}p < 0.01$).

factors, we quantified apical and basal VEGF secretion using the primary RPE cell culture system described above. Cell culture medium was collected from the apical and basal compartments of each well. VEGF165 was quantified using a validated ELISA. The apical VEGF concentration did not differ between groups (**Figure 5A**). Compared to WT-Fibulin-3, basal VEGF secretion was significantly lower in the R345W-Fibulin-3 group at both 9 and 12 weeks post-infection (n = 8, $p < 0.01$; **Figure 5B**). These results suggest that expression of mutant Fibulin-3 alters the polarized secretion of VEGF.

Expression of R345W-Fibulin-3 Alters Its Polarized Secretion in Primary RPE Cells

To monitor the secretion of WT-Fibulin-3 and R345W-Fibulin-3, cell culture media were collected once a week for 12 weeks

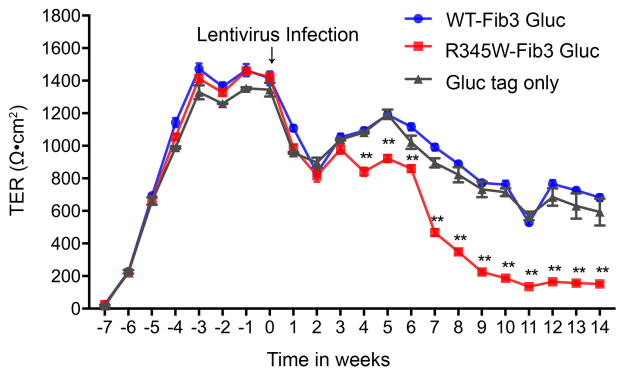

FIGURE 4 | Expression of R345W-Fibulin-3 decreases TER in primary RPE cells. Cell permeability was assessed by monitoring TER in RPE cells once a week for 21 weeks. At the beginning of the 4th week (week-3 on *x*-axis), RPE cells reached maturity with TER values maximizing between 1,300 and 1,500 Ω cm^2. In the beginning of the 7th week (week 0 on *x*-axis), RPE cells were infected with lentivirus with either luciferase-tagged WT-Fibulin-3 (blue), luciferase-tagged R345W-Fibulin-3 (red), or luciferase-tag only (gray) for five consecutive days. At 3 weeks post-infection, TER decreased to approximately 800 Ω cm^2 in all groups. At 4 weeks post-infection, TER continuously dropped in the mutant group (n = 8) and reached values significantly lower than the WT (n = 8) and tag-only groups (n = 2). (Values are mean ± SEM of individual wells, $^{**}p < 0.01$).

following infection. The results revealed that luciferase activity in basal cell culture media was significantly higher than that of apical cell culture media from RPE cells expressing both WT and mutant forms of Fibulin-3 (n = 8, $p < 0.01$; **Figure 6A**). Luciferase activity in the cell culture media of primary RPE cells expressing mutant Fibulin-3 was significantly lower than that of cells expressing the WT form on both apical and basal sides. Moreover, we found that R345W-Fibulin-3 secretion was severely impaired in the basal direction and moderately impaired apically. The decreased basal:apical secretion ratio of R345W-Fibulin-3 suggests that R345W-Fibulin-3 expression impairs polarized Fibulin-3 secretion in RPE cells (n = 8, $p < 0.01$; **Figures 6A,B**).

At 14 weeks post-infection, cell lysates and cell culture media were harvested, and luciferase activity was quantified via a luciferase assay. Our results showed no significant differences in primary RPE cell lysate luciferase activities between WT and mutant groups, which is not consistent with previous studies in RPE-J cells or ARPE-19 cells that reported greater amounts of mutant Fibulin-3 within cells relative to the WT protein (Marmorstein et al., 2002; Roybal et al., 2005). One possible explanation for the differing results is the differences between these cell culture systems. Consistent with previous studies (Hulleman et al., 2011), luciferase activity in the cell culture media of RPE cells expressing mutant Fibulin-3 was 73.6 and 88.8% lower on the apical and basal sides, respectively, than that of cells expressing the WT form after 14 weeks of infection (n = 8, $p < 0.01$; **Figure 6C**). The reduction in total luciferase activity including both cell lysates and cell culture media in the mutant group may due to the increased degradation of mutant Fibulin-3 and/or RPE dysfunction at later time points.

Expression of R345W-Fibulin-3 Leads to Downregulated Expression of RPE Signature Genes and Upregulated Expression of EMT Markers

To determine the differentiation state of the RPE cells in culture, we evaluated the expression levels of validated RPE cell signature genes. These genes were defined as mean expression values upregulated 10-fold or greater in native adult RPE cells relative to other cell types (Liao et al., 2010; Strunnikova et al., 2010). BEST1 (bestrophin-1), CRALBP (retinaldehyde-binding protein), RPE65 (retinal pigment epithelium-specific 65 kDa protein), and TRPM1 (transient receptor potential cation channel) are four RPE cell signature genes with functions in membrane transport, the visual cycle, and pigmentation

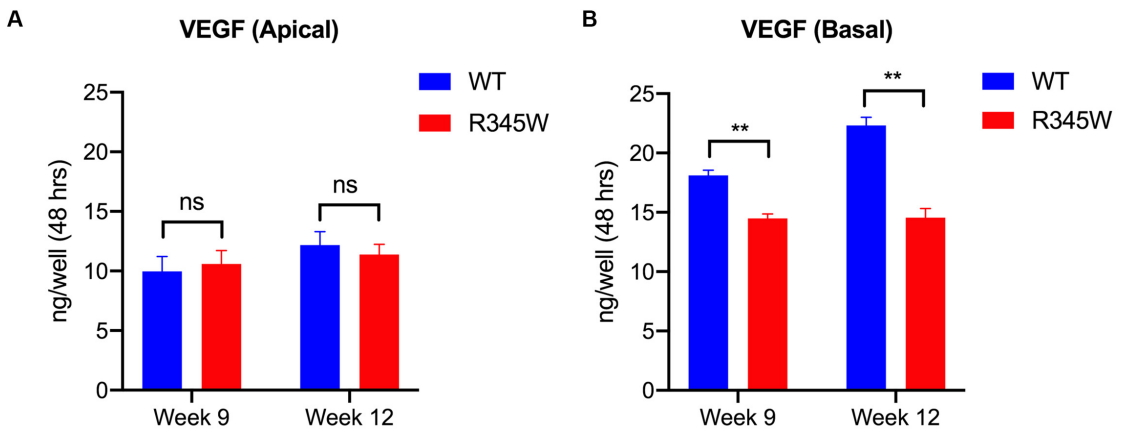

FIGURE 5 | VEGF secretion is impaired in primary RPE cells expressing R345W-Fibulin-3. VEGF secretion levels were quantified by ELISA. Cell culture media were collected from the apical and basolateral sides of primary RPE cells on Transwell filters at 9 and 12 weeks post-infection. **(A)** Apical VEGF secretion was not significantly different in the R345W-Fibulin-3 cells compared to WT, **(B)** Basal VEGF secretion was significantly lower in the mutant group compared to the WT group at 9 and 12 weeks post-infection. Values are the mean ± SEM of individual wells (n = 8, $^{**}p < 0.01$).

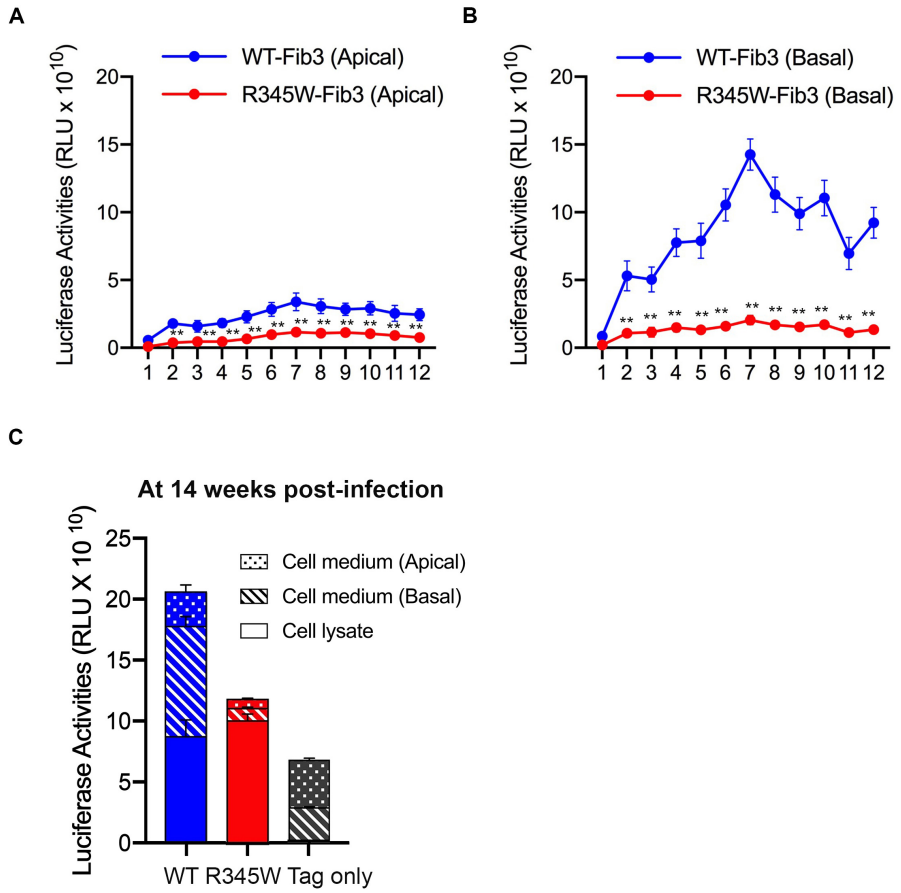

FIGURE 6 | Secretion of R345W-Fibulin-3 is impaired in both apical and basolateral directions. Fibulin-3 secretion levels were monitored by luciferase assay from media collected from the apical and basolateral sides of primary RPE cells. **(A,B)** Fibulin-3 was preferentially secreted to the basal side in both WT and mutant cells. R345W-Fibulin-3 secretion levels in mutant cells were significantly lower than those of WT cells over time ($n = 8$, $^{**}p < 0.01$). **(C)** After 14 weeks of infection, primary RPE cells were harvested and cell media were collected. No significant differences in luciferase activities between the WT and mutant groups were observed. Luciferase activities were decreased in RPE cells expressing R345W-Fibulin-3 in both the apical and basolateral directions.

pathways. We investigated whether R345W-Fibulin-3 expression influences RPE signature gene expression using the same cell culture system as above. After 14 weeks of infection, primary RPE cells were harvested for total RNA isolation. RT-PCR was conducted to examine signature gene expression in each group. BEST1, CRALBP, RPE65, and TRPM1 expression levels were normalized to GAPDH expression. RT-PCR results showed that BEST1, CRALBP, RPE65, and TRPM1 expression levels were significantly lower in the GLuc-tagged R345W-Fibulin3 group than in the GLuc-tagged WT-Fibulin-3 group ($n = 3$, $p < 0.01$), indicating that the expression of R345W-Fibulin-3 attenuates RPE cell differentiation (**Figure 7A**).

It is thought that cell dedifferentiation is usually followed by trans-differentiation, namely, EMT, due to stress-induced cellular reprogramming which includes increased cell migration (Pallet, 2012; Johno and Kitamura, 2013; Lamouille et al., 2014). Using RT-PCR, we compared the expression levels of TGFB2 (transforming growth factor beta-2), ZEB1 (Zinc Finger E-Box Binding Homeobox 1), VIM (Vimentin), and CDH2 (Cadherin 2) across groups using the primary RPE cell culture system described above. Compared to the WT group, the expression levels of all four EMT markers were significantly higher in the mutant group ($n = 3$, $p < 0.01$). Notably, the magnitude of EMT induction was similar to prior work (Wang et al., 2010; Adijanto et al., 2012), suggesting that expression of R345W-Fibulin-3 facilitates EMT in RPE cells (**Figure 7B**).

Expression of R345W-Fibulin-3 Leads to Enhanced Migration Ability in RPE Cells

To evaluate whether the expression of R345W-Fibulin-3 leads to migratory changes in RPE cells, scratch assays were conducted in ARPE-19 cells overexpressing either a dox-inducible GLuc-tagged WT-Fibulin-3 or R345W-Fibulin-3. We examined WT-Fibulin-3 and R345W-Fibulin-3 expression in this Tet-On ARPE-19 cell system by western blot. We found that the non-induced mutant group displayed a small but detectable amount of leaky expression of R345W-Fibulin-3 (**Supplementary Figure 1**). In the cell culture media, the amount of "leaky" expression in the absence of dox is 7% (GLuc-tagged WT) and 34% (GLuc-tagged

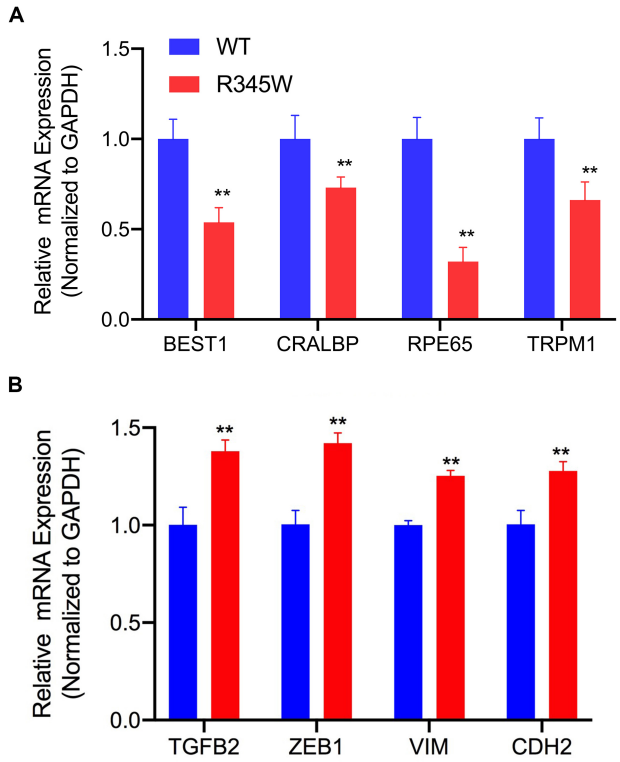

FIGURE 7 | RPE signature genes are downregulated and EMT markers are upregulated in primary RPE cells expressing R345W-Fibulin-3. **(A)** At 14 weeks post-infection, primary RPE cells were harvested and total RNA was isolated. RT-PCR was performed to quantify four RPE signature genes (BEST1, CRALBP, RPE65, and TRPM1). All expression levels are normalized to GAPDH expression. Relative to the WT group, mRNA for BEST1, CRALBP, RPE65, and TRPM1 were significantly reduced in RPE cells expressing R345W-Fibulin-3 ($n = 3$, $^{**}p < 0.01$). **(B)** At 14 weeks post-infection, primary RPE cells were harvested, and total RNA was isolated. RT-PCR was performed to quantify four EMT markers, (TGFB2, ZEB1, VIM, and CDH2). All expression levels were normalized to GAPDH expression. The experimental results showed that, relative to the WT group, TGFB2, ZEB1, VIM, and CDH2 were significantly upregulated in RPE cells expressing R345W-Fibulin-3 ($n = 3$, $^{**}p < 0.01$).

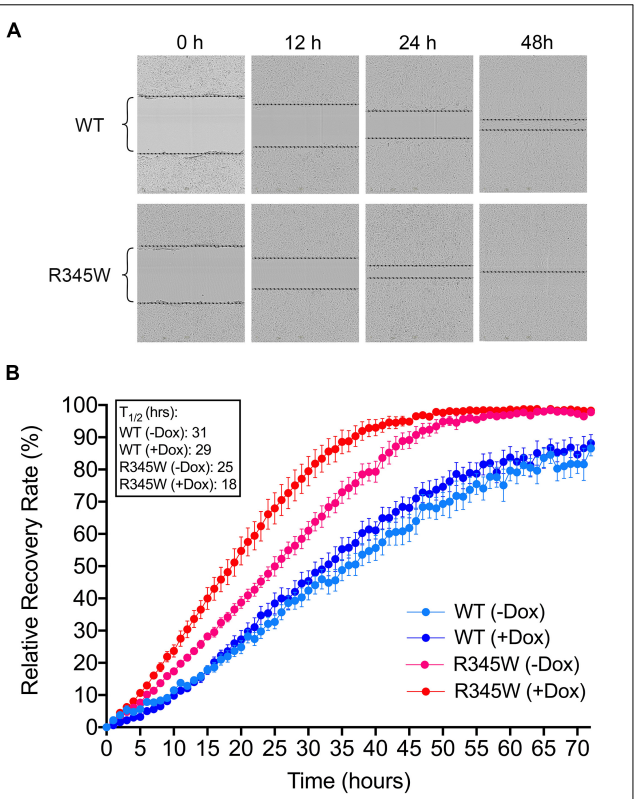

FIGURE 8 | Expressing R345W-Fibulin-3 increases cell migration. Scratch assays were conducted in ARPE-19 cells overexpressing either WT-Fibulin-3 or R345W-Fibulin-3. **(A,B)** The experimental results showed that, relative to the WT group, ARPE-19 cells overexpressing R345W-Fibulin-3 had a significantly faster scratch recovery rate in would healing assays. Brackets represent wound distances ($n = 8$, $p < 0.01$).

R345W) of the respective western blot signal with dox treatment for 48 h. In the cell lysate, the amount of "leaky" expression in the absence of dox is 3% (GLuc-tagged WT) and 3% (GLuc-tagged R345W) of the respective western blot signal with dox treatment for 48 hours, consistent with previous study (Hulleman et al., 2013).

Expression of R345W-Fibulin-3 enhanced migration ability in a dose-dependent manner. Scratches were made after 48 h of dox-induced expression of either WT-Fibulin-3 or R345W-Fibulin-3 in ARPE-19 cells. Relative wound confluence was calculated automatically by the IncuCyte^{TM} software system at one-hour intervals for 72 h (**Figure 8**). Scratch recovery rate was calculated as time to 50% wound closing ($T_{1/2}$). We found that dox-induced mutant group had the fastest time for closing 50% of the wound ($T_{1/2} = 18$ h), suggesting that the expression of R345W-Fibulin-3 increased the migration ability of RPE cells. Compared to the non-induced WT ($T_{1/2} = 31$ h) and dox-induced WT groups ($T_{1/2} = 29$ h), the non-induced mutant group had a faster scratch recovery rate ($T_{1/2} = 25$ h), and, relative to the dox-induced mutant group, it had a slower scratch recovery rate ($n = 8$, $p < 0.01$; **Figures 8A,B**).

DISCUSSION

The current study sheds light on the potential impact of misfolded protein accumulation due to the R345W mutation in fibulin-3 in RPE cells. In this study, we cultured primary RPE cells on Transwells and observed their morphology under TEM. As in studies presented by others, our primary RPE cells showed robust tight junctions and structural polarity under TEM (Maminishkis et al., 2006; Sonoda et al., 2009; Maminishkis and Miller, 2010). Unfolded protein response activation involves several mechanisms aimed at reducing the load of aberrant protein accumulation, including attenuated protein translation to avoid worsening the accumulation, increased transcription of ER chaperones, including GRP-78 and GRP-94 to aid in the folding process, and an increase in ER-associated degradation (ERAD). Three ER transmembrane sensors, IRE1, PERK, and

ATF6, regulate the UPR and determine the appropriate adaptive response, directing the cell to proliferate, change shape, or undergo apoptosis. Prior studies have also shown that mutant Fibulin-3 is misfolded and accumulated in the ER (Marmorstein et al., 2002; Roybal et al., 2005). Our study showed that expression of mutant Fibulin-3 induces increased expression of ER chaperones and IRE1/XBP1 pathway. We found that, in primary RPE cells expressing R345W-Fibulin-3, barrier function was impaired, as evidenced by disrupted ZO-1 distribution and decreased TER. This is in line with a prior study which showed that RPE cells retained a differentiated phenotype if they maintained cell-cell contacts, whereas EMT was initiated when RPE cells lost tight junctions, suggesting that cell-cell contact plays an important role in the RPE transition from a well-differentiated phenotype to a fibroblast phenotype. This phenotypic switch may contribute to the development of fibrotic complications (Tamiya et al., 2010).

We next examined the effects of R345W-Fibulin-3 expression on the polarized secretion of proteins by RPE cells. In fully polarized RPE cells, VEGF is preferentially secreted to the basal side of the RPE monolayer for choroidal vasculogenesis, a function that may be impaired in AMD. For example, decreased thickness of the choroidal layer has been observed in AMD patients (Lee et al., 2013), while a previous study showed that RPE de-differentiation causes atrophy of the choriocapillaris (Ohlmann et al., 2016). Our data are consistent with these findings, whereby the levels of basally secreted VEGF were significantly lower in the mutant group, indicating that the polarized secretion of VEGF was disrupted in RPE cells expressing R345W-Fibulin-3.

To our knowledge, this is the first study to delineate the directionality of impaired R345W-Fibulin-3 secretion. We found that WT-Fibulin-3 is preferentially secreted in the basal direction, substantiating the hypothesis that Fibulin-3 plays a role in maintaining the RPE cell basement membrane (Giltay et al., 1999; Segade, 2010). We further found that R345W-Fibulin-3 secretion is severely impaired on the basal side and moderately impaired in the apical direction. These results suggest that expression of R345W-Fibulin-3 impairs polarized Fibulin-3 secretion in RPE cells. The reduction in total secretion of R345W Fibulin-3 may be due to accelerated degradation or to excessive intracellular accumulation.

We next showed that RPE cell signature genes were downregulated in primary RPE cells expressing R345W-Fibulin-3. Moreover, our data showed that four EMT markers, TGFB2, ZEB1, VIM, and CDH2, were upregulated and migration ability was enhanced in the mutant group, suggesting that the expression of R345W-Fibulin-3 not only attenuates RPE cell differentiation, but also facilitates EMT of RPE cells. This may explain the formation of sub-RPE deposits and increased thickness of Bruch's membrane in macular degeneration, as more extracellular matrix proteins are secreted by mesenchymal-like cells than epithelial cells. In addition, increased migration ability in mutant RPE cells may explain the presence of HRF in OCT images, which have been shown to display characteristics of RPE migration in published studies. **Figure 9** illustrates our working model for RPE cell dedifferentiation and EMT.

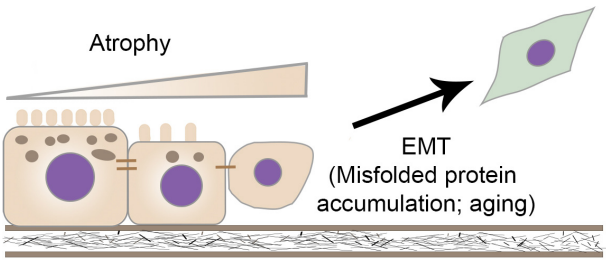

FIGURE 9 | Working model for RPE cells dysfunction and undergoing EMT. RPE cells exhibit a well-differentiated epithelial phenotype, characterized by integrated tight junctions, a high degree of polarity, and proper RPE signature gene expression. Expression of R345W-Fibulin-3 in RPE cells leads to a mesenchymal-like phenotype with cytoskeletal changes, a transcriptional shift, and an increased capacity for migration. Potential mechanisms contributing to EMT in RPE cells include aging and misfolded protein accumulation.

The current study shows that Fibulin-3 mutation leads to a phenotypic shift in RPE cells. However, the specific mechanisms by which mutant Fibulin-3 leads to EMT of RPE cells are unclear. Previous studies have shown that expression of mutant Fibulin-3 causes activation of the UPR in ARPE-19 cells (Roybal et al., 2005). Emerging evidence suggests that the accumulation of misfolded proteins drives EMT via activation of the UPR (Mo et al., 2015; Cuevas et al., 2017; Santamaria et al., 2019). Thus, UPR activation may constitute one of the underlying mechanisms by which RPE cells undergo EMT.

In summary, our experimental results have shown that native and WT-fibulin-3 overexpressing RPE cells exhibit a terminally differentiated epithelial phenotype with continuous, barrier-forming tight junctions, high polarization, and high expression of RPE signature genes. The expression of R345W-Fibulin-3 causes RPE cells to undergo EMT, as evidenced by upregulated EMT markers and an increased migration ability. The findings from this study will help us gain a better understanding of the role of misfolded proteins in RPE dysfunction.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MZ, YZ, and JS conceived and planned the experiments. MZ, YZ, and HC carried out the experiments. MZ, SW, YZ, AB, SG, CW, HW, JH, and JS contributed to sample preparation and interpretation of the results. MZ, SW, and JS took the lead in writing the manuscript. JS conceived the original idea and supervised the project. All authors

provided critical feedback and helped shape the research, analysis and manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00469/full#supplementary-material

FIGURE S1 | Western blot analysis of Fibulin-3 secretion and expression levels in ARPE-19 cells. Secretion and expression levels of WT-Fibulin-3 and R345W-Fibulin-3 were quantified in the cell culture media and cell lysate, respectively, from the Tet-On ARPE-19 cells by western blot. The results showed that, relative to R345W-Fibulin-3, more WT-Fibulin-3 was secreted into the cell culture media. Relative to WT-Fibulin-3, more R345W-Fibulin-3 was retained within the cells. Without dox induction, GLuc-tagged WT-Fibulin-3 and R345W-Fibulin-3 were still weakly detected in both cell culture media and cell lysates, suggesting slightly leaky Fibulin-3 expression in this Tet-On ARPE-19 cell system.

TABLE S1 | PCR primers used in this paper.

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REVIEW
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Role of Epithelial-Mesenchymal Transition in Retinal Pigment Epithelium Dysfunction

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Retinal pigment epithelial (RPE) cells maintain the health and functional integrity of both photoreceptors and the choroidal vasculature. Loss of RPE differentiation has long been known to play a critical role in numerous retinal diseases, including inherited rod-cone degenerations, inherited macular degeneration, age-related macular degeneration, and proliferative vitreoretinopathy. Recent studies in post-mortem eyes have found upregulation of critical epithelial-mesenchymal transition (EMT) drivers such as TGF-β, Wnt, and Hippo. As RPE cells become less differentiated, they begin to exhibit the defining characteristics of mesenchymal cells, namely, the capacity to migrate and proliferate. A number of preclinical studies, including animal and cell culture experiments, also have shown that RPE cells undergo EMT. Taken together, these data suggest that RPE cells retain the reprogramming capacity to move along a continuum between polarized epithelial cells and mesenchymal cells. We propose that movement along this continuum toward a mesenchymal phenotype be defined as *RPE Dysfunction.* Potential mechanisms include impaired tight junctions, accumulation of misfolded proteins and dysregulation of several key pathways and molecules, such as TGF-β pathway, Wnt pathway, nicotinamide, microRNA 204/211 and extracellular vesicles. This review synthesizes the evidence implicating EMT of RPE cells in post-mortem eyes, animal studies, primary RPE, iPSC-RPE and ARPE-19 cell lines.

Keywords: RPE, dedifferentiation, RPE dysfunction, EMT, UPR, retinal degeneration

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INTRODUCTION

The Epithelium to Mesenchyme Continuum

Epithelial cells and mesenchymal cells exhibit different characteristics and functions in the human body. Major hallmarks of terminally differentiated epithelial cells are expression of junctional complexes and apical-basal polarization. Epithelial cells reside on a basal membrane that separates them from other tissue components. In contrast to epithelial cells, mesenchymal cells are non-polarized cells with invasive and migratory behavior. Throughout embryogenesis, the capacity of cells to alternate between epithelial and mesenchymal states is vital for the development of the human body (Acloque et al., 2009; Kalluri and Weinberg, 2009). These processes are known as epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET). In healthy tissues, fully differentiated epithelial cells typically exert specific functions after

development and are thought not to oscillate between two states. However, EMT can be activated under pathological circumstances, such as inflammation, wound healing, and carcinogenesis, enabling epithelial cells to obtain an enhanced migration ability and increase their production of extracellular matrix components.

Historically, EMT is classified into three subtypes: type I EMT occurs in the early stages of embryogenesis; type II EMT is associated with tissue regeneration and organ fibrosis; type III occurs in cancer cells and enables invasion and metastasis (Kalluri, 2009). Loss of epithelial markers, including zona-occludens-1 (ZO-1), E-cadherin, and cytokeratin, and gain of expression of mesenchymal drivers, including vimentin, N-cadherin, and fibronectin, are encompassed in the classical definition of EMT. However, this definition remains heavily debated and is thought to be oversimplified. Indeed, gain of invasive and migratory abilities is not necessarily accompanied by the complete loss of epithelial traits. Moreover, Huang et al. (2012, 2013) showed that ovarian cancer cells are heterogeneous, as some cells lose E-cadherin but do not gain N-cadherin. As such, it has been proposed that EMT and MET exist on a continuum, and an intermediate phenotype exists (Nieto et al., 2016). Across this continuum, factors that drive the transition from epithelial to mesenchymal remain to be determined. Herein, the transition from fully differentiated epithelial cells toward mesenchymal cells (including the intermediate states) is defined as RPE dysfunction. Potential mechanisms involved in RPE dysfunction may include aging, loss of tight junctions, accumulation of misfolded protein, and inflammation (**Figure 1**).

Retinal Pigment Epithelial (RPE) Cells

The RPE form a single layer of highly polarized cells juxtaposed between the photoreceptors and choroid. There are approximately 3.5×10^6 RPE cells in each adult human eye. Classically, RPE cells are thought to be terminally differentiated throughout life (Panda-Jonas et al., 1996). Several signaling pathways have been reported to be involved in RPE differentiation, including Sonic hedgehog (Shh), Wnt/β-catenin, and Notch (Perron et al., 2003; Burke, 2008; Schouwey et al., 2011; Amirpour et al., 2012). MicroRNAs (miRNAs) also play a role; previous studies have shown that miRNA204/211 are critical for maintaining RPE differentiation (Wang et al., 2010; Adijanto et al., 2012). RPE cells display morphological polarity, with apically located microvilli, tight junctions, and melanosomes, and basally located nuclei and basal infoldings. Melanin pigment granules in RPE cells absorb light, contributing to visual function and protecting against photo-oxidative stress (Sundelin et al., 2001). Basally, RPE cells attach to Bruch's membrane, which consists of a mixture of collagen type IV, laminin, and fibronectin that is similar to other basement membranes and functions to separate the RPE from the choriocapillaris (Libby et al., 2000).

The structure of RPE cells is optimized for their many functions. Proper RPE function requires specific polarized distribution of transmembrane proteins. For example, Na^+/K^+-ATPases (Wimmers et al., 2007), chloride intracellular channel 4 (CLIC4) (Wimmers et al., 2007), mannose receptors (Tarnowski et al., 1988), and proton-coupled monocarboxyate transporters 1 (MCT1) (Deora et al., 2005) are restricted to the apical aspect of RPE cells. CD36 that functions in phagocytosis is also apically located (Ryeom et al., 1996). In contrast, integrins, MCT3 (Yoon et al., 1997), and Bestrophin-1 (Milenkovic et al., 2011), a chloride anion channel, are located basally. In addition to the polarized distribution of membrane proteins, RPE cells secrete proteins in a polarized manner. Vascular endothelial growth factor (VEGF) is primarily secreted in the basal direction to promote the growth of the choroidal vasculature, whereas pigment epithelium-derived factor (PEDF), an angiogenic inhibitor, is apically secreted (Bhutto et al., 2006). Some proteins lack a polarized distribution, including ezrin (Bonilha et al., 1999) and Glucose transporter (GLUT) 1 (Senanayake et al., 2006), which exist on both apical and basal aspects of the cell (**Figure 2**).

CLINICAL AND PRECLINICAL EVIDENCE OF EMT IN RPE CELLS

Overview

Together, photoreceptors, retinal pigment epithelial cells (RPE), and the choroid form a functional unit that is required for proper visual function. RPE cells are the central component of this unit, as they maintain the health and functional integrity of both photoreceptors and the choroid. RPE dysfunction is often an initiating or early factor in retinal disease, manifesting as a loss of RPE barrier function, disrupted RPE polarization, and downregulated microRNA-204/211 expression. Emerging evidence shows that RPE cells become less differentiated and subsequently undergo EMT with upregulated mesenchymal cell markers and enhanced migration ability in several degenerative retinal diseases (Tamiya and Kaplan, 2016; Ghosh et al., 2018; Wu et al., 2018; **Figure 3**). Clinical evidence suggesting that RPE cells undergo EMT in macular degenerations and proliferative vitreoretinopathy (PVR), as well as potential underlying mechanisms, are discussed in the following sections.

Hyperreflective Foci

Hyperreflective foci (HRF) are well-circumscribed lesions with equal or greater reflectivity than the RPE band that spread over various retinal and choroidal layers in spectral domain optical coherence tomography (SD-OCT) images (Liu S. et al., 2019; Roy et al., 2019). Numerous clinical studies have shown that retinal HRF appear in optical coherence tomography OCT images in retinal diseases, including age-related macular degeneration (AMD), inherited rod-cone degenerations, and inherited macular degenerations (Kuroda et al., 2014; Piri et al., 2015; Miura et al., 2017). HRF in choroid have been reported in Stargardts disease (Piri et al., 2015) and diabetic macular edema (DME) (Roy et al., 2019). The cellular origins of HRF were initially hypothesized to be migrated RPE cells, macrophages, or hard exudates. Emerging evidence confirms that a portion of HRF are RPE cells (Chen et al., 2016; Miura et al., 2017). A previous study investigated the origin of HRF by combining polarimetry with auto-fluorescence imaging, enabling differentiation between RPE cells, inflammatory cells, and hard exudates. The results

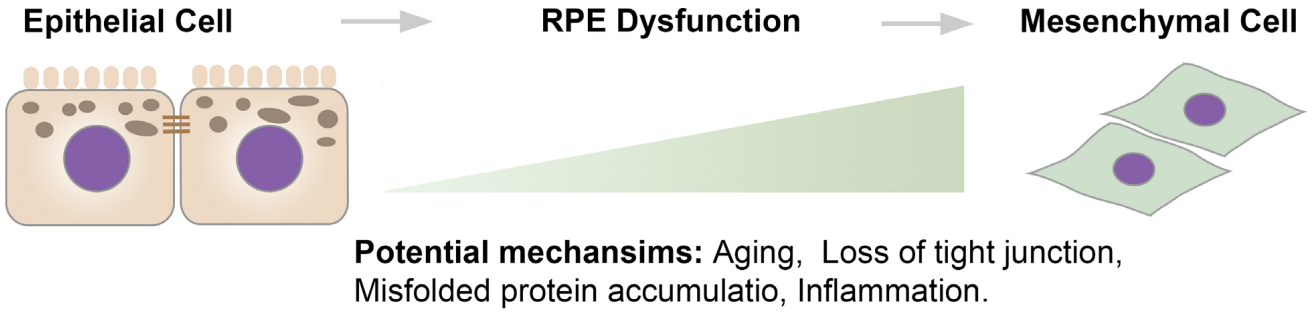

FIGURE 1 | The epithelium to mesenchyme continuum. There is a continuum between epithelial cells and mesenchymal cells. Across the continuum, the transition starts from well-differentiated RPE cells that exhibit barrier integrity and cellular polarity to intermediate stages, where RPE cells become dedifferentiated and lose functions, to mesenchymal-like cells that exhibit an increased proliferative rate and migrate into the neuroretina.

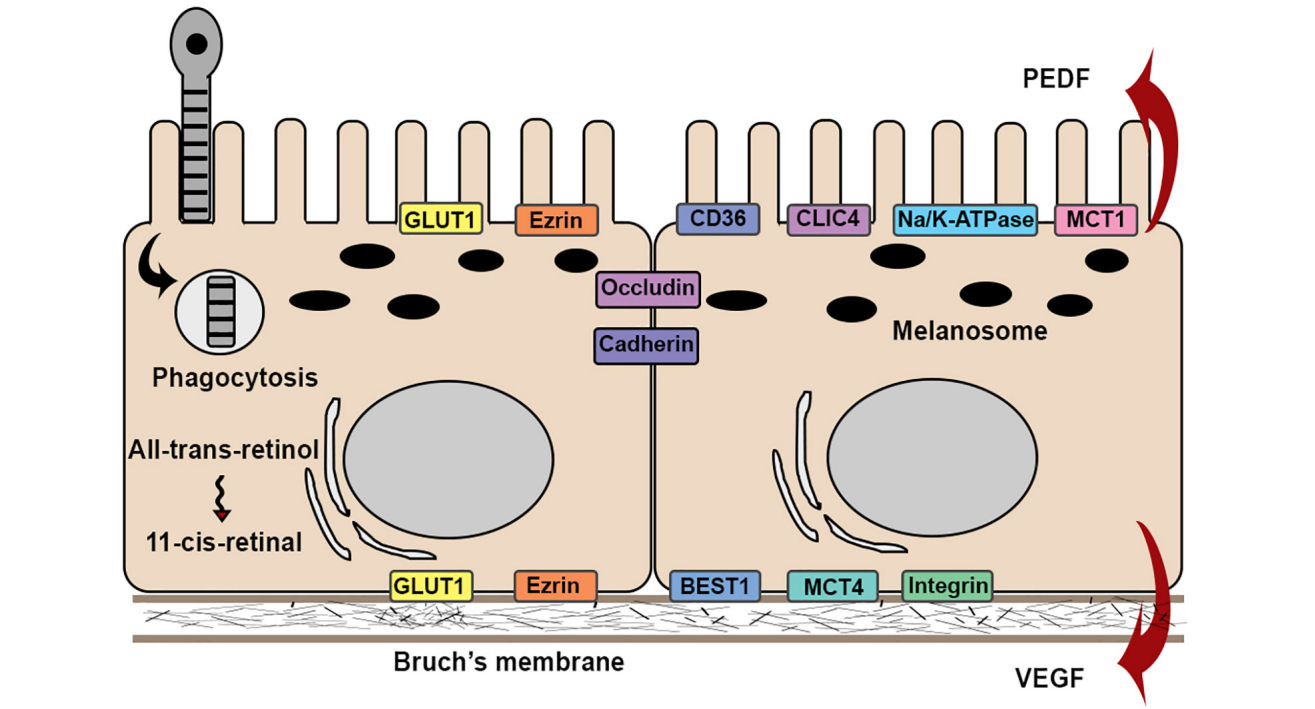

FIGURE 2 | Schematic illustration of the structures and functions in RPE cell. RPE cells attach to Bruch's membrane in a single monolayer with apical microvilli and melanosomes, and basal nuclei. Barrier function is maintained by expression of occludin and cadherins. RPE cells exhibit physiological functions, such as phagocytosis and visual cycles. RPE cells exhibit functional polarity by polarized secretion of proteins and apical-basal distribution of membrane proteins.

revealed that, in early stages of AMD, a portion of HRF are likely secondary to RPE migration (Miura et al., 2017). Moreover, histopathological studies confirmed that HRF represent cells of RPE origin, substantiating the idea that RPE cells have the capacity to migrate into neuroretina (Chen et al., 2016).

In numerous retinal degenerative diseases, HRF were found to correlate with disease stage. In AMD, HRF were found to correlate with pigmentary changes on fundus imaging and were seen with increased frequency in advanced forms of AMD, including geographic atrophy and choroidal neovascularization (CNV) (Christenbury et al., 2013). In the latter case, anti-VEGF treatment has been shown to significantly decrease the amount of HRF (Abri Aghdam et al., 2015). In addition to AMD, HRF appear to hold clinical significance in inherited retinal degenerations. For example, in Stargardts disease, an inherited macular degeneration caused by a single mutation in the *ABCA4* gene, the appearance of HRF was found to correlate strongly with poor visual acuity, decreased central macular thickness, and increased disease duration (Piri et al., 2015). In retinitis pigmentosa, HRF were observed more often in the inner nuclear layer in early stages, whereas they were more frequently observed in the outer nuclear layer in later stages, indicating that HRF location correlates with disease stage (Kuroda et al., 2014). Thus, HRF may be viewed as a predictive marker for disease

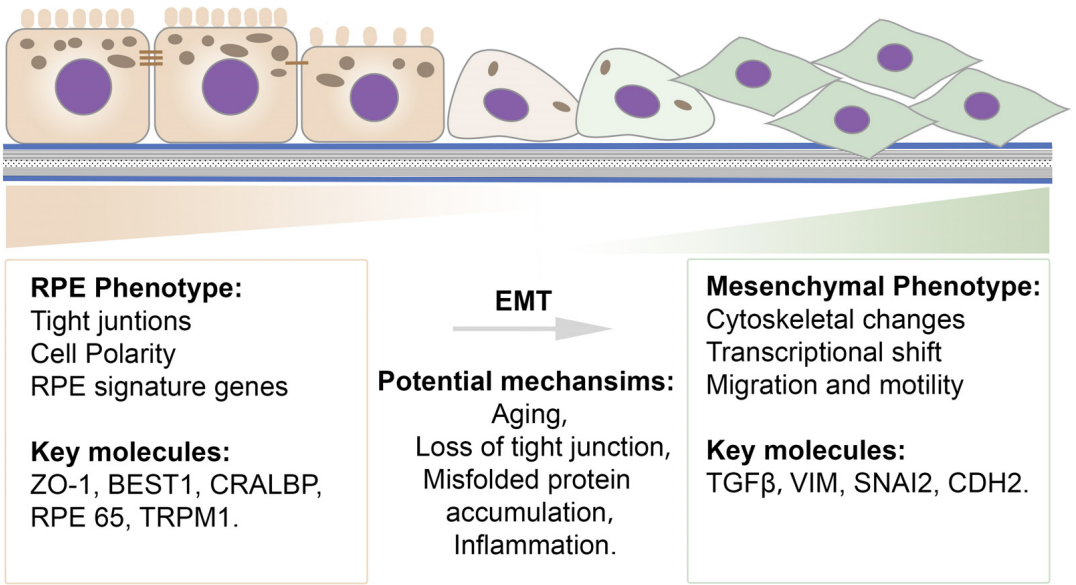

FIGURE 3 | Schematic illustration of the transition spectrum in RPE cells. Common epithelial and mesenchymal cell features and key molecules are listed. RPE cells exhibit a well-differentiated epithelial phenotype that is characterized by integrated tight junctions, high polarization, and proper RPE signature gene expression. A mesenchymal-like phenotype exhibits cytoskeletal changes, a transcriptional shift, and an increased ability for migration. Potential mechanisms contributing to EMT in RPE cells include aging, loss of tight junctions, misfolded protein accumulation and inflammation.

progression, and migratory RPE cells can be seen as a sign of poor prognosis.

Evidence of EMT in Age-Related Macular Degeneration (AMD)

Clinical Evidence

Age-related macular degeneration is a leading cause of irreversible vision loss, accounting for 13.4 million cases worldwide (Friedman et al., 2004). The primary pathology of AMD occurs at the level of the RPE cells. As RPE cells become atrophic, the hallmark clinical lesions of AMD ensue; sub-RPE lipoprotein deposits, known as drusen and drusenoid deposits, and RPE pigment disruption become visible within the macula. Most AMD begins after the age of 55 as the "dry" form of macular degeneration. In 10–15% of AMD cases, it progresses to the "wet" form of the disease, which is defined by CNV. Drusen deposits typically form between the RPE and Bruch's membrane, which can create a mechanical tension that negatively impacts cell-cell contacts. A prior study showed that the volume of drusenoid pigment epithelium detachment (PED) was inversely correlated with visual acuity (Balaratnasingam et al., 2016). An increase in drusenoid PED size promotes the disintegration of RPE layer and facilitates RPE migration (Balaratnasingam et al., 2016). Likewise, in wet AMD patients, increased area of serous PEDs is strongly associated with RPE layer disruption (Miura et al., 2019). In CNV, abnormal vessel growth and retinal hole formation directly disrupt cell-cell contact between RPE cells.

The Age-Related Eye Disease Study (AREDS) identified two clinical risk factors for disease progression, namely, drusen burden and the presence of pigment abnormalities (Ferris et al., 2005). Patients with either large drusen or macular pigment are at a 13 and 12.5% risk of developing advanced AMD, respectively. Interestingly, when the drusen burden is large and pigment is present, the risk of progression increases synergistically to 47.3%. However, the molecular mechanisms that drive RPE dysfunction and lead to pigment accumulation in macular degeneration remain to be determined.

As mentioned in the previous section, HRF are observed on OCT in AMD patients and correlate with abnormal pigment on fundus imaging, suggesting that HRF and pigmentary changes represent RPE cells that have migrated into the neuroretina (Folgar et al., 2012; Christenbury et al., 2013; **Figure 4**). This concept is substantiated by studies demonstrating EMT of RPE cells in AMD. Ghosh et al. (2018) demonstrated upregulated expression of the EMT transcriptional markers vimentin and Snail1 and downregulated expression of E-cadherin in RPE cells in post-mortem human dry AMD eyes relative to age-matched controls, suggesting that RPE cells in dry AMD patients had undergone EMT. Hirasawa et al. (2011) showed that Snail co-localized with RPE65-positive cells in 11 human CNV eyes. Additionally, this study found that Snail- and α-SMA-double-positive RPE cells were strongly associated with RPE fibrotic changes, indicating that RPE cells undergo epithelial-myofibroblast transition, and that this transition leads to retinal fibrosis, an end-stage manifestation of wet AMD (Ishikawa et al., 2016a; Roberts et al., 2016; **Figure 5A**).

Here, we hypothesize that pigment abnormalities represent RPE cells that have undergone EMT and migrated into the neuroretina. A contradiction to this hypothesis is that RPE cells should, in theory, become less pigmented with EMT, and, therefore, would not result in a pigmented macula. However, it

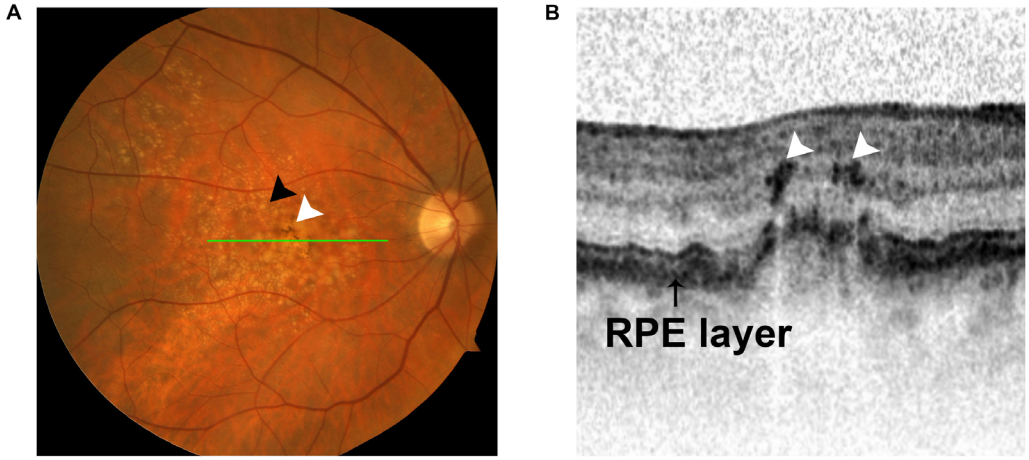

FIGURE 4 | Intraretinal hyperreflective foci (HRF) appear in OCT images in intermediate AMD. **(A)** Fundus photograph showing two hallmarks of intermediate AMD: sub-RPE deposits (black arrow) and pigment changes (white arrow). **(B)** OCT image demonstrating the presence of intraretinal HRF (white arrows) in intermediate AMD. The HRF correlate with pigment on funduscopic imaging (green line).

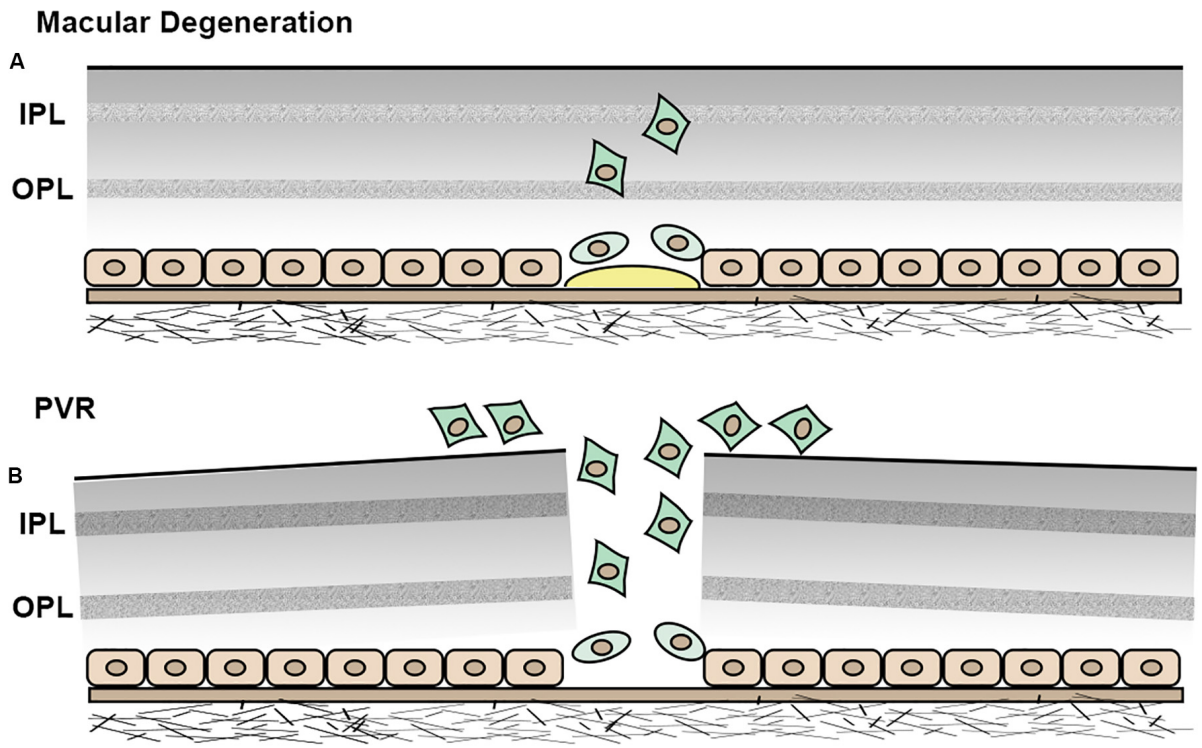

FIGURE 5 | Schematic illustration of EMT in RPE cells. **(A)** In macular degeneration, RPE cells become less differentiated, undergo EMT and migrate into neuroretina. **(B)** In PVR, RPE cells undergo EMT and migrate into the epiretinal area.

is possible that migrating RPE cells become less differentiated, but remain pigmented to, perhaps, a lesser degree. It is also possible that macular hyperpigmentation results from a cluster of RPE cells that have migrated to this area in order to make up for the reduction in pigmentation. Another possibility is that migrating RPE cells undergo MET in the neuroretina and become more pigmented. Further studies should focus on the molecular mechanisms that drive RPE dysfunction and lead to pigment accumulation in macular degeneration.

Preclinical Evidence

Published studies using animal models of AMD have shown that proteins, such as PCG-1-alpha, MRTF-A and PTEN, are involved in the EMT of RPE cells, as inhibiting their activity

attenuated the severity of disease progression (Kim et al., 2008; Kobayashi et al., 2019; Rosales et al., 2019). Interestingly, Ishikawa et al., 2016b found that αβ-crystallin, a protein linked to AMD pathophysiology, was found to modulate EMT through SNAIL and SLUG. They showed that suppressing αβ-crystallin results in the inhibition of EMT development in RPE cells (Ishikawa et al., 2016b).

Evidence of EMT in Proliferative Vitreoretinopathy (PVR)

Clinical Evidence

Proliferative vitreoretinopathy is a scarring process that occurs following the treatment of rhegmatogenous retinal detachment. The incidence of post-operative PVR is estimated to be 5–10% (Bonnet et al., 1996; Rodriguez de la Rua et al., 2003). PVR is initiated by a retinal break, which is followed by persistent inflammation and wound healing. RPE cells are believed to contribute to the healing process by undergoing EMT and migrating to the epiretinal area (Yamashita et al., 1986; Morino et al., 1990; Casaroli-Marano et al., 1999). The resultant formation and contraction of PVR membranes ultimately lead to retinal folds and loss of vision (**Figure 5B**).

Several studies have shown that cytokeratin, an RPE cell marker, co-localized with vimentin in PVR membranes (Yamashita et al., 1986; Morino et al., 1990; Casaroli-Marano et al., 1999). Additionally, Feist et al. (2014) showed that, in human PVR membranes, cells positive for cytokeratin were co-expressed with α-SMA. By counting the cells positive for both cytokeratin and α-SMA, the study showed that the majority of myofibroblasts in human PVR membranes originated from RPE cells, suggesting that RPE cells are capable of undergoing EMT and migrating into neuroretina, and that this process plays a major role in the pathogenesis of PVR.

Preclinical Evidence

A number of PVR animal studies have shown that RPE cells undergo EMT when PVR is induced (Saika et al., 2004; Nagasaki et al., 2016; Yoo et al., 2017; Zhang et al., 2017). Following retinal detachment, one study found that RPE cells would stain positive for alpha-SMA in wild-type mice, but RPE cells in Smad3-null mice would be negative, indicating that EMT is attenuated when Smad3 is absent (Saika et al., 2004). The same investigation also found that Smad3-null mice had decreased residual subretinal fibrosis. Additionally, PVR in mice induced by dispase injection has been shown to increase retinal alpha-SMA-positive cells (Yoo et al., 2017). Together, these *in vivo* studies suggest that RPE cells undergo EMT following PVR, whereby suppressing the EMT process can greatly reduce the severity of PVR.

Evidence of EMT in Inherited Retinal Degenerations (IRDs)

Clinical Evidence

A subtype of Best disease, autosomal dominant vitreoretinochoroidopathy (ADVIRC) is a chorioretinal degeneration caused by a mutation in the Bestrophin-1 (*BEST1*) gene, and evidence suggests that EMT of RPE cells plays a role in its pathogenesis. In a study of ADVIRC post-mortem human eyes, Goldberg et al. demonstrated minimal expression of TGF-β within the RPE cell monolayer (Goldberg et al., 2018). In contrast, RPE cells that had migrated into the neuroretina have been characterized as having downregulated RPE-65 and upregulated expression of TGF-β, suggesting that RPE cells become dedifferentiated once they start to migrate (Goldberg et al., 2018). In inherited retinitis pigmentosa, proliferative and displaced RPE cells have been observed in regions up to the internal limiting membrane in several patient samples (Szamier and Berson, 1977; Fox, 1981; Flannery et al., 1989; Li et al., 1995). One study showed that RPE cells in a spared region of retina had apically displaced nuclei (loss of polarization) and abundant melanolysosomes, whereas RPE cells in areas of more severe disease were flattened and depigmented (Szamier and Berson, 1977). In photic maculopathy, transmission electronic microscopy (TEM) indicated that RPE cells were displaced and proliferative with depigmentation, loss of infoldings, and irregular shape (Tso, 1973). Another study demonstrated RPE cell proliferation in retinal detachment and found that new cells did not display typical RPE cell polarity (Anderson et al., 1981). In choroideremia and chorioretinal atrophies, regions of proliferating and attenuated RPE cells were observed, with abrupt transitions in between them (Curcio et al., 2000; Jonasson et al., 2007; MacDonald et al., 2009).

MECHANISMS OF EMT IN RPE CELLS

Overview

The molecular mechanisms that drive RPE dysfunction and lead to retinal degeneration remain to be determined. Emerging evidence suggests that the impairment of tight junctions and accumulation of misfolded proteins drive EMT in RPE cells. Moreover, upregulation of TGF-β and Wnt pathway appears to to play a critical role in RPE de-differentiation and promoting EMT. In contrast, Nicotinamide and microRNA 204/211 have been shown to enhance the RPE phenotype and prevent EMT in multiple RPE cell model systems. Extracellular vesicles that have been shown to regulate EMT in numerous other tissues are also discussed in the following sections.

Roles of Junctional Proteins in EMT

Cell-cell contact is critical for maintaining an epithelial phenotype. The RPE acts as part of the outer blood-retina barrier by way of tight junctions (TJs) and adherens junctions (AJs) between neighboring RPE cells (Foulds et al., 1980). At a molecular level, the major proteins that constitute TJs in RPE cells are occludin and claudins, while cadherins serve as the major AJ proteins (Erickson et al., 2007; Tash et al., 2012). The extracellular domains of these molecules form an intact blood-retinal barrier that results in a high transepithelial resistance.

A prior study showed that RPE cells in the center of cultured sheets maintained a well-differentiated phenotype with proper expression of RPE signature genes and pigment, whereas RPE cells at the edges of the sheet lost epithelial morphology and were less pigmented (Tamiya et al., 2010). Moreover, this study showed upregulated expression of vimentin and N-cadherin in RPE cells at the edges, suggesting that loss of cell-cell contact leads to RPE cell de-differentiation and facilitates EMT of RPE cells. Georgiadis et al. (2010) performed *in vivo* studies in C57/Bl6 mice, utilizing shRNA to target and disrupt junctional proteins, like ZO-1, which induced RPE cell proliferation and hyperpigmentation of the retina (Georgiadis et al., 2010). The RPE cells were found to be undergoing EMT, whereby the absence of ZO-1 induced the expression of key EMT markers and reduced the expression of epithelial markers in the affected RPE cells (Georgiadis et al., 2010). These data show that the loss of the tight junction protein causes RPE cell dedifferentiation and induces EMT.

The mechanism by which TJs and AJs maintain an epithelial phenotype involves sequestration of EMT signals within their complexes. The junctional protein ZO-1 binds to ZO-1–associated Y-box factor (ZONAB), a transcription factor that is able to upregulate cell proliferation rate by regulating nuclear expression of cyclin-dependent kinase 4 (CDK4) (Ikenouchi et al., 2003; Gonzalez-Mariscal et al., 2014). Loss of ZO-1 results in a release of ZONAB into the cytoplasm, enabling a subsequent translocation of ZONAB (Erickson et al., 2007). ZONAB then binds to the transcriptional factor for CDK4, increasing expression of CDK4 and promoting cell proliferation. Similarly, E-cadherin sequesters β-catenin on the cell membrane, and downregulation of E-cadherin leads to a release of β-catenin into the cytoplasm. β-catenin subsequently translocates into the nucleus and activates promoters of EMT and proliferation, including Snail and Cyclin D1 (Gonzalez and Medici, 2014; **Figure 6A**). The Hippo-YAP pathway, an important regulator of RPE cell differentiation, depends strongly on cell junction complexes. TJ and AJ complexes inhibit YAP/TAZ translocation (two effectors of the Hippo pathway) into the nucleus. Loss of TJs or AJs enables activation of YAP/TAZ. Activated YAP/TAZ bind to TEAD and upregulate ZEB1, promoting EMT (Lei et al., 2008). A previous study showed that knockdown of YAP in primary mouse RPE cells downregulated MITF expression and upregulated ZEB1 expression, indicating that loss of YAP attenuates RPE differentiation and induces RPE cells to undergo EMT (Liu et al., 2010). Thus, RPE cell-cell junctions are critical regulators of EMT.

Roles of Misfolded Proteins in EMT

Emerging evidence suggests that the unfolded protein response (UPR) and EMT signaling interact in several different organs (Zhong et al., 2011; Lenna and Trojanowska, 2012; Pang et al., 2016; Santamaria et al., 2019; **Figure 6B**). Mutations in *BEST1* cause its mislocation and results in retinitis pigmentosa and other retinal dystrophies (Johnson et al., 2014, 2017). Milenkovic et al. (2018) showed that recessive mutations in *BEST1* activate the UPR with upregulated expression of XBP1. Morton et al. showed that in ADVIRC patient, a chorioretinal degeneration caused by a mutation in the *BEST1* gene, RPE cells that had migrated into the neuroretina have been characterized as having downregulated RPE-65 and upregulated expression of TGF-β (Goldberg et al., 2018). A previous study showed that accumulation of intracellular amyloid-β attenuates TJs of RPE cells by downregulating occludin and claudin-1 proteins, supporting the idea that accumulation of abnormal proteins in RPE cells attenuates RPE differentiation (Park et al., 2014). Crystallin is a protein expressed in the eye and is classified into three types: α, β, and γ crystallins. βA3/A1-crystallin localizes to the lysosome and plays a critical role in the clearance functions of lysosomes, including phagocytosis and autophagy (Zigler and Sinha, 2015). A prior study demonstrated that the expression level of βA3/A1-Crystallin in polarized primary human RPE cells is 23.75-fold higher than that of non-polarized primary human RPE cells (Ghosh et al., 2018). Moreover, knockdown of βA3/A1-Crystallin in human and murine RPE cells results in upregulated expression of Snail and vimentin and an enhanced migration ability (Ghosh et al., 2018). This evidence suggests a connection between the protein misfolding and EMT in RPE cells. Doyne honeycomb macular dystrophy (DHC) results from an R345W mutation in Fibulin-3 (Marmorstein, 2004; Narendran et al., 2005), an extracellular matrix protein and downstream target of HTRA1 (Lin et al., 2018). The R345W mutation causes Fibulin-3 misfolding, poor Fibulin-3 secretion, and activation of the UPR (Marmorstein et al., 2002; Hulleman and Kelly, 2015). In our lab, we found that overexpression of R345W-Fibulin-3 in primary human RPE cells activates the UPR via the IRE1α/XBP1 pathway, attenuates RPE differentiation and facilitates EMT in RPE cells (Zhou et al., 2020; accepted in Frontiers in Cell and Developmental Biology). Taken together, these data suggest that the accumulation of misfolded proteins in RPE cells contributes significantly to EMT and likely accelerates the disease process in patients who have inherited macular degenerations.

UPR activation involves several mechanisms aimed at reducing the load of aberrant protein accumulation, including attenuated protein translation to avoid worsening the accumulation, increased transcription of endoplasmic reticulum (ER) chaperones to aid in the folding process, and an increase in ER-associated degradation (ERAD). Three ER transmembrane sensors, IRE1α/XBP1, PERK, and ATF6, regulate the UPR and determine the appropriate adaptive response, directing the cell to proliferate, change shape, or undergo apoptosis (Lin et al., 2007; Walter and Ron, 2011). Several studies have shown that the UPR and TGF-β-induced EMT signaling pathways interact at the level of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (JNK/p38-MAPK) in an IRE1-dependent manner (Urano et al., 2000; Santibanez, 2006; Liu Z. et al., 2019). The activation of IRE1α/XBP1 signaling promotes EMT by upregulating JNK and EMT drivers, including Snail and Zeb family members, in different organs and tissues, including the breast, lung, and liver (Li et al., 2015; Mo et al., 2015; Cuevas et al., 2017; Liu Z. et al., 2019). Future studies should focus on the mechanisms by which ER stress regulates EMT in RPE cells.

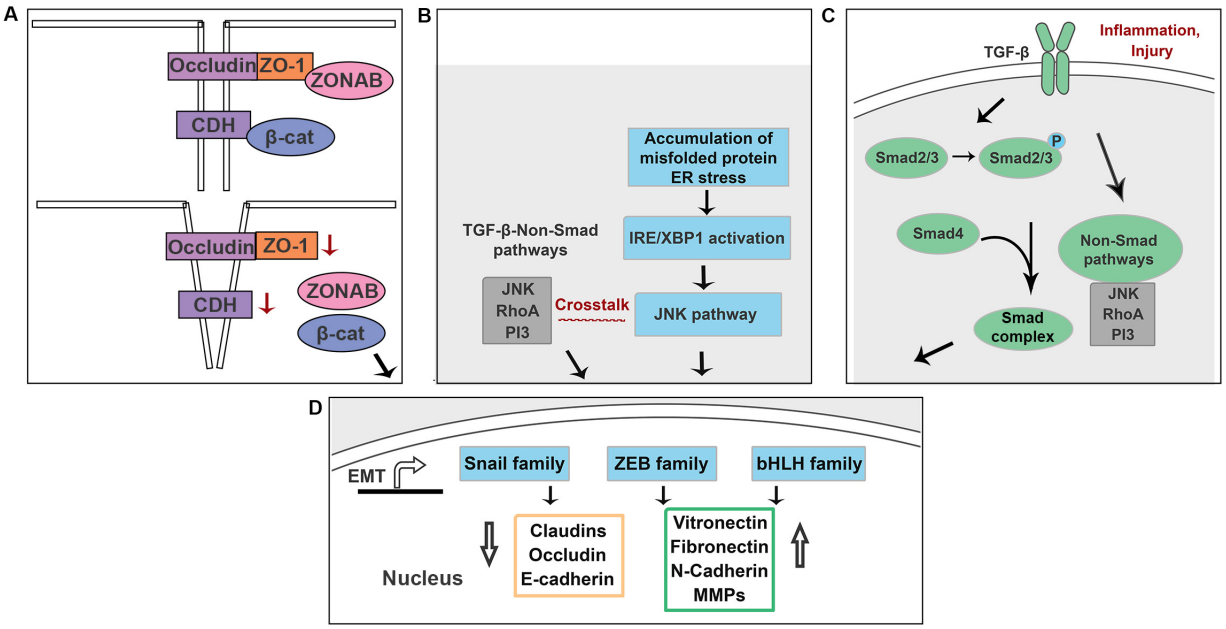

FIGURE 6 | Schematic illustration of the potential mechanisms underlying EMT in RPE cells. **(A)** Loss of ZO-1 and E-cadherin lead to a release of ZONAB and β-catenin. **(B)** Accumulation of misfolded proteins activate NK/p38-MAPK pathway that crosstalks with TGF-β-induced non-Smad pathways in an IRE-dependent manner. **(C)** Increased production of TGF-β is associated with inflammation and injury in RPE cells. **(D)** Above signals promote EMT by upregulating EMT drivers including Snail, Zeb and bHLH family.

Key Pathways and Molecules

TGF-β Pathway

TGF-β acts as an anti-inflammatory cytokine; increased production of TGF-β is associated with injury and inflammation. TGF-β signaling induces EMT by activating either Smad or non-Smad pathways (Xu et al., 2009). In the Smad-dependent pathway, phosphorylation of TGF-β receptor (Type I and Type II) recruits Smad2 and Smad3 (Valcourt et al., 2005). The phosphorylation of Smad2 and Smad3, then recruits Smad4 and facilitates the formation of the Smad-complex. The Smad-complex translocates into the nucleus and binds regulatory elements that in turn induce the transcription of several key genes associated with EMT (Gonzalez and Medici, 2014). In the Smad-independent pathway, TGF-β cross-talks with the JNK/p38-MAPK pathway to regulate EMT in an IRE1α-dependent manner (Engel et al., 1999; Zhou et al., 2004; **Figure 6C**).

Previous studies have shown that the amount of TGF-β in the vitreous from eyes with PVR is three times higher than that of eyes without intraocular fibrosis (Connor et al., 1989; Kita et al., 2008). Subconfluent culturing of primary human RPE cells, which mimics a wound stimulus, has been shown to result in acquisition of a mesenchymal phenotype by activating the TGF-β pathway (Radeke et al., 2015). Treatment with TGF-β and TNF-α has been found to accelerate EMT in adult human RPE stem cell – derived RPE cell cultures (Boles et al., 2020). Blockage of TGF-β and FGF/MAPK pathways has been shown to markedly promote RPE differentiation efficiency during induced pluripotent stem cell (hiPSC)-derived RPE cell culture (Kuroda et al., 2019).

Most of the *in vivo* investigations of EMT mechanisms in RPE cells involve genetic (Saika et al., 2004; Wu et al., 2019) or pharmacological (Ishikawa et al., 2015; Yoo et al., 2017; Zhang et al., 2017) manipulation of the TGF-β pathway. Ishikawa et al. (2015) used a PVR rabbit model to pharmacologically inhibit transforming growth factor-β2(TGF-β2)-induced EMT of RPE cells using Resveratrol, a compound that deacetylates SMAD4 (Ishikawa et al., 2015). To determine the effect of notch inhibition on TGF-β1-induced EMT, Zhang et al. (2017) injected ARPE-19 cells that had been pre-incubated with a γ-secretase Notch inhibitor (LY411575) in a PVR mouse model, and found EMT was attenuated when Notch signaling was inhibited (Zhang et al., 2017). In a PVR *in vivo* rat model, miR-194 decreased the ZEB1 protein. ZEB1 can synergize with SMAD and lead to TGF-β-dependent gene transcription; therefore, suppression of this process repressed EMT in RPE cells (Cui et al., 2019). The absence of Galactin-1, a galactoside-binding lectin family protein which modifies the TGF-β pathway as well as others, in a knockout mouse following CNV resulted in reduced CNV severity, level of subretinal fibrosis and expression of EMT-related markers in RPE cells (Wu et al., 2019). Taken together, these results show the importance of the different components of the TGF-β pathway in the RPE-EMT process (**Figure 6D**).

Wnt Pathway

Wnt-β-catenin is another well-characterized pathway that mediates EMT in the eye. A previous study showed that, in mouse eyes, laser photocoagulation activated the Wnt/β-catenin pathway and facilitated RPE proliferation and

EMT (Han et al., 2015). In ARPE-19 cells, EMT was promoted by the overexpression of β-Catenin and was blocked by a Wnt Inhibitor (XAV939) (Chen et al., 2012). Light exposure has also been shown to induce EMT in RPE cells by activating the Wnt/β-catenin pathway (Iriyama et al., 2008).

β-catenin is a key element in the Wnt signaling pathway. Without the activation of Wnt signaling, β-catenin is sequestered by a complex of glycogen synthase kinase-3 beta (GSK-3β) and Axin. Activation of Wnt signaling leads to a release of β-catenin from the complex, enabling the subsequent translocation of β-catenin. Nuclear β-catenin then binds to the transcriptional factors for Snails, leading to increased expression levels of Snail and promotion of EMT.

Nicotinamide

Nicotinamide (NAM), a vitamin B3 derivative, has both antioxidant and anti-inflammatory properties. NAM has been shown to enhance the RPE phenotype and prevent EMT in multiple RPE cell model systems (Saini et al., 2017; Meng et al., 2018; Hazim et al., 2019; Boles et al., 2020). ARPE-19 cell line is not typically thought to be well differentiated but does offer relative convenience and consistency. NAM has been shown to rapidly promote ARPE-19 cell differentiation (Hazim et al., 2019). In human adult RPESC-RPE, NAM prevents and reverses RPE EMT that is induced by TGF-β and TNF-α treatment (Boles et al., 2020). In a human iPSC model of AMD, NAM ameliorates disease phenotype by inhibiting drusen proteins and inflammatory and complement factors (Saini et al., 2017). Potential mechanisms by which NAM promoted RPE cell survival and differentiation include inhibiting Rho-associated protein kinase (ROCK) and casein kinase 1 (CK1) (Meng et al., 2018).

microRNA-204/211

miRNA-204/211 plays a critical role in RPE cell differentiation. Prior studies demonstrated that the TGF-β receptor is a direct target of miRNA-204/211 in RPE cells. Transient receptor potential cation channel (TRPM)1 and TRPM3 are two signature genes of RPE cells. miRNA-204 resides in the sixth intron of TRPM3, and miR-211 resides in the sixth intron of TRPM1. miRNA-204/211 and TRPM3/1 co-translate in RPE cells (Adijanto et al., 2012). miRNA-204 and miRNA-211 are highly expressed in fully differentiated RPE cells, allowing maintenance of RPE terminal differentiation (Wang et al., 2010). In contrast, both TRPM1/3 and miRNA-211/204 are downregulated in dedifferentiated RPE cells (Wang et al., 2010). Further, anti-miRNA-204/211 leads to elevations in the levels of several EMT transcriptional factors, including TGFBR2, JNK, SNAIL1, SNAIL2, Smad3, and Smad4. Together, these data demonstrate the importance of miRNA-204/211 in preventing EMT in RPE cells.

Extracellular Vesicles

Extracellular vesicles (EVs) play a critical role in cell-cell communication, modulate cellular differentiation, and promote aggregate formation (Yuyama et al., 2008; Alvarez-Erviti et al., 2011). EVs can originate from either multivesicular bodies (MVBs) or from the plasma membrane. They have recently been shown to be a major constituent of the vitreous body in the eye (Zhao et al., 2018). Prior studies have shown that alterations in EV cargo are representative of the phenotypic status of their parental cells (Vella, 2014; Kim et al., 2016). EVs contribute to the regulation of EMT and promote cancer metastasis in numerous tissues, including the lungs, breasts, liver and brain (Vella, 2014; Kim et al., 2016; Chen et al., 2017; van de Vlekkert et al., 2019). Chen et al. (2017) showed that EVs derived from p85α$^{-/-}$ fibroblasts that possess greater mesenchymal features promoted breast cancer cells migration and invasion compared with EVs from WT fibroblasts (Chen et al., 2017). van de Vlekkert et al. (2019) showed that myofibroblast-derived EVs are sufficient to induce normal fibroblasts to become myofibroblasts that possess greater mesenchymal features, by upregulating TGF-β pathways and EMT drivers. Although the specific role of EVs in mediating RPE cell EMT remains to be determined, they appear to be involved in numerous mechanisms relating to EMT in RPE cells.

THERAPEUTIC IMPLICATIONS

Many preclinical studies using promising therapeutic interventions have been shown to effectively rescue RPE cells from EMT, including TGF- β receptor inhibitors, peroxisome proliferator-activated receptor (PPAR)-γ agonists, retinoic acid receptor-γ (RAR-γ), and anti-inflammation agents. The TGF-β receptor I inhibitor LY-364947 has been shown to reduce RPE transdifferentiation *in vitro* and prevent PVR development in a rabbit model (Nassar et al., 2014). By injecting a negative TGF-β receptor II, the severity of PVR was significantly attenuated in the rabbit eye (Yamada et al., 2007). PPAR-γ and RAR-γ possess anti-inflammatory properties and regulate EMT as a result of their attenuation of TGF-β actions. In rats, PPAR-γ agonists have been shown to attenuate fibrosis in several organs, including the heart, liver, lungs, and kidney, by inhibiting the TGF-β pathway (Galli et al., 2002; Aoki et al., 2009; Higashi et al., 2010; Elrashidy et al., 2012). Previous studies showed that the PPAR-γ agonists Troglitazone and Pioglitazone prevent TGF-β2-induced EMT in RPE cells by inhibiting Smad phosphorylation (Cheng et al., 2008; Hatanaka et al., 2012). Similar to PPAR-γ, RAR-γ also plays a role in mediating fibrosis in several organs. A prior study showed that an RAR-γ agonist inhibits the development of subretinal fibrosis in mice by inhibiting the TGF-β pathway (Kimura et al., 2015). Bone morphogenetic proteins (BMPs) are pluripotent growth factors which have anti-fibrotic activity (Yao et al., 2019). Injections of BMP7 in a rabbit PVR model maintained RPE cell phenotypes and prevented TGF-β2-induced EMT, migration and gel contraction (Yao et al., 2019). Anti-inflammatory agents, including Bortezomib, a proteasome inhibitor that regulates the NF-κB pathway, and resveratrol, a polyphenol phytoalexin and heavy chain-hyaluronan/pentraxin3, inhibit EMT in RPE cells and prevent PVR development by downregulating the TGF-β pathway (Ishikawa et al., 2015; He et al., 2017; Moon et al., 2017). Thus, many possible avenues exist for the application of

therapeutics aiming to alleviate EMT of RPE cells, potentially preventing vision loss in retinal disease.

CONCLUSION

Emerging evidence suggests that RPE cells undergo EMT and migrate into the neuroretina in certain pathological conditions, manifesting clinically as HRF in OCT that correlate with pigmentary changes on funduscopy. The data summarized here indicate that EMT of RPE cells is a significant predictor for disease prognosis. We summarized recent advances and potential mechanisms underlying this process. These advances may help clarify the role of EMT in retinal disease states and point to avenues that can be exploited for the development of new therapeutic targets.

AUTHOR CONTRIBUTIONS

MZ, JG, SG, SW, WW, YZ, and JS contributed in writing the review.

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REVIEW
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Pluripotent Stem Cells for the Treatment of Retinal Degeneration: Current Strategies and Future Directions

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Stem cells have been part of the biomedical landscape since the early 1960s. However, the translation of stem cells to effective therapeutics have met significant challenges, especially for retinal diseases. The retina is a delicate and complex architecture of interconnected cells that are steadfastly interdependent. Degenerative mechanisms caused by acquired or inherited diseases disrupt this interconnectivity, devastating the retina and causing severe vision loss in many patients. Consequently, retinal differentiation of exogenous and endogenous stem cells is currently being explored as replacement therapies in the debilitating diseases. In this review, we will examine the mechanisms involved in exogenous stem cells differentiation and the challenges of effective integration to the host retina. Furthermore, we will explore the current advancements in *trans*-differentiation of endogenous stem cells, primarily Müller glia.

Keywords: stem cell, pluripotent, IPSCs, retinal regeneration, embryonic stem cell

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INTRODUCTION

Amongst vertebrates, the retina is a highly conserved tissue (Mitashov, 2004). However, unlike zebrafish and many amphibian species, the singular inability to regenerate damaged tissue in the mammalian retina has drastic ramifications. Retinal diseases or visual impairment inflict over 237 million people worldwide and may impact more than 587 million by 2050 (Bourne et al., 2017). Therefore, optimizing stem cell integration and developing strategies that encourage retinal regeneration are critical for preempting this massive rise in retinal diseases in the near future.

Cellular potency is defined as a cell's ability to give rise to different cell types (Binder et al., 2009). Pluripotent cells under standard conditions will continue to propagate, producing daughter cells that are identical to the mother cell (Kolios and Moodley, 2013). However, under developing or inductive conditions, pluripotent cells can escape the replicative cycle, and produce differentiated cells, completely dissimilar to its progenitor (Kolios and Moodley, 2013).

Embryonic, induced, and reprogrammed stem cells are pluripotent cells currently being explored for retinal cell replacement therapies. Embryonic stem cells are derived from the blastocyst of a developing embryo and can generate cells of the three germ layers – the mesoderm, endoderm, and ectoderm (Vazin and Freed, 2010). Contrarily, induced pluripotent stem cells originate from adult somatic cells and are reprogrammed by the transfection of critical transcription factors, such as SOX2, Klf4, c-Myc, and Oct4. Once expressed, these factors induce the transformation of adult somatic cells into cells of the germ layers and can be re-differentiated into any desired cell type (Takahashi and Yamanaka, 2006). Reprogramming of endogenous cells is another viable method of stem cell

generation (Jeon and Oh, 2015). The adult eye is actually comprised of cells with dormant potency (Bernardos et al., 2007). These cells include Müller glia (Turner and Cepko, 1987; Bernardos et al., 2007) and cells derived from the ciliary pigment epithelia (Ballios et al., 2012; Jeon and Oh, 2015). As these cells possess neurogenic genes, amplification of specific pathway mediators can induce regenerative pathways (Hamon et al., 2019), mobilizing these cells to replace damaged tissue (Yao et al., 2018).

Historically, treatment for degenerative diseases of the retina are marginally effective (Hamel, 2006; Ambati and Fowler, 2012). In late stage retinopathies and inherited malignancies, extensive cellular dysfunction and oxidative stress ultimately lead to cell death (Wert et al., 2014), structural damage, neuronal rewiring (Marc and Jones, 2003; Jones and Marc, 2005) and vision loss. Vision loss is an incredible challenge for treatment, especially in the post-mitotic environment of the retina. Additionally, retinal diseases, such as retinitis pigmentosa (RP), tend to be heterogenic and highly varied, even in the event of mutations on the same locus (Wert et al., 2014; Verbakel et al., 2018). Clinical trials in molecular therapies such as gene and protein therapies are currently underway and have some promising results, especially for patients with defects in the retinal pigment epithelium (RPE) (Smith et al., 2009). The FDA approved Luxturna^{TM} (voretigene neparvovec-rzyl) for gene therapy for RP and Leber's Congenital Amaurosis (LCA) caused by mutations in retinoid isomerohydrolase (RPE65) (Weng, 2019). But for the most part, gene therapy is ineffective when the majority of the cell population is absent after end stage degeneration (He et al., 2014). Furthermore, individual therapies will have to be developed to address each disease. Therefore, exploring the mechanism of exogenously and endogenously generated pluripotent stem cells is critical for the treatment of retinal degenerative diseases. In this review, we will explore the advancements made in the generation of exogenous pluripotent stem cells and their translation to viable replacement therapies. Additionally, we will present the progress of elucidating the regenerative mechanisms involved in activating regenerative pathways in endogenous stem cells of the retina.

ENVIRONMENT OF THE DEGENERATIVE RETINA

Certain properties of the retina make it highly amenable to stem-cell therapies. The retina is self-contained, isolated from critical tissues that may contribute to treatment-related systemic effects, and the RPE and tight vasculature of the retina create an immune-privileged environment (Eveleth, 2013; Ramsden et al., 2013). Therefore, delivery and implantation are not the most prohibitive challenges to stem cell therapy; instead the most difficult obstacles are cellular survival and integration into the cellular network of the host retina (Wong et al., 2011; He et al., 2014), which are dependent outcomes. So, in order to properly address the advancements in stem cell retinal therapy, our exploration will begin with a discussion of the retinal environment before and after the onset of disease.

Briefly, the retina is a highly specialized stratified tissue, each layer containing constituents integral for light capture and signal propagation. The RPE is a monolayer of polarized epithelial cells that forms the primary layer and is critical for supporting photoreceptor cells (Strauss, 2005). The cells of the RPE have a host of functions not limited to, retinoid recycling, nutrient delivery, and light absorption (Strauss, 2005). The primary sensory cells of the retina are the photoreceptors (PRs), which sit directly below the RPE, but within its microvilli. PRs are primary neurons with specialized cilia that house the chromophore necessary for visual transduction. Photoreceptors can be either rods or cones, which have significant structural, functional and molecular differences. The most consequential difference, however, is the chromophore. The cone chromophores are important for colored vision, while rods are more sensitive and are responsive in low light conditions (Kawamura and Tachibanaki, 2008). Once the chromophore has been activated by light, an electro-chemical signal is propagated to the synaptic terminal which activates second order neurons. Horizontal, bipolar, and ganglion cells, in that order, integrate and transmit the signal to the brain, were it is finally perceived as vision (Remington, 2012). Müller glia, astrocytes, and microglia surround both primary and second order neurons (Remington, 2012), and are important for maintaining retinal homeostasis.

Consequently, any disruption to this delicate architecture can lead to deleterious changes to the retinal environment. RP and age-related macular degeneration (AMD) are emblematic retinal atrophic pathologies that highlight the major themes of retinal degeneration and are ideal candidates for stem cell therapies.

Retinitis Pigmentosa

Retinitis pigmentosa is the most common form of inherited retinal diseases (Wert et al., 2014) whereby about 190 genes have been identified that are directly linked to the disease (Daiger et al., 2013). However, despite its etiological diversity, disease pathogenesis is similar between patients (Wert et al., 2014) and can be used to highlight the changes to the retinal environment as the disease progresses. In early stages of RP, patients may experience night blindness, which is indicative of rod photoreceptor dysfunction or loss. Mutant genes associated with RP disrupt proteins involved in photo-transduction or proteins integral to photoreceptor structure (Ferrari et al., 2011). As a result, the continuous insult of gain or loss of function mutations within photoreceptor proteins ultimately induces apoptosis of the cell (Wert et al., 2014). Generally, photoreceptors loss begins in the periphery, and slowly progresses to the mid-periphery and center of the retina. At this late stage, patients may report "tunnel vision" where sight is predominately attenuated at the periphery, and central vision is all that remains (Wert et al., 2014). Depending on the mutation, rod loss is proceeded by the loss of cone photoreceptors, causing complete vision loss (Verbakel et al., 2018).

Cone photoreceptor degeneration is the most debilitating aspect of RP. Though many mutations are rod-specific, death of cones generally ensues (Wert et al., 2014). Many suggest that rods provide critical trophic components into the retinal environment

that ensure cone survival (Kelley et al., 2017) and because rods are the major cellular component of the photoreceptor layer, they contribute essential infrastructure for the cones (Steinberg, 1994; Ripps, 2002). Nevertheless, RP has demonstrated that cone survival is contingent upon rod photoreceptor health. As photoreceptors disappear, RPE cell death follows. RPE cells are highly myelinated and the deposition of melanin is a characteristic feature observed in the fundoscopy of RP patients (Wert et al., 2014).

Many RPs are monogenic (Weleber, 2005), which highlights the broad interconnectivity of the retina. One mutation can cause a cascade of negative events, eventually rendering most of the tissue completely dysfunctional. Dysregulation and degeneration of RPE cells as a consequence of mutations in photoreceptors is an example of one of these secondary effects (Stuck et al., 2014). These effects extend to the choroidal and inner retinal vasculature, which are markedly attenuated in RP patients (Yang et al., 2018). Under normal conditions the oxygen levels in the retinal choroid are near arterial levels (Yang et al., 2018). In the degenerative environment of RP, the oxygen levels are even higher as oxygen consumption is reduced as a result of reduced photoreceptors (Yang et al., 2018). Vascular changes reduce nutrient bioavailability which may further perpetuate RPE and photoreceptor dysfunction. These drastic changes to metabolism and homeostasis encourages the recruitment of glial cells to the outer retina and the secretion of tumor necrosis factor alpha (TNF-α) (Roesch et al., 2012) and other proinflammatory factors from Müller glia.

Mutations in rhodopsin form the majority of autosomal dominant RP cases in North America and serve as an example of monogenic RPs (Mendes et al., 2005). Different mutations within the gene can cause a host of varying dominant negative and gain-of-function phenotypes. For instance, class I mutations do not affect the tertiary structure of rhodopsin, but cause mislocalization of the protein. Class II mutations cause misfolding of the protein, and the large majority of these aberrant proteins aggregate in the cytosol and ER, generating a cytotoxic environment (Mendes et al., 2005). Irrespective of the mutation, over time photoreceptor and retinal atrophy significantly attenuates function, damages the cellular infrastructure, and the inner retina undergoes significant remodeling to accommodate these drastic changes (discussed in a subsequent section).

Because the pathogenesis of RP is multifactorial, many therapies have been explored to improve prognoses, however, many can only slow disease progression and are not curative. Therapy with vitamin A and DHA has a short-term positive effect, but effective dosage of vitamin A were approximately 15,000 units/day, which can be toxic (Sahni et al., 2011). Gene therapy is a strategy that has had promising results, but still has major challenges considering the vast number of different genes involved in RP, the diversity of clinical presentations, and the difficulties in transfecting PRs (Smith et al., 2009).

Neuroprotection involves the use of trophic factors such as ciliary neurotrophic factor (CNTF) or brain derived neurotrophic factor (BDNF) to enhance cell survival and curb the effect of pro-apoptotic factors (Sahni et al., 2011). Encapsulated cells secreting CNTF were delivered to the vitreous in degenerative animal models and preservation of retinal structure was observed. This modality of trophic delivery to the retina advanced to clinical trials. However, the large majority of trophic therapies are in very nascent stages since these factors have a short half-life requiring frequent applications through invasive means. Although beyond the scope of this review, stem cells can also be used to elicit this neurotrophic effect (Xu and Xu, 2011). Recently, intravitreal injection of mesenchymal cells altered to overexpress BDNF has been shown to attenuate apoptosis in RD6 mice (Lejkowska et al., 2019), demonstrating the versatility of stem cell approaches.

Age Related Macular Degeneration

Analogous to RP, age related macular degeneration (AMD) has a multi-faceted etiology and devastates the aging population. Recent indications suggest that by 2040 over a quarter of a billion people worldwide may be affected by AMD (Wong et al., 2014). AMD is marked by chronic malignant changes to structures that support the macula, such as the choroid, RPE, and Bruch's membrane. As structures are compromised, patients report loss of central vision (Zając-Pytrus et al., 2015).

Based upon symptom onset and clinical presentation, AMD is divided into early and late stage AMD (García-Layana et al., 2017). Initially, before patients experience any disruption to vision, the Bruch's membrane will start to thicken due to lipid-protein deposits (Young, 1987). These deposits may occur under normal conditions, however, when these deposits aggregate to approximately 125 μm or larger they become pathogenic (The Eye Diseases Prevalence Research Group, 2004). These deposits (aka drusen) inhibit proper flux and communication between the Bruch's membrane and the RPE (The Eye Diseases Prevalence Research Group, 2004). Like the inner retina, the RPE serves as an integration junction, responding to stimuli from the outer retina, choroid, and Bruch's membrane. The deposits critically injure the RPE and debilitate its important functions. Without the proper flux of nutrients and environmental signals, the RPE is now susceptible to a host of injurious events, such as oxidative stress, metabolic dysfunction, reduced photoreceptor interconnectivity, and inflammation (Young, 1987).

In fact, inflammation plays an important role in AMD pathology (Akhtar-Schäfer et al., 2018). Previous studies have indicated that over reactive immune responses by the innate immune system of the retina (Ambati et al., 2013) and slow chronic infiltration of inflammatory immune components promote degeneration of the RPE and photoreceptors. As previously mentioned, the RPE is part of an intricate architecture that imparts immune privilege to the outer retina (Sugita, 2009). However, in cases of AMD, where autoimmunity, aberrations in microglia behavior (Ambati et al., 2013), and RPE degradation allow for dendritic and macrophagic invasion (Ambati et al., 2013), the mechanisms that sequester the retina from circulating immune elements break down creating a compromised microenvironment (Ambati et al., 2013), further complicating treatment, as immune privilege is an aspect of the retina that makes it highly amenable to stem cell therapies.

Nevertheless, photoreceptor atrophy, specifically in the macula, is the worst eventuality (Young, 1987). In 10% of cases (Halpern et al., 2006), this RPE dysfunction can cause the upregulation of pro-angiogenic factors like vascular endothelial growth factor (VEGF) (Hernández-Zimbrón et al., 2018). The promotion of angiogenesis in this already fragile environment will ultimately cause blindness when left untreated. Angiogenic presentation in AMD, is called "exudative" or "wet" AMD (Hernández-Zimbrón et al., 2018). VEGF causes neovascularization in the choroidal strata. As the oxygen demand is highest at the macula, most of this neovascular invasion may occur at the macula. The newly formed vascular infrastructure is weak and immature, causing excessive leakage which reaches the macula, causing detachment and RPE and PR atrophy and ultimately blindness (Hernández-Zimbrón et al., 2018).

Mitigating degeneration in AMD is challenging as genomics have unearthed many potential genes and mechanisms that may contribute to its pathogenesis (Fritsche et al., 2016; Yan et al., 2018). By improving diet, discontinuing smoking, and reducing exposure to environmental risk factors, patients can slow disease progression (Khan et al., 2006; Moutray and Chakravarthy, 2011). Additionally, photocoagulation laser therapy had been previously implemented to prevent neovascular damage, but relapses were not uncommon (Moutray and Chakravarthy, 2011). More efficacious treatments are VEGF inhibitors, such as ranibizumab which is a monoclonal antibody binding to VEGF-A (Moutray and Chakravarthy, 2011). Most recently, a better FDA approved drug, Aflibercept, has become a common treatment of wet AMD. It is a fusion protein that functions similarly to ranibizumab or bevacizumab, only with improved inhibition of VEGF and requires less frequent dosing (Sarwar et al., 2016). VEGF inhibition, however, is only effective in a third of patients (Moutray and Chakravarthy, 2011), thus additional investigations are exploring therapies that address pro-angiogenic factors upstream and downstream of VEGF (Moutray and Chakravarthy, 2011). Despite partial successes with wet AMD, there are still no curative treatments for dry AMD (Zając-Pytrus et al., 2015).

Retinal Remodeling

As previously mentioned, integration of transplanted cells into the host retina remains a substantial challenge for stem cell therapy. The retina is a dynamic system that undergoes histological, molecular, and genetic remodeling to accommodate the degenerative environment under pathogenic conditions as illustrated in **Figure 1**. As photoreceptors apoptose, the inner retina loses sensory input (deafferentation) which is the primary reason for remodeling (Jones et al., 2012). Remodeling is composed of three phases. Phase I is characterized by photoreceptor dysfunction and stress which is the result of the initial pathological insult. In phase II photoreceptor apoptosis encourages glial migration, remodeling in the outer retina, and morphological changes to second order neurons. Müller cells and astrocytes leave behind a glial seal between the remaining RPE and neural retina. Phase III is a period of constitutive neural rewiring, and glial and vascular remodeling. These phases are highly variable as degeneration varies between diseases and pathogenesis and rate of decline are dictated by disease mechanism and the individual patient. For example, RPs with delayed cone degeneration can extend the onset of phase III.

Many of the changes that happen in phase I occur on a molecular level and do not manifest clinically (Jones et al., 2012). Contrarily, phase II and phase III are quite dynamic. As a consequence of photoreceptor apoptosis, bipolar cells can exhibit either telodendria or dendritic retraction (Jones et al., 2012). Investigations using mouse models, have determined that rod bipolar cells are the first to demonstrate significant structural changes, followed by ON bipolar cone cells, and finally OFF bipolar cells (Strettoi, 2015). Because bipolar cells express metabotropic glutamate receptor (mGluR6) and undergo glutamatergic synapses with photoreceptors, these cells are the first to experience the effects of deafferentation as photoreceptors die (Strettoi, 2015). Bipolar cells can rearrange and migrate forming aberrant connections and ectopic synapses (Strettoi, 2015). Continued photoreceptor atrophy causes hypertrophic glial cells to migrate to the outer retina and begin gliotic remodeling (Jones and Marc, 2005). They digest dying cells, remodel the subretinal space, and rearrange molecular components of the extracellular space to develop a gliotic "seal" (Marc and Jones, 2003). Large amounts of chondroitin sulfate proteoglycans are deposited into retinal extracellular matrix (ECM) by glial cells, which inhibits axonal growth (Fawcett, 2009). Horizontal cells and amacrine cells undergo slower remodeling exhibiting the same axonal sprouting or retraction that may be observed by bipolar cells (Jones et al., 2012). Surprisingly, ganglion cells are the least affected by photoreceptor loss (Strettoi, 2015) whereby major aberrations are only seen long after outer retinal disintegration (Strettoi, 2015).

As the pathophysiological changes in the retina occur, the inner retinal remodeling can be overlooked, as photoreceptor degeneration is the primary event. However, understanding of the topographical and molecular changes in the inner retina are critical to developing the most efficacious stem cell therapeutics. This remodeling timeline must be carefully considered, to ensure that connection between bipolar cells can be re-established and extensive gliosis has not occurred. Nevertheless, in order to advance transplantation of exogenous stem cells, which for the most part have only been marginally successful, therapies should consider the amelioration to the degenerative environment in conjunction with the delivery of stem cells.

EXOGENOUS STEM CELLS

Embryonic Stem Cells and Retinal Differentiation of Pluripotent Cells

Embryonic stem cells (ESCs) are pluripotent cells that originate from the blastocysts of a developing embryo (Itskovitz-Eldor et al., 2000). Their pluripotent capacity has been extensively examined in mouse and in *in vitro* models (Itskovitz-Eldor et al., 2000), and it is this potent ability that has garnered excitement for the development of therapies for a wide range of neurodegenerative diseases. However, in order to generate a viable therapeutic, ideal inductive conditions must exist for ESCs to differentiate and acquire attributes of retinal cells.

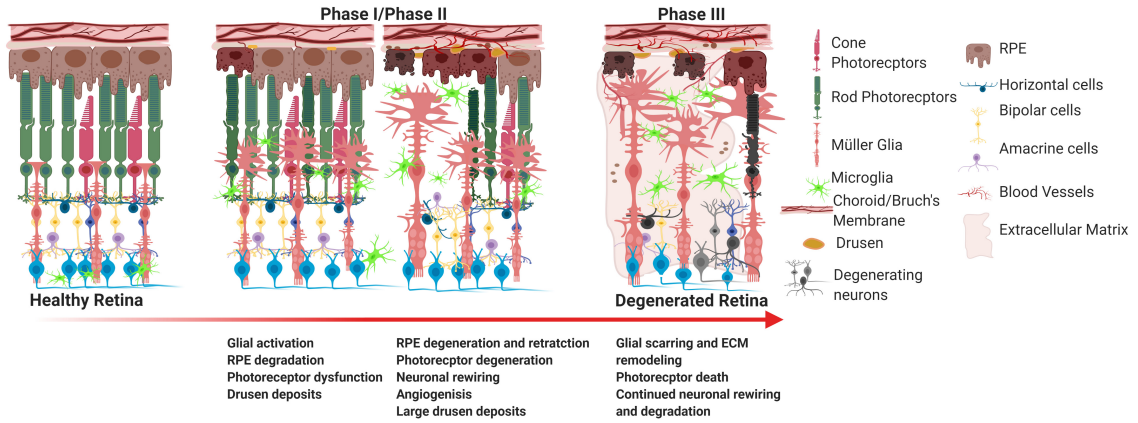

FIGURE 1 | Progression of retinal degeneration. The major hallmarks of degeneration include RPE degradation and photoreceptor atrophy. In the case of wet AMD and some forms of RP, choroidal neovascularization may occur. In the last phase of degeneration, the large majority of the subretinal space undergoes drastic glial remodeling and scarring, as represented in phase III, which significantly obstructs cellular integration of stem cells.

To establish induction methodology, it was important to identify critical factors of retinal development which may encourage ESC differentiation. Previous data from chick retina has elucidated the role of WNT signaling in neural determination (Cho and Cepko, 2006; Lamba et al., 2006). Identified sequential steps of retinal development which were determined to be sufficient for ESC induction. In order to begin what was coined by Lamba "retinal determination," human embryonic stem cell (hESC) aggregates were initially cultured with Dickkopf-1 (dkk1), noggin, and insulin-like growth factor-1 (IGF-1) (Lamba et al., 2006). Dkk1 is a WNT/β catenin antagonist, and noggin inhibits the bone morphogenic pathway (BMP) (Lamba et al., 2006). IGF-1 was identified to promote ocular development in *Xenopus* embryos (Pera et al., 2001). Eye field associated transcription factors (e.g., Paired box protein and PAX6) were identified in ESCs after 3 weeks, and after longer exposure to inductive media, analysis indicated that the large majority of cells had ganglion, horizontal and amacrine cell characteristics. Co-culturing with retinal explant encouraged the population of ESCs to develop rudimentary photoreceptor attributes, as a small population of cells expressed recoverin (Lamba et al., 2006).

However, requiring retinal explant for photoreceptor differentiation would significantly limit the use of ESCs for clinical applications. Osakada et al. (2008) developed a strategy that implemented γ-secretase inhibitor, DAPT, to effect Notch signaling pathways and Left-Right Determination Factor A (Lefty A) to inhibit WNT signaling. By using these inhibitors, Osakada et al. (2008) produced a small population of cone rod homeobox positive (CRX^+) cells, significantly more than in the absence of DAPT. CRX^+ positive cells do not only indicate photoreceptor precursor cells, but they also suggest post-mitoses. Furthermore, step-wise treatment with taurine, retinoic acid, Sonic hedgehog (Shh), and fibroblast growth factor (FGF), continued to push ESCs toward a photoreceptor-like cell as they expressed rhodopsin and recoverin following exposure to these morphogens.

Both the work of Osakada and Lamba created a framework in which to induce retinal cells from embryonic stem cells and further validated that the inhibition of BMP and WNT pathways is critical for retinal determination. In the developing optic cup, centralized progenitor cells transform to become the different retinal cell types, while progenitor cells at the periphery become non-neural cells, i.e., the ciliary body and iris (Cho and Cepko, 2006). Since WNT signaling is integral to these determinations, upregulation of WNT activity is indicative of a non-neural fate, while inhibition of WNT may encourage differentiation to neural cells. In the developing retina SOX2 plays a modulatory role restricting WNT activity (Heavner et al., 2014).

In addition to retinal determination, the combined inhibition of WNT and BMP pathways can be modulated to control rod or cone photoreceptor lineages. Both noggin and chordin are antagonists to BMP and are frequently used in retinal determination of IPSCs and ESCs (Smith and Harland, 1992; Messina et al., 2014). BMP, in a neural context, is important for glial cell development (Ueki et al., 2015b). Inhibition of WNT and BMP may yield 12% CRX expressing cells (Lamba et al., 2006), while only 4% of the cells may express rod photoreceptor markers, and 0.01% may express cone photoreceptors proteins (Lamba et al., 2009). Zhou et al. (2015) reported that the use of Coco (a factor from the Cerberus family), as an effective WNT and BMP inhibitor, not only promoted photoreceptor neurogenesis in hESCs, but also increased the propensity for cone photoreceptors, up to 60%. These cone photoreceptors were transplanted into 2-day old pups, whereby they were able to differentiate and integrate into the host retina and exhibit similar morphology to the endogenous photoreceptors (Zhou et al., 2015). The use of Coco for cone differentiation presents a viable method for developing cone photoreceptors cell replacement therapies in RP and macular degeneration.

The aforementioned strategies, however, generated retinal precursor cells in substantially low yields, over an extensive period of time, up to 120 days in some instances (Mellough et al., 2012). Proceeding studies have ventured to improve upon this groundwork by ameliorating culture conditions. Yields demonstrated considerable improvement by simply controlling the size and population of embryoid bodies. By limiting the size

to approximately 200 μm, the total population of CRX^+ cells was 78%, and further increased to 93% by negative selection of hESCs (Yanai et al., 2013). Moreover, stepwise analysis of morphogens and media supplements has shortened the time frame for differentiation, and isolated specific supplements (such as B27) that encourage a neural or retinal fate more expeditiously (Mellough et al., 2012).

Induced Pluripotent Cells and Retinal Differentiation

The use of embryonic stem cells, specifically human embryonic stem cells, comes with ethical and sourcing limitations. As a consequence, Takahashi and Yamanaka developed a method for inducing pluripotency in somatic cells by viral-vector mediate transfection using the four transcription factors previously mentioned (Takahashi and Yamanaka, 2006). After transfection of these factors, cells from the three germ layers should be present (Takahashi and Yamanaka, 2006). Originally, established using adult mouse fibroblasts, advancement in the technology have indicated that virtually any somatic cell can be used, and as they can be patient-derived, once differentiated, these cells appear to show no or limited immune rejection (Mandai et al., 2017). Transplanted RPE sheets of autologous induced pluripotent stem cells (IPSCs) were tested in patients with wet AMD and demonstrated no immune rejection a year after transplantation (Mandai et al., 2017).

The differentiation systems discussed in the prior section primarily employed serum-free embryoid body like (SFEB) culture conditions, where cellular aggregates are grown in serum free media for the preliminary stages of differentiation followed by a transfer to coated plates for attachment (Lamba et al., 2006). The resultant cells express photoreceptor specific markers and demonstrate reduced expression of mitotic proteins, suggesting the presence of photoreceptor precursors (Lamba et al., 2006). However, many of the cells do not possess the specialized cilia of the photoreceptor, the photoreceptor outer segment, which is inherent to photoreceptor functionality. From developmental studies, it has been well established, that retinal development depends not only on transcription and trophic factors, but a host of spatial, environmental, and temporal cues, which cannot be entirely recapitulated in 2D culture (DiStefano et al., 2018). Consequently, many IPSC protocols now involve the generation of retinal organoids by growing IPSCs in a three-dimensional environment, to establish an ECM and multi-dimensional cell-cell interactions. These organoid cultures can even expand to accommodate a bioreactive system that can simulate oxygen exchanges, and vascular delivery of nutrients, and waste removal (DiStefano et al., 2018).

Successful retinal differentiation of IPSCs is contingent upon establishing a 3D microenvironment in addition to the proper sequential additions of morphogens (Zhong et al., 2014). At early differentiation stages of *in vitro* differentiation, as the eye field develops, IPSCs will organize themselves and migrate to form a rudimentary architecture (Zhong et al., 2014). The eye field will continue to develop and begin expressing the transcription factors visual system homeobox 2 (VSX2) and microphthalmia-associated transcription factor (MITF) which indicate a neural retina and RPE fate, respectively. Following expression of VSX2, cells committed for retinal formation, continue finessing retinal lamination and will also start to form a retinal cup with a pseudo-RPE attached. Once retinoic acid and taurine are added at this stage, neurogenesis continues with the formation and self-organizing lamination of Müller glia and ganglion cells preliminarily, followed by photoreceptors, horizontal cells, amacrine cells, and finally bipolar cells, which in part recapitulates cellular development *in vivo*. *In vitro* recapitulation of embryonic retinal neurogenesis is itself a successful feat, however, the formation of functional outer segments demonstrates the importance of accounting for the microenvironment when differentiating IPSCs. In the laminated organoid, photoreceptors express both rod and cone opsins and present with distal-end budding structures emanating from the outer retina, similar to what is observed in the developing outer segment. Patch clamp responses indicated that maturing photoreceptors in the organoid are light responsive (Zhong et al., 2014).

Induced Pluripotent Cells as Models of Retinal Pathologies

Considerable clinical barriers still remain for the use of IPSCs, such as fear of oncogenicity, lack of homogeneity in differentiated cell populations, genetic instability, and the massive resources mandated for generating safe and effective lines from individual patients (Singh et al., 2018). Despite these limitations, they have become vital research tools for modeling retinal disease pathology and developing personalized therapies, bearing in mind the difficulty of recapitulating diseases in rodent models. As an example, Yoshida et al. (2014) generated IPSCs from a patient's skin punch that presented with retinitis pigmentosa caused by a heterozygous rhodopsin mutation, were a glutamic acid was substituted with a lysine (E181K). The skin fibroblasts were transfected with the indicated transcription factors, and the presence of each germ layer was determined by generating teratomas. The genetic construct of the mutagenic IPSCs were introduced into a healthy patient cell line to determine that the point mutation in rhodopsin was the singular cause of degeneration. After this validation, the same culture conditions developed by Lamba were implemented by Yoshida to induce retinal determination and rod photoreceptor differentiation. Analysis of these generated rod photoreceptor precursors confirmed disease pathogenesis; primarily, they determined that ER stress and autophagy contributed to photoreceptor death. Using rapamycin and a host of other drugs, patients derived rod photoreceptors precursors responded well to drug cocktails. These critical findings can be taken to the clinic for further assessment.

A successful IPSC model, as in the example above, is able to confirm the genetic cause of disease, recapitulate disease phenotype, elucidate cellular mechanisms of the disease, and ultimately validate pathogenesis by positively responding to interventions (Doss and Sachinidis, 2019). Another example involved the generation of 3D organoids from patients with frame

shift mutations in the retinitis pigmentosa GTPase regulator gene (RPGR) which causes a form of autosomal dominant retinitis pigmentosa effecting cilia function and morphology (Deng et al., 2018). Thus far, animal models have not been able to categorically reflect the disease phenotype exhibited by patients, therefore these *in vitro* models are critical to understanding disease pathogenesis (Hong et al., 2001). 3D retinal organoids were generated from urinary cells of three diseased patients and three healthy donors (Deng et al., 2018). Cells differentiated and organized into retinal strata, as they would in the fetal retina and became electrophysiologically responsive. The diseased retina demonstrated a thin outer segment and inner segment layer, aberrant outer segment morphology and rhodopsin transport, and mislocalization of opsins, providing significant insight into the causes of photoreceptor loss exhibited by patients.

To correct the frame shift mutations, the highly effective bacterial gene editing system, clustered regularly interspersed short palindromic repeats (CRISPR/Cas9), was implemented to repair the RPGR gene (Deng et al., 2018). Briefly, guided RNA bound to the Cas9 nuclease, excises the complementary sequence in the target DNA, where it can be replaced with the desired sequence (Baliou et al., 2018). Here, exon 14 of the RPGR gene was replaced with the healthy sequence (Deng et al., 2018). Resultant IPSCs were differentiated and showed significant biochemical and physiological improvement over the mutant IPSCs (Deng et al., 2018).

Therefore, genes with single mutations can be effectively modeled with IPSCs and treated with CRISPR gene editing technology or drugs and small compounds to target specific cellular mechanisms. However, limitations exist in the context of more complex polygenic diseases, as in the case of AMD or systemic diseases such as diabetic retinopathy. Disease can be the result of gene variants compounded with environment and systemic insults which cannot be recapitulated in the dish. So, rather than using IPSCs to model a disease of a single genetic etiology, Golestaneh et al. (2016) performed a study of IPSCs derived from patients of multiple genetic backgrounds and exposures, to provide an exhaustive analysis of AMD disease features and mechanisms. AMD patient samples with risk alleles for complement factor H (CFH), age-related maculopathy susceptibility 2/serine peptidase 1 (ARMS2/HTRA 1), LOC alleles, and complement factor B (FACTOR B) were used in this study. Physiological and biochemical assays indicated common features despite the different mutations, and a common underlying pathway (silent information regulator 1 (SIRT1)/peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1 α) pathway) effecting mitochondrial biogenesis, which can be a potential therapeutic target that can be further explored using these RPE cells lines differentiated from patient samples.

Transplantation and Factors Affecting Integration

As previously discussed, the degenerative environment is not amenable to cellular integration, so effective cell replacement therapy must overcome these environmental challenges. A host of characteristics must be considered before transplantation, such a degree of differentiation, number of cells, the cell types that may promote cellular health and integration, and whether the cells will be transplanted as a sheet or a suspension. Previously, there had been very few successful attempts of stem cell integration in the adult post-mitotic retina, not to mention, degenerative retinas (Young et al., 2000). In these cases, cells that do not integrate did not survive. Many observed a lack of long-term survival, eventual degeneration of the stem cell as result of chronic immune responses (Zhu et al., 2017), and in these advanced stages where a small number of endogenous cells remain, integration becomes a lot more difficult because islands of non-integrated cells coalesced and formed rosettes (Chacko et al., 2000).

In order to highlight the importance of the microenvironment to successful stem cell integration, Barber et al. (2013) conducted an experiment, in which stem cells were transplanted into six different genotypes with varying pathologies and rates of deterioration. In this experiment, age or extent of deterioration was accounted for. The phenotypes tested include $Prph2^{+/\Delta 307}$, $Prph2^{rd2/rd2}$, $Rho^{-/-}$, $PDE6\beta^{rd1/rd1}$, $Crb1^{rd8/rd8}$, and $Gnat1^{-/-}$, the first four being models of retinitis pigmentosa, the following, a model of LCA, and the latter a model of stationary night blindness. RP models and $Gnat1^{-/-}$ showed integration similar to what was observed in wild type mice, where an average of 4,000 of 200,000 delivered cells were able to integrate into the host retina. The model of LCA demonstrated the greatest number of integrated cells, an average of approximately 10,000 cells. The rhodopsin null mice had the worst integration patterns, where almost no cells showed integration. This preliminary data suggested that even though the animals were injected at the same time point each disease had varying levels of degeneration at the time of transplantation which affected the success of integration.

Barber et al. (2013) also wanted to ascertain whether outer limiting membrane (OLM) integrity and gliosis had any effect on successful integration. In the $Crb1^{rd8/rd8}$ model, where OLM integrity is already compromised, an interesting interplay between gliosis and OLM integrity was observed. It appears that the reduction in tight junctions in the OLM may actually improve stem cell integration as long as gliosis is relatively mild. At an intermediate time point where gliosis is not as rampant and OLM integrity is fully compromised, successful integration of stem cells in the $Crb1^{rd8/rd8}$ was seen, even more integration than what was observed in samples 3 months younger. It is important to note, that the $Crb1^{rd8/rd8}$ model had the largest number of integrated cells.

The rhodopsin null mice had the least number of integrated cells (Barber et al., 2013). To buttress the idea that modulating the microenvironment can improve integration, rhodopsin null mice were treated with siRNAs that target ZO-1 and disrupt OLM integrity. Chondroitinase was used to digest chondroitin sulfate proteoglycans which are inhibitory ECM molecules deposited as a result of glial scarring. With these modifications, transplantation was significantly more successful in this model, as well as in the wild type control, suggesting that simple transplantation may not be sufficient in highly degenerative models and that modulation

of the microenvironment can significantly improve the success of stem cell integration and consequent long-term survival.

In addition to the microenvironment, dynamics of cell transplantation are also important aspect to consider. The degree of degeneration should dictate how cells are introduced into the environment whether as a cell suspension or as a retinal sheet. When there is still a remaining cohort of endogenous cells these pluripotent stem cells tend to fuse with the endogenous cells remaining (Decembrini et al., 2017). However, in advanced degenerative models, where most photoreceptors have died and the interphotoreceptor matrix and subretinal space are largely reduced, a more robust delivery system is required to supplement the highly disintegrated outer nuclear layer. Assawachananont et al. (2014) developed a retinal sheet to graft into PDE6$\beta^{rd1/rd1}$ mice where most rods are absent by 3 weeks. Here, integration efficiency was tested at varying levels of stem cell differentiation and variation to sheet construction. What was observed was that younger, less differentiated cells that were not accompanied by a fully developed inner nuclear layer (INL), fared a lot better *in vivo* than the more highly differentiated organized sheets. These sheets, made of less differentiated cells, were able to form proper outer segments containing well aligned disk almost identical to wild type and were able to form synapses with host bipolar cells. In more differentiated retinal sheets, more rosette formation and less integration were observed. There was no significant difference between sheets developed from ESCs or IPSCs. Though this assessment did not include electrophysiological examination of the integrated stem cells, it provided important properties that can be taken forward to be implemented for the development of retinal sheets in advanced retinal degeneration.

Another feature the degenerative environment is the pro-inflammatory factors that are upregulated as result of the pathology (such as in the case of AMD) and the immunogenicity caused by transplantation itself (Nazari et al., 2015). A chronic immune response contributes to the lack of long-term survival of stem cells, especially with hESCs (Zhu et al., 2017). Immune suppressed mice where used to determine the effect of immunity on stem cell integration and longevity, using a compound animal that has a mutation in the CRX gene and is null for interleukin 2γ (IL2γ) ($Crx^{tvrm65}/IL2r\gamma^{-/-}$). This mutation in CRX recapitulates some of the visual symptoms described by patients with LCA. This investigation demonstrated that immunosuppression improved integration of hESCs; cells were able to mature, integrate with the existing INL, and were functional even after 9 months. Though the degree of integration was still less than the immunosuppressed wildtype control, it does indicate immune activity plays a role in long-term stem cell survival; additionally, as the number of integrated cells in the compounded animals was reduced from the wildtype (Zhu et al., 2017), it does imply that other elements also contribute to the death of grafted stem cells.

The reduced integration observed by Zhu et al. (2017) may attest to the influence of the inherent dysfunction of the retinal immune system under pathogenic conditions (Akhtar-Schäfer et al., 2018). As part of the retina's immune privilege, circulating soluble elements such as macrophage inhibitory factors (MIFs) and transforming growth factor β2 (TGFβ2), and additional membranous components, play an important role in dampening and mitigating damage from the retina's internal innate immune system or from other injury (Perez and Caspi, 2015). RPE cells, in particular, are integral to this homeostasis (Sugita et al., 2016). Specifically, they express complement components, minor histocompatibility molecules, anti-inflammatory cytokines (Holtkamp et al., 2001), and T-cell mediators and response elements (Mandai et al., 2017). In degenerative diseases, this ability to modulate immune activity becomes progressively dysfunctional and ineffectual as cellular atrophy worsens. Therefore, in addition to cell replacement, it is imperative that pluripotent stem cells retain the immunomodulatory properties of the cell type they become, in order to restore the immune regulatory mechanisms and prevent further insult to retinal tissues. IPSCs derived from healthy donors have demonstrated the ability to suppress T-cell activation, induce T-regulatory cells, and secrete immune suppressive factors such as MIF and TGFβ (Sugita et al., 2015). HESCs have also indicated a similar capacity, and appear to have a better immunomodulatory response in the absence of immune suppressive drugs, such as cyclosporine (Idelson et al., 2018).

As we will discuss in the following sections, allogenic transplantation using hESCs have made further strides to clinical translation than IPSCs for many reasons. One reason is that the polygenic and multifactorial nature of many retinal pathologies, make genetic corrections difficult, leaving allogenic transplantation from healthy donors the only viable option, currently. Nevertheless, genetic strategies to enhance immunomodulatory effects and reduce immunogenicity can significantly improve the disease microenvironment and stem cell survival, concurrently. Successful attempts using recombinant adeno-associated viruses (AAV) and CRISPR/Cas9 have yielded IPSCs with reduced immunogenicity by targeting human leukocyte antigen (HLA) polymorphisms, potentiating the availability of universal IPSCs for allogenic transplantation that will elicit no immune response (Gornalusse et al., 2017; Xu et al., 2019).

Translation Capacities of IPSCs and ESCs and Current Clinical Trials

In order to meet clinical standards, many pluripotent stem cells should meet manufacturing standards that may pose challenging for some therapies. Propagation of stem cells may introduce genetic instability or phenotypic changes (Whiting et al., 2015). Moreover, though differentiation and induction occur under controlled settings, it is difficult to ensure 99% population homogeneity (a small sub-population may remain undifferentiated) (Whiting et al., 2015). These are just two of many requirement researchers must met before ESCs and IPSCs can be implemented in a clinical setting.

Nevertheless, a host of clinical trials using hESCs and IPSCs are underway for dry AMD, Stargardt's disease and RP (Jayaram et al., 2014). One trial involves the subretinal transplantation of

human derived RPE cells (OpRegen®) (NCT02286089, 2015). OpRegen had proven long term effectivity in Royal College of Surgeon (RSC) rat retina by significantly improving optokinetic thresholds and preventing further degeneration of the outer retina (McGill et al., 2017). The trial involved 24 participants 50 and older with dry AMD that presented without neovascularization (Banin et al., 2019). Interim reports indicate that the cells were well tolerated, and any adverse events were relatively mild (Banin et al., 2019). Most adverse events were post-operative problems and the formation of epiretinal membranes (Banin et al., 2019). However, some evidence suggested improvement in visual acuity as well (Banin et al., 2019).

In 2014, RIKEN undertook treatment of AMD using autologous IPSCs in two patients. The study was abruptly halted as three DNA deletions were identified in the male patient's IPSCs. Though not proven to be tumorigenic, concerns regarding the deletions and the implementation of strict regulatory measures in Japan prevented further advancement of the study (Mandai et al., 2017) so a study using allogenic transplantation replaced it soon after (Garber, 2015). Recent changes to the IPSC induction cocktail and non-integrating episomal transfection strategies have been introduced, significantly diminishing worries concerning oncogenic potential of IPSCs. Consequently, NEI has entered phase I/IIa clinical trials for patients with dry AMD to test the safety and efficacy of autologous IPSC-derived RPE cells (NCT04339764, 2020). In 2012, 20 RP patients were recruited for phase I/IIa trials to determine the effectiveness of intravitreally administered bone marrow-derived stem cells (Reticell) (NCT01560715, 2011). Bone marrow-derived stem cells are adult stem cells, which are quite different from ESCs and extend beyond the scope of this review. However, patients reported improvement in the short term, but there was no long-term sustained improvement (Siqueira et al., 2015), suggesting use of stem cells in the ocular environment still have barriers to address.

ENDOGENOUS STEM CELLS

Thus far our exploration has examined the viability of pluripotent stem cells for cell replacement therapies in degenerative pathologies of the retina. However, the hostility of the degenerative environment and the low frequency of integration between graft and host remain prohibitive to clinical translation, as most of past and present clinical trials are focused on rescuing the RPE to preserve retinal health and not the photoreceptor. As a result, exploration of the retinal regenerative capacities has become another stem cell approach for addressing retinal pathologies.

The retina, for the most part, is entirely post-mitotic. Cells are terminally differentiated, and it has been shown that cell cycle reentry of photoreceptors may even induce apoptosis (al-Ubaidi et al., 1992). However, after analysis of injured retina in lower vertebrates, Müller glial and ciliary pigment epithelial cells have indicated some latent regenerative ability (Vihtelic and Hyde, 2000) and will be discussed in the subsequent sections.

Müller Cells and *Trans*-Differentiation

In lower vertebrate, such as zebrafish, after acute injury, Müller glial will enter the cell cycle and produce retinal progenitor cells that can differentiate into any lineage. However, elucidating the molecular factors involved in this regenerative process has proven somewhat enigmatic. Following injury in zebrafish retina, reactive Müller cells (MC) dedifferentiate and produce neural progenitor cells (NPCs) (Pearson and Ali, 2018). This has an expansion effect, further recruiting more MCs and encouraging dedifferentiation (Pearson and Ali, 2018). NPCs increase in number, and migrate to the sight of injury, there they differentiate to the required cell type an reintegrate into the cellular network (Pearson and Ali, 2018). It is important to note, that microglia are essential to this MC response. When microglia were ablated in zebrafish using pharmacological agents, MCs had a delayed glial fibrillary acid protein (GFAP) response (indicating a delay in reactive gliosis) and the retina did not properly recover (Conedera et al., 2019).

What is the molecular basis for Müller cell *trans*-differentiation after injury? One hypothesis suggests a "negative-modulatory" model, where neural cell death may reduce the expression of an inhibitory factor that represses Müller regeneration, while another postulates that neural death may cause expression of a regenerative signal (Gorsuch and Hyde, 2014). Changes to expression levels of WNT signaling elements has provided some insight. After neural injury, WNT components are upregulated and inhibitors are drastically downregulated (Gorsuch and Hyde, 2014). It is interesting to note that WNT inhibition was critical to retinal differentiation in pluripotent retinal determination, indicating the modulation of WNT signaling is integral to retinal programming. In addition to WNT elements, TNF-α and TGFβ are important factors that mediate neurogenesis in zebrafish after injury (Sharma et al., 2020). TNF-α is released by the dying neural cells and is produced by Müller cells as well (Nelson et al., 2013), while TGFβ is released from the ECM by metalloproteases after injury. Both cytokines induce downstream expression of factors such as Achaete-scute homolog 1a (Ascl1a), Signal transducer and activator of transcription 3 (STAT3), and hairy-related 4 (her4.1), all of which are indispensable to NPC propagation from the Müller cell (Ueki et al., 2015a; Sharma et al., 2020). TGFβ also promotes cell-cycle exit of Müller cells after progenitor cell proliferation (Sharma et al., 2020). By understanding these factors, they can be implemented in mammalian retina to stimulate regeneration. Overexpression of Ascl1a in conjunction with deacetylase inhibitor allowed for Müller regeneration to INL neurons after injury in mouse retina (Ueki et al., 2015a). However, this was only successful after injury.

Under pathological conditions in the mouse retina, Müller cells reenter the cell cycle at G1 phase (Dyer and Cepko, 2000), but they do not replicate, instead reactive gliosis ensues. Nevertheless, this partial reentry indicates a latent regenerative ability which can be exploited. Yao et al. (2018) transfected Müller glial of adult mice with β-catenin under the control of a

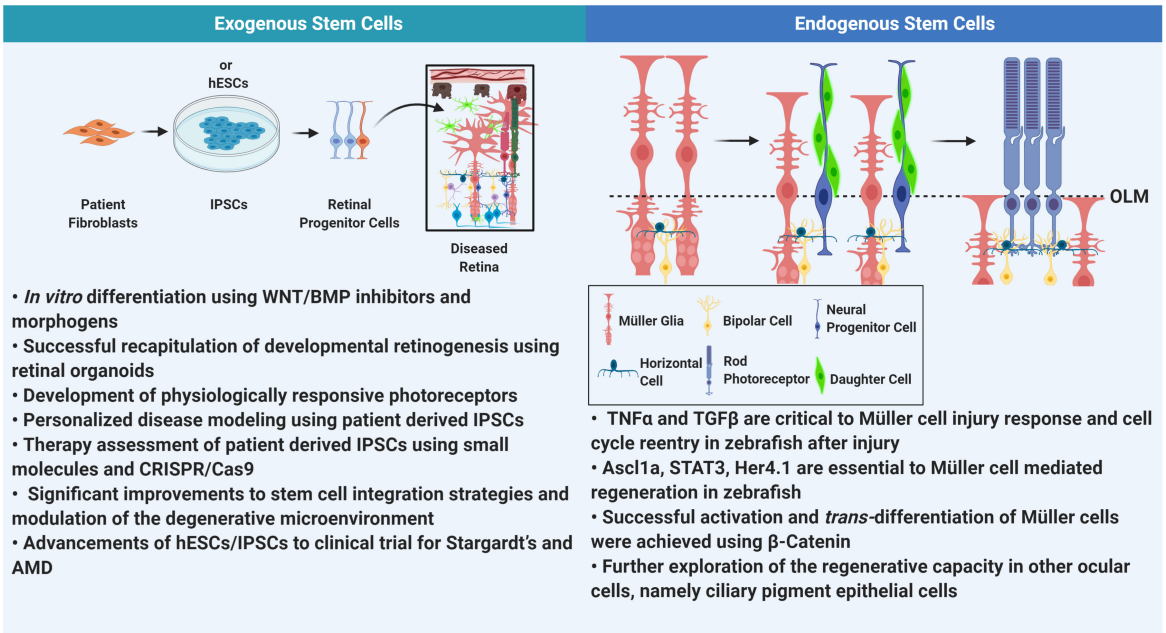

FIGURE 2 | Comprehensive summary. In this review we address some of the major advancements in stem cell technology as it relates to retinal degeneration. Understanding the degenerative milieu has improved stem cell integration into the retina. However, thus far only RPE derived from hESCs and IPSCs have progressed to clinical trials. On the other hand, retinal regeneration in mammalian tissue is in very nascent stages. Elucidation of pathways involved in regeneration in zebrafish has been successful in isolating elements that can be used to mobilizing Müller glia for regeneration in mammalian retina. (OLM, outer limiting membrane).

GFAP promotor using AAV (AAV; ShH10-GFAP-β-catenin) to promote cell cycle reentry (Yao et al., 2018). This injection was followed by another injection of OTX2, CRX, and NRL under the same promotor to stimulate rod photoreceptor regeneration. With this second injection another construct was delivered in order to express a reporter gene, td-tomato, under the control of the rhodopsin promotor. It was determined that Müller glial underwent one complete cell cycle before producing the daughter precursor cell. By examining td-tomato expression, the rod precursor initially had Müller-like morphology with radial processes. This cell produced two daughter cells, one of which maintained this Müller cell morphology and remained in the INL and eventually stopped expressing TD-tomato, as it became more Müller-like. While the other, Müller-derived rod-precursor, maintained td-tomato expression, was localized to the ONL, and matured into a rod photoreceptor that integrated into the cellular network of $Gnat^{rd17}Gnat^{cpfl3}$ mutants. Single cell recordings suggested presence of functional outer segments. This seminal work by Yao et al. (2018) provides strong evidence of the regenerative capacity of Müller glial using WNT signaling effector β-catenin. Given these findings, studies should employ a similar approach in highly degenerative mutants such as Rho^{P23H} or $PDE6\beta^{rd1/rd1}$ where retinas undergo extensive remodeling and gliosis, then substantiate any recovery with more stringent physiological data.

Additionally, many studies have attempted to harness Hippo pathway effectors for Müller cell regeneration. The Hippo pathway is a highly conserved pathway necessary for mediating organ growth, and has been associated with hepatic regeneration (Grijalva et al., 2014). In order to inhibit organ growth in the mammalian cell, Hippo pathway effectors, Yes-associated protein (Yap) and Tafazzin (TAZ) undergo constitutive proteasomal degradation in the cytosol to prevent translocation to the nucleus where they would mediate the expression of mitogenic factors (Moya and Halder, 2019). Instead of inducing regeneration by gene transfer like Yao et al. (2018), Rueda et al. (2019) took a different approach by disrupting the mechanism inhibiting regeneration. Briefly, Rueda et al. (2019) demonstrated that by using an inducible transgenic model (Yap5SA), in which Yap is no longer degraded, cells can be reprogrammed to a progenitor-adjacent state. Once induced, a subset of cells ($Yap5SA^{+}$ cells) were absent for GFAP, but significantly upregulated cyclin D1 and other cell cycle factors after retinal damage by *N*-methyl-D-aspartate (NMDA).

Thus far, investigations of Müller cell regeneration has yielded a superficial understanding of Müller cell response after injury, potentiating pathways to induce *trans*-differentiation or to block mechanisms that prevent regeneration in the mammalian retina. However, questions remain regarding the potential for regeneration during or after reactive gliosis. What changes? Does the retina have regenerative capacity long after the retinal environment has deteriorated? Current studies have validated the idea of retinal regeneration. Now, further evaluation of the regenerative capacity of the retina in severe degenerative models or models of end stage degeneration will move the field closer to clinical translation.

Ciliary Pigment Epithelial Cells

The ciliary pigment epithelium (CPE) is an extension of the RPE and forms part of the ciliary body (Chang et al., 2016). Many have suggested that CPE cells may also have some regenerative capacity. In lower vertebrates the ciliary margin zone is a site of continued neurogenesis (Centanin et al., 2011), and the localization of the CPE stem cells suggests an analogous region of stem cells may also exist in the mammalian eye (Fischer and Reh, 2001; Raymond et al., 2006). Not only have there been clinical reports of neoplasm (though rare) forming in the ciliary body (Chang et al., 2016), but CPE cells have demonstrated a capacity for clonal expansion *in vitro* (using spheroid assay) where cells express retinal progenitor markers (nestin, CHX 10) and can *trans*-differentiate to neural cell types (Tropepe et al., 2000). Furthermore, cells at the ciliary margin of the mouse eye have shown relatively increased expression of cyclin D2, which is critical for G1/S phase transition (Marcucci et al., 2016).

Nevertheless, the stem cell population amongst these cells is quite small, and some even dispute this characterization of CPE cells as stem cells (Frøen et al., 2013). Gualdoni et al. (2010) demonstrated that CPEs were able to expand as a monolayer and as spheroids (to a lesser degree). Their expression profiles indicated the presence of progenitor markers, visual homeobox (CHX10) and retinal homeobox (RX), however, they never became stem cells because they concomitantly expressed and maintained epithelial characteristics. Additionally, exposure to the morphogens discussed in the prior section "Embryonic Stem Cells and Retinal Differentiation of Pluripotent Cells" did not induce CPEs to *trans*-differentiate and become photoreceptors precursors as was previously reported.

Many discrepancies exist regarding the regenerative capacity of CPE cells. RPE cells have also shown some capacity for self-renewal and can dedifferentiation to express progenitor markers. Transformed lines such hTERT-RPE1 can also be induced to express IRBP, recoverin, cone opsin, arrestin and transducin (Yan et al., 2013). Therefore, CPEs and RPE cells do appear to have multipotent capacities, and further research is required to understand the mechanisms involved in *trans*-differentiation. However, considering the small number of endogenous putative CPE stem cells in the mammalian retina, expansion and propagation is a prohibitive factor that may discourage the use of CPE cells for retinal regeneration.

CONCLUDING REMARKS

Figure 2 highlights the major themes addressed in this review. However, considering these advances, RPE cells derived from hESCs are the first to show a strong translational capacity, as therapies are currently under clinical trials. However, ethics and limited sourcing are still important barriers to consider. This approach has proven effective, but the ideal therapy, would be to simply replace the dying photoreceptors themselves. This treatment would be ubiquitous, and completely independent of the underlying pathology or injury. Recent developments have provided a strong framework in which we can successfully induce retinal cells from pluripotent stem cells. Next steps should involve further exploration of the nuances of the degenerative environment in order to combine cell replacement therapies with drugs or molecules that can improve the environment and facilitate integration, though integration will always remain a limiting factor to this form of treatment. The most promising advent, however, is the elucidation of mechanisms that induce retinal regeneration. A regenerating retina will not face graft rejection, and because the cells emanate from the retina itself, there are no challenges to integration. However, understanding how this regenerative capacity may be affected by pathological insult still remains a key component needed to make the advancement toward therapeutics. Changes to cellular plasticity and increasing ECM rigidity may negatively impact the retina's ability to regenerate. Furthermore, in the case of RP and AMD, where most photoreceptor loss is late onset, newly regenerated PRs must sustain and function in a harsher microenvironment than the first iteration of cells, so perhaps in addition to induction, patients may have to consistently supplement, to maintain retinal health. In the antithetical instance, where degeneration happens early in life, other options have to be contemplated. A younger retina could be more amenable to regeneration, but will gene therapies have to be delivered in conjunction with regenerative cues? Considering the vast number of acquired and inherited retinal diseases, cell replacement therapy would be the most ubiquitous and efficacious therapy. The advancements discussed here suggest a viable future, should the challenges of integration and regeneration and its remaining complexities be met.

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Integrated Microarray and RNAseq Transcriptomic Analysis of Retinal Pigment Epithelium/Choroid in Age-Related Macular Degeneration

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We report for the first time an integrated transcriptomic analysis of RPE/choroid dysfunction in AMD (mixed stages) based on combining data from publicly available microarray (GSE29801) and RNAseq (GSE135092) datasets aimed at increasing the ability and power of detection of differentially expressed genes and AMD-associated pathways. The analysis approach employed an integrating quantitative method designed to eliminate bias among different transcriptomic studies. The analysis highlighted 764 meta-genes (366 downregulated and 398 upregulated) in macular AMD RPE/choroid and 445 meta-genes (244 downregulated and 201 upregulated) in non-macular AMD RPE/choroid. Of these, 731 genes were newly detected as differentially expressed (DE) genes in macular AMD RPE/choroid and 434 genes in non-macular AMD RPE/choroid compared with controls. Over-representation analysis of KEGG pathways associated with these DE genes mapped revealed two most significantly associated biological processes in macular RPE/choroid in AMD, namely the neuroactive ligand-receptor interaction pathway (represented by 30 DE genes) and the extracellular matrix-receptor interaction signaling pathway (represented by 12 DE genes). Furthermore, protein-protein interaction (PPI) network identified two central hub genes involved in the control of cell proliferation/differentiation processes, *HDAC1* and *CDK1*. Overall, the analysis provided novel insights for broadening the exploration of AMD pathogenesis by extending the number of molecular determinants and functional pathways that underpin AMD-associated RPE/choroid dysfunction.

Keywords: age-related macular degeneration, retinal pigment epithelium, neurodegeneration, transcriptome, neuroactive ligand-receptor, extracellular matrix

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INTRODUCTION

The pathogenesis of age-related macular degeneration (AMD), a leading cause of irreversible blindness in the world, is linked to degenerative changes in the retina, retinal pigment epithelium (RPE) and choroid. Major risk factors for AMD are advanced age, family history and smoking (Klein et al., 2007; Wang et al., 2007). At the cellular level, DNA damage, oxidative stress, inflammation, mitochondrial dysfunction, cellular senescence, abnormal metabolism, and aberrant proteolysis contribute to AMD development (Kay et al., 2014; Wang et al., 2019; Blasiak, 2020).

Located between the neuroretina and choriocapillaris, the RPE is a major tissue involved in pathogenesis sustaining retinal function through metabolite exchanges, protein secretion, phagocytosis of spent photoreceptor outer segments, and immune barrier function through interaction with Bruch's membrane, the basement membrane of the RPE (Strauss, 2005; Sparrow et al., 2010). Impaired RPE function has been shown to precede photoreceptors' death in AMD, leading to progressive degeneration of the neuroretina. Accumulation of medium and large-size drusen, lipo-proteinaceous deposits found below the RPE's basement membrane (Mitchell et al., 2018; Wang et al., 2019; Blasiak, 2020) is a significant factor in AMD progression from early to the advance disease, evidenced by population-based cohorts (Klein et al., 2007; Wang et al., 2007). The choriocapillaris, a vascular endothelium situated just beneath the RPE and Bruch's membrane provides nutrients and oxygenation to the RPE (Whitmore et al., 2015) and also represents a major site of age-related degenerative changes with reduced vascular endothelial density (Ramrattan et al., 1994), vulnerability to inflammation through the membrane attack complex with increasing age, together contributing to AMD (Mullins et al., 2014). However, to date the precise molecular mechanisms of AMD pathogenesis and progression from early to advanced stages are incompletely understood (Ardeljan and Chan, 2013). Significant amount of research in recent years has concentrated on the complement pathway and inflammatory processes, but new emerging treatments targeting only the complement pathway failed to improve clinical outcomes in phase 3 trials (Mitchell et al., 2018). Clearly, an integrated research approach considering other contributing pathogenic mechanisms is needed to identify novel and viable therapeutic targets.

Transcriptomic data, gathered by microarray (Booij et al., 2009; Newman et al., 2012; Whitmore et al., 2013), RNAseq (Whitmore et al., 2014; Kim et al., 2018) or very recently advanced single-cell (sc)RNAseq (Voigt et al., 2019; Orozco et al., 2020) studies provide a solid starting point for the study of the molecular determinants of RPE/choroid dysfunction in AMD (Morgan and DeAngelis, 2014; Tian et al., 2015). Publicly available transcriptomic datasets allow targeted analyses of specific cellular processes, pathways, and their interactions. To date, transcriptomic RPE/choroid analyses focused on topographic regions, specifically macular versus non-macular retinal regions, have revealed different transcription profiles in these regions associated with various macular dystrophies and degenerative retinal diseases, including Best disease, Stargardt's disease and retinitis pigmentosa (Whitmore et al., 2014; Ashikawa et al., 2017). However, identification of the causative differentially expressed genes between AMD and age-matched controls from individual experiments is far from conclusive to date, conceivably due to the relatively small sample sizes of many datasets often compounded by AMD phenotype heterogeneity within the datasets [early and advanced AMD, geographic atrophy (GA), and neovascular (NV) AMD samples] and further confounded by the transcriptomic characteristics of aging biology (De Magalhães et al., 2009; Whitmore et al., 2013; Orozco et al., 2020). This is reflected in the generally small overlap between differentially expressed genes from specific AMD datasets. Other confounding factors may also include different sample preparation methods, transcriptomic platforms and data analysis methods employed across different studies (Tian et al., 2015).

An integrating quantitative method of analysis of combined datasets can eliminate bias between transcriptomic studies and increase the power of detection of differentially expressed genes (Zhou et al., 2016; Brown et al., 2017; Ma et al., 2017; Alimadadi et al., 2020). Here, we describe such an analysis approach applied to investigate different platforms of publicly available transcriptomic datasets of post-mortem human AMD RPE/choroid. The differential gene expression patterns, pathway analysis and networks of protein-protein interactions (PPI) were explored in the combined datasets.

MATERIALS AND METHODS

Data Collection

Publicly available post-mortem human AMD RPE/choroid transcriptome datasets were accessed through the NCBI GEO and ArrayExpress databases combined with a literature review for individual datasets. The post-mortem human AMD RPE/choroid transcriptome data generated by microarrays and RNAseq were selected and filtered using the following criteria: (1) data published between January 2010 and February 2020; (2) complete gene expression data available (raw or normalized); (3) sample size equal or higher than 10 in each group (AMD and control); (4) original specimens divided into macular and non-macular samples. Only two datasets passed these criteria and were included in our study, GSE135092 and GSE29801. GSE135092 originated from an RNAseq study performed by Illumina HiSeq2500. The respective gene expression data provided by this dataset was quantified by HTSeqGenie as reads per kilobase of gene model per million total reads (RPKM), then normalized by DESeq2 (Orozco et al., 2020). GSE29801 dataset originated from a study using the Agilent G4112F array, obtained after quality control, background subtraction, and normalization as described by Newman et al. (2012).

Data Analysis

To integrate the different study platforms, we used the two-step conventional metanalysis approach described by Ma et al. (2017) For each platform, individual analyses were performed separately using the appropriate and specific bioinformatics pipeline for the respective application (e.g., edgeR or DESeq2 or limma for RNAseq and limma for microarray). We then combined the *p*-values obtained, setting the statistical significance threshold for each gene based on the result of this combined *p*-value (Tseng et al., 2012). The combined *p*-value is widely used in meta-analysis statistics of differential expressed genes since it is simple and versatile – it was shown to be applicable to analysis of both multiple microarray datasets and combined microarray and RNAseq datasets (Tseng et al., 2012; Ma et al., 2017). The diagram of data processing is shown in **Figure 1**. The gene expression table from each individual dataset was annotated and analyzed by the

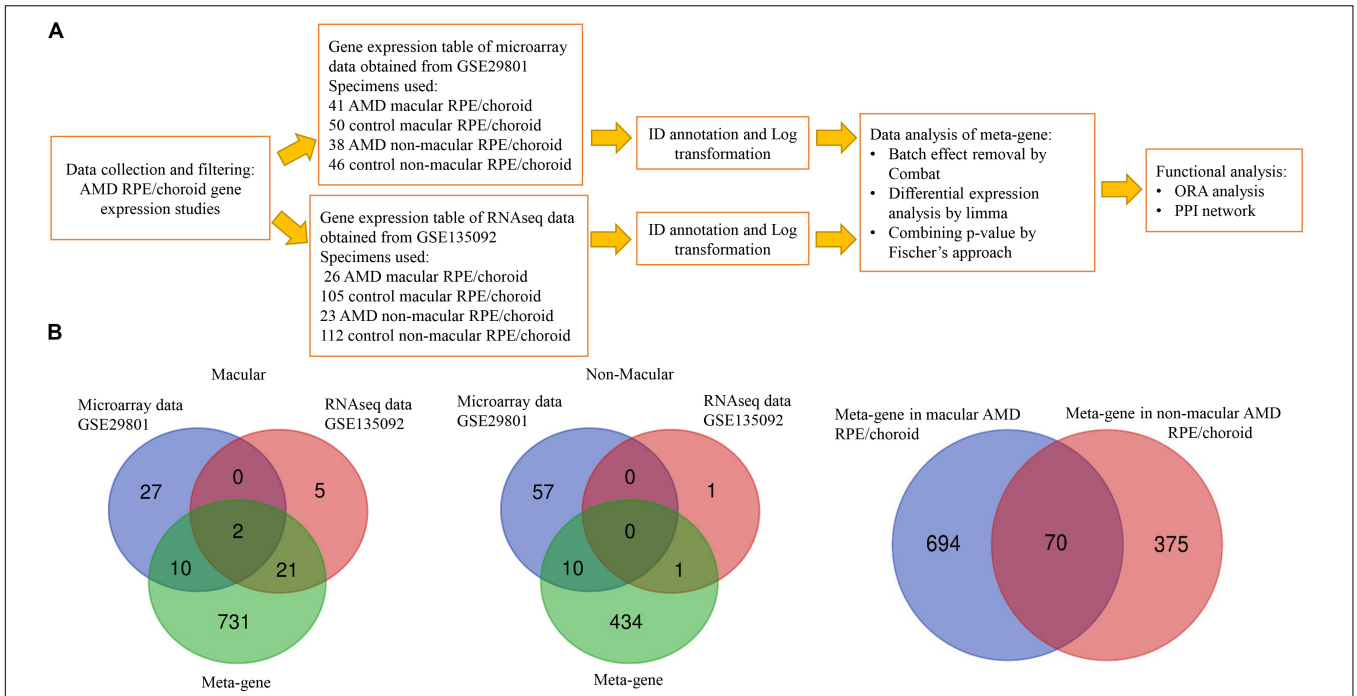

FIGURE 1 | Data processing workflow and common genes between the datasets analyzed. **(A)** Data processing workflow. **(B)** Venn diagram showing the overlap of differentially expressed genes identified by microarrays, RNAseq and meta-gene in macular and non-macular locations of RPE/choroid in AMD.

web-based analysis tool Networkanalyst[1] (Xia et al., 2014, 2015; Zhou et al., 2019). The identifiers (IDs) from different platforms (ENSEMBL gene IDs for RNAseq and probe IDs for microarrays) were converted to Entrez gene IDs. The log transformation by variance stabilizing normalization (VSN) in combination with quantile normalization was performed for microarray data. Similarly, RNAseq data were transformed to log2 counts per million by the log2 count procedure. Differential expression (DE) analysis of each study was performed by limma using adjusted $p < 0.05$ from Benjamini-Hochberg's False Discovery Rate (FDR) (Ritchie et al., 2015). To make data comparable, the batch effect between studies was minimized using the ComBat algorithm and then examined by principal component analysis (PCA) (**Supplementary Figures S1**, **S2**; Johnson et al., 2007). The batch effect removal algorithm (ComBat) was also beneficial in background noise reduction, through the removal of genes with totally absent expression in more than 80 percent of samples whilst equally reducing the variability of gene expression levels between batches (Johnson et al., 2007; Zhou et al., 2016). Using Fischer's approach for meta-analysis, each study p-value was combined together using the formula below.

$$F_g = -2\sum_{s=1}^{s}\left(\ln(P_{gs})\right)$$

A calculated combined p-value for each gene was considered significant if lower than 0.05 (Fisher, 1992; Xia et al., 2015; Alimadadi et al., 2020). The differential significant gene list obtained was then called the meta-gene dataset in this paper.

Data Interpretation and Functional Analysis

The resulting meta-gene list was compared with the original DE gene list in each of the original studies. To identify significant pathways from the meta-gene list, over-representation analysis (ORA) was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) and p-values were adjusted by Benjamini-Hochberg's False Discovery Rate (FDR). A protein-protein interaction (PPI) network was constructed based on STRING database (Szklarczyk et al., 2019) and then visualized by a web-based tool[1] (Xia et al., 2014, 2015; Zhou et al., 2019). Hub nodes were identified by high degrees and high centrality from the PPI network. The results were then compared with the network constructed by WEB-based GEne SeT AnaLysis Toolkit (Zhang et al., 2005; Wang et al., 2013, 2017; Liao et al., 2019).

RESULTS

RPE/Choroid AMD Transcriptomic Datasets

Five post-mortem human RPE/choroid AMD transcriptome studies were identified through the literature review shown in **Supplementary Table S1**: two microarrays studies, one RNAseq, one scRNAseq and a recent study using both RNAseq and

[1]https://www.networkanalyst.ca/

scRNAseq. All of these datasets were accessible through the NCBI GEO database but only two fulfilled our inclusion criteria, as follows. The dataset GSE29801 was generated by a study using Agilent Whole Human Genome 4 × 44K *in situ* oligonucleotide array platform (G4112F array) (Newman et al., 2012). In this study eyes with either a clinical or pathological diagnosis of AMD and with age ranging from 43 to 101 years were analyzed making use of 41 AMD macular RPE/choroid specimens [9 advanced AMD, 16 intermediate, 10 early and 6 undefined stage using the Age Related Eye Diseases (AREDS) classification], 50 control macular RPE/choroid specimens, 38 AMD non-macular RPE/choroid specimens (9 advanced, 14 intermediate, 9 early and 6 undefined stage) and 46 control non-macular RPE/choroid specimens. The GSE135092 dataset was provided by an RNAseq study of eyes with a clinical diagnosis of AMD using the AREDS classification and ages ranging from 59 to 98 years, performed using the Illumina HiSeq2500 platform (Orozco et al., 2020). The study analyzed 26 AMD macular RPE/choroid specimens (mixed advanced stages), 105 control macular RPE/choroid specimens, 23 AMD non-macular RPE/choroid specimens and 112 control non-macular RPE/choroid specimens (**Figure 1**).

Meta-Gene Dataset

The DE genes identified as a result of the combined analysis are referred to as meta-genes. DE genes were analyzed by integration of the two selected datasets through Networkanalyst web-based software. Initial analysis of individual datasets by limma with an FDR < 0.05 found only 10 DE genes (**Supplementary Table S2**) in macular and 57 DE genes in non-macular AMD RPE/choroid (**Supplementary Table S3**). To further interrogate the differences between AMD and control RPE/choroid, a more sensitive method involving Fischer's approach was then applied to the integrated data. After data normalization and batch effect adjustment, the PCA plot did not show major differences between studies, which indicated that the batch effect was reduced between the two studies (**Supplementary Figures S1**, **S2**). By using Fisher's approach for combining *p*-value, 764 significant meta-genes (366 down-regulated and 398 up-regulated) were detected in macular AMD RPE/choroid (**Supplementary Table S4**) and 445 meta-genes (244 down-regulated and 201 up-regulated) in non-macular AMD RPE/choroid (**Supplementary Table S5**). By ranking the combined *p*-values, the top 20 significant genes in macular and non-macular AMD RPE/choroid, respectively, were obtained and shown in **Table 1**.

The extent of overlap between meta-genes and original DE genes detected in each study (**Supplementary Tables S2**, **S3**) is shown in the Venn diagrams in **Figure 1**. A higher degree of overlap was identified in macular AMD RPE/choroid, with *PRSS33* and *SMOC1* detected as common DE genes in all datasets. No overlap of DE genes was detected between all three groups of genes in non-macular AMD RPE/choroid. Thirty-one genes were common between the microarray or RNAseq datasets, and the meta-genes of macular AMD RPE/choroid, while 11 common genes were detected in non-macular AMD RPE/choroid. In our analysis, 731 genes were newly detected as DE genes in macular and 434 genes in non-macular AMD RPE/choroid.

TABLE 1 | Meta-gene list showing top differentially expressed genes.

EntrezID	Gene symbol	F_g	Combined *p*-value	Effect
Differential expressed genes identified in macular AMD RPE/choroid vs. macular control RPE/choroid				
84624	FNDC1	−47.991	1.72E-05	Up-regulated
4060	LUM	−46.502	1.75E-05	Up-regulated
131578	LRRC15	−40.042	0.00019	Up-regulated
5803	PTPRZ1	−38.086	0.00032	Up-regulated
9547	CXCL14	−38.22	0.00032	Up-regulated
8148	TAF15	−35.072	0.00102	Up-regulated
4804	NGFR	−34.669	0.00109	Up-regulated
3381	IBSP	−31.756	0.00272	Up-regulated
3371	TNC	−31.912	0.00272	Up-regulated
1118	CHIT1	−31.852	0.00272	Up-regulated
1515	CTSV	−31.612	0.00272	Up-regulated
84466	MEGF10	−31.106	0.00278	Up-regulated
2224	FDPS	−31.247	0.00278	Up-regulated
6695	SPOCK1	−30.827	0.00287	Up-regulated
55827	DCAF6	40.05	0.00019	Down-regulated
64093	SMOC1	37.387	0.00039	Down-regulated
7066	THPO	31.517	0.00272	Down-regulated
100128731	OST4	31.747	0.00272	Down-regulated
2619	GAS1	32.158	0.00272	Down-regulated
83473	KATNAL2	31.124	0.00278	Down-regulated
Differential expressed genes identified in non-macular AMD RPE/choroid vs. non-macular control RPE/choroid				
54108	CHRAC1	−40.355	0.00066	Up-regulated
10648	SCGB1D1	−36.412	0.00216	Up-regulated
64116	SLC39A8	−31.469	0.00826	Up-regulated
84656	GLYR1	−30.629	0.00826	Up-regulated
79095	C9orf16	−30.731	0.00826	Up-regulated
6422	SFRP1	−28.224	0.01414	Up-regulated
1974	EIF4A2	32.756	0.0081	Down-regulated
58155	PTBP2	30.667	0.00826	Down-regulated
400073	C12orf76	31.225	0.00826	Down-regulated
146225	CMTM2	29.187	0.0118	Down-regulated
65982	ZSCAN18	29.314	0.0118	Down-regulated
23564	DDAH2	29.244	0.0118	Down-regulated
115761	ARL11	28.924	0.01223	Down-regulated
6404	SELPLG	27.816	0.01414	Down-regulated
84695	LOXL3	27.903	0.01414	Down-regulated
8936	WASF1	27.635	0.01414	Down-regulated
8675	STX16	27.52	0.01414	Down-regulated
8803	SUCLA2	27.535	0.01414	Down-regulated
54816	ZNF280D	28.257	0.01414	Down-regulated
3187	HNRNPH1	27.798	0.01414	Down-regulated

Among the meta-genes, 70 genes were similarly differentially expressed in both macular and non-macular AMD RPE/choroid (**Supplementary Table S6**).

Furthermore, because AMD samples in GSE135092 consisted of mixed advanced stages of AMD (GA and NV AMD), and samples in GSE29801 consisted of advanced stages (GA and NV

AMD), intermediate, and early stage of AMD, we performed subgroup analysis combining each AMD stage subgroup (early, intermediate, "mixed" advanced AMD) from GSE29801 with all GSE135092 samples (**Supplementary Table S7**). Interestingly, the presence of advanced AMD predominantly influenced the expression of genes included in the 764 meta-genes identified as DE in macular RPE/choroid, a stepwise reducing trend identified in intermediate then early stage of AMD, respectively (**Supplementary Figure S3**). However, to maximize the number of samples and therefore power in this analysis, we used the meta-genes from all RPE/choroid samples in further downstream analyses.

KEGG Pathway Analysis

To interrogate the functional significance of meta-genes, over-representation analyses (ORA) of KEGG pathways were applied to both macular and non-macular meta-genes identified. Applying FDR < 0.05, the interactions with the neuroactive ligand-receptor and the extracellular matrix (ECM)-receptor interaction pathways were statistically significant in macular AMD RPE/choroid, while there was no statistically significant pathway identified in non-macular AMD RPE/choroid. **Table 2** shows the top 5 KEGG pathways and meta-genes in each pathway found in macular and non-macular AMD RPE/choroid.

Among the identified significant genes associated with the neuroactive ligand-receptor interaction, 13 genes were found down-regulated in macular AMD RPE/choroid including *ADRA1A, LEPR, PENK, SCT, BDKRB1, ADRB3, PTGDR, BDKRB2, RLN3, C5, EDN3, GABRE*, and *NPY1R*. *LEPR* or Leptin Receptor Factor was the second highest significant down-regulated gene. LEPR was initially identified as a satiety factor, but was subsequently shown to play a role in normal aging and neuroprotective processes (Gorska et al., 2010; Seshasai et al., 2015; Wauman et al., 2017). Other genes upregulated in the neuroactive ligand-receptor interaction pathway included *GRIK3, GRPR, CHRNA1, ADRA1D, OXTR, NPFFR1, P2RY2, MC5R, GABRB1, GRIA1, TRH, GCGR, MTNR1A, HTR2A, GRIN2C, CHRNB4*, and *GABRG3*.

All 12 significant genes associated with the ECM-receptor interaction pathway were upregulated in macular AMD RPE/choroid, with a distinct sub-pathway represented by a group of collagen genes including *COL6A3, COL9A3*, and *COL9A2*. The most statistically significant gene in the ECM group was *TNC* or Tenascin C, which encodes a key ECM component in the nervous system altered in various eye diseases (Kobayashi et al., 2016). Tenascin C also plays a role in inflammation process by regulating transforming growth factor β (TGFβ) (Reinhard et al., 2017). Noteworthy, *TGFB2* gene, an isoform of TGFβ, was also identified as up-regulated in both macular and non-macular meta-gene lists. Although not reaching statistical significance in this analysis, the fatty acid metabolism pathway was also among the enriched pathways in macular AMD RPE/choroid. Remarkably, all meta-genes associated with this pathway, consisting of *ELOVL3*, *FASN, ACAT2, FADS2, HADHB, HSD17B4, SCD*, and *FADS1*, were not differentially expressed in non-macular AMD RPE/choroid.

PPI Network Analysis

Since the macula is the primary anatomical area affected in AMD, we sought to get more insight into the genes differentially expressed in macular AMD RPE/choroid by further exploring them by through a PPI network. For this purpose, a PPI network was constructed using STRING database and Networkanalyst web-based tools, with the input of 764 significant genes from macular AMD RPE/choroid meta-gene list. Initially, a first order network created an extensive network comprising 1718

TABLE 2 | ORA analysis showing top KEGG pathways involving the meta-genes.

Pathway	*p*-value	FDR	Differential expressed gene (gene symbol)
Macular AMD RPE/choroid vs. macular control RPE/choroid			
Neuroactive ligand-receptor interaction	0.000126	0.0297	CHRNA1; GRIA1; OXTR; GABRB1; NPFFR1; SCT; GRIK3; ADRA1D; TRH; HTR2A; GRPR; ADRA1A; C5; P2RY2; PENK; LEPR; BDKRB2; BDKRB1; GABRE; PTGDR; CHRNB4; EDN3; GCGR; NPY1R; GRIN2C; GABRG3; MTNR1A; ADRB3; MC5R;RLN3
ECM-receptor interaction	0.000187	0.0297	COMP; RELN; IBSP; ITGB4; ITGA3; TNC; SPP1; COL6A3; COL9A3; COL9A2; THBS2; THBS4
AMPK signaling pathway	0.000626	0.0664	SREBF1; CAB39L; IRS2; PPP2R3A; FOXO3; EEF2; ADRA1A; G6PC2; PFKL; SCD; FASN; LEPR; PPARG; PCK2
Wnt signaling pathway	0.00126	0.0999	APC2; CAMK2B; MMP7; FZD9; WNT9B; CACYBP; DKK1; DKK2; SFRP1; SFRP2; APC; TBL1XR1; BAMBI; RSPO3; GPC4; LGR5
Fatty acid metabolism	0.0018	0.102	ELOVL3; FASN; ACAT2; FADS2; HADHB; HSD17B4; SCD; FADS1
Non-macular AMD RPE/choroid vs. non-macular control RPE/choroid			
Choline metabolism in cancer	0.00445	0.363	WASF1; WAS; PLA2G4C; AKT2; PIK3R3; DGKH; MAPK10
Regulation of actin cytoskeleton	0.00495	0.363	WASF1; DIAPH2; WAS; TMSB4X; ITGA3; SPATA13; PIK3R3; ITGA6; ITGAE; ARHGEF7; FGD3
Osteoclast differentiation	0.00511	0.363	OSCAR; IFNAR1; TYROBP; AKT2; PIK3R3; TGFB2; MAPK10; LCK
Influenza A	0.00796	0.363	HLA-DRB5; DNAJB1; IFNAR1; XPO1; AKT2; IL18; PIK3R3; PYCARD; IFNA10
Hypertrophic cardiomyopathy (HCM)	0.00836	0.363	PRKAB2; ITGA3; ITGA6; TGFB2; CACNA1C; DAG1

nodes and 2578 edges. To improve the clarity of the network and obtain more important nodes, we created a zero order PPI network (**Figure 2**). This network contains 14 nodes with the highest degree of 7. Two downregulated genes with the highest degrees and high centrality were Histone Deacetylase 1 (*HDAC1*) and Cyclin-dependent kinase 1 (*CDK1*). HDAC1 and CDK1 are both cell cycle regulators (Göder et al., 2018) suggesting altered cell proliferation responses in macular AMD RPE/choroid. We also input these 764 DE genes in AMD macular RPE/choroid into the WEB-based pathway analysis tool "GEne SeT AnaLysis Toolkit." GEne SeT AnaLysis Toolkit constructs networks by using Network Topology-based Analysis method and used PPI BIOGRID as its reference list (Liao et al., 2019). The result revealed HDAC1 and CDK1 among the top five per cent of these genes when ranked by random walk probability (**Supplementary Table S8**).

DISCUSSION

The increasing microarray and RNAseq transcriptomic datasets available provide an important resource for exploring, at a molecular level, the pathogenic machinery of AMD through bioinformatics approaches (Morgan and DeAngelis, 2014; Tian et al., 2015). However, analysis of individual AMD transcriptomic datasets with conventional statistical approaches may not enable comprehensive identification of DE genes and pathways in functionally impaired RPE/choroid. For example, the microarray analysis undertaken by Whitmore et al. (2013) concluded that there were no significantly DE genes when FDR was applied to the respective AMD RPE/choroid dataset. Similarly, the RNAseq analysis described by Orozco et al. (2020) also highlighted less than 30 putative causal genes for AMD RPE/choroid. Analysis approaches combining different transcriptomic datasets obtained from different platforms were recently used to detect more DE genes in various diseases, such as dilated cardiomyopathy (Alimadadi et al., 2020), Alzheimer's disease (Su et al., 2019), tuberculosis (Wang et al., 2018), rheumatoid arthritis (Badr and Häcker, 2019), and helminth infection (Zhou et al., 2016). These integrated analyses expand the number of specimens analyzed and are also well suited for AMD, given the multifactorial nature of the disease.

Here we report an analysis of normal and AMD RPE/choroid transcriptome data performed by integrating microarray

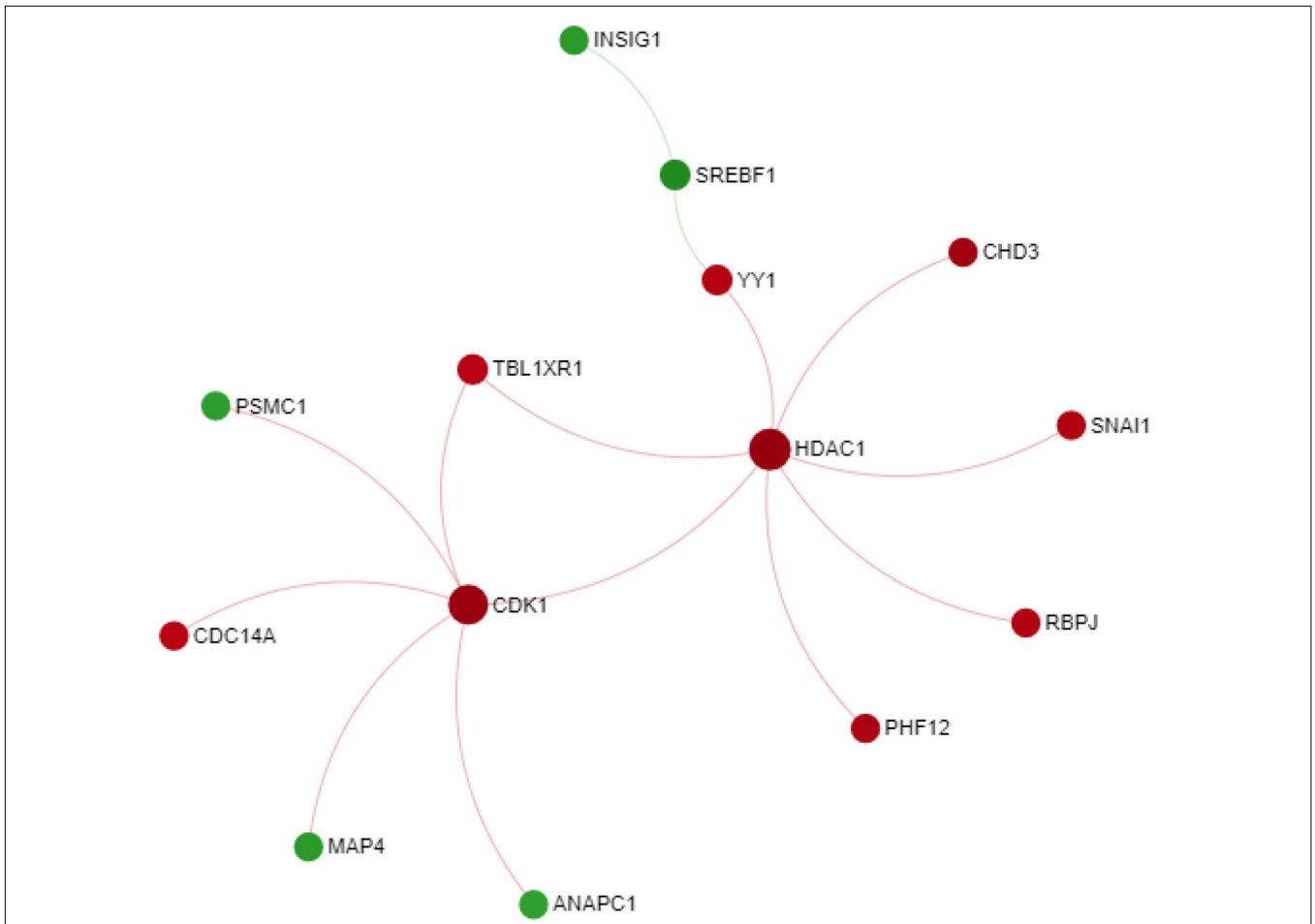

FIGURE 2 | Zero order PPI network of meta-gene in macular AMD RPE/choroid. Downregulated nodes in red; upregulated nodes in green.

and RNAseq datasets employing the web-based tool Networkanalyst (Xia et al., 2015) with Fischer's method (Fisher, 1992; Alimadadi et al., 2020). Our analysis extended the number of statistically significant differentially expressed RPE/choroid genes in AMD to 764 in macular RPE/choroid, and 445 in non-macular AMD RPE/choroid. The resulting meta-genes identified as significantly differentially expressed in macular AMD RPE/choroid in comparison with normal RPE/choroid highlighted two significantly enriched pathways of potential functional importance in AMD pathogenesis, the neuroactive ligand-receptor interactions and extracellular matrix (ECM)-receptor interactions.

The most significant pathway in macular AMD RPE/choroid, the neuroactive ligand-receptor interactions had a FDR equal to 0.0297 by ORA analysis. This pathway regulates multiple neuroreceptors and their associated distant signaling molecules such as leptin, thyrotropin releasing hormone (TRH) and epinephrine (Biernacka et al., 2013; Kanehisa et al., 2016). It was previously shown to be functionally significant in neurotransmitter-mediated disorders such as alcohol dependence disorder (Biernacka et al., 2013), autism spectrum disorders (Wen et al., 2016), Parkinson's disease (Hardy, 2010; Hamza et al., 2011; Kong et al., 2015), as well as some types of lung cancer (Ji et al., 2018). Our analysis suggested that 30 genes associated with this pathway may be linked to AMD, including *LEPR*, a receptor of leptin, which was initially identified in adipocytes (Gorska et al., 2010). Noteworthy, decreased serum leptin was observed in AMD patients in a case-control study and leptin was hypothesized to have a neuroprotective function and to lower the risk of AMD by removing extracellular β-amyloid in drusen deposits, decreasing triglyceride fatty acid synthesis and downregulating genes such as lipogenic enzyme, oxidative stress and inflammation related genes (Seshasai et al., 2015; Wauman et al., 2017). Our integrated data analysis identified the downregulation of leptin receptor in macular RPE/choroid in AMD for the first time. Cholinergic Receptor Nicotinic Alpha 1 Subunit (*CHRNA1*) and Cholinergic Receptor Nicotinic Beta 4 Subunit (*CHRNB4*), encoding two of the twelve gene subunits of the nicotinic acetylcholine receptor (Conti-Fine et al., 2000; Barrie et al., 2016), were found upregulated among the AMD meta-genes. The increased expression of these genes is associated with higher risk of lung cancer in smokers as the binding of the receptor by nicotine can stimulate angiogenesis especially within a context of inflammation and tumorigenesis (Yoo et al., 2014). The upregulation of *CHRNA1* and *CHRNB4* in AMD RPE/choroid may underlie one mechanism that contributes to the increased risk of AMD in smokers. Thyroid releasing hormone (TRH) has a central role in the thyroid hormone pathway that is found abnormal in some AMD patients. (Gopinath et al., 2016; Yang et al., 2018; Ma et al., 2020). Our analysis also showed that TRH, another gene linked to the neuroactive ligand receptor pathway, is upregulated in the AMD RPE/choroid.

Genes associated with the ECM-receptor interaction pathway in AMD, highlighted by our analysis, have previously been shown to have high variability of expression between individuals (Booij et al., 2009). The finding of multiple significantly upregulated genes associated with this pathway in AMD RPE/choroid underpins wound healing responses as putative pathophysiological mechanisms implicated in AMD (Newman et al., 2012). Tenascin C, the most statistically significant differentially expressed gene in this pathway, can upregulate TGFβ and promote inflammatory processes (Reinhard et al., 2017), in line with the increased level of Tenascin C identified in surgically excised choroidal neovascular membranes (Nicolò et al., 2000) and observation of its secretion in neovascular AMD (Kobayashi et al., 2016; Reinhard et al., 2017). Furthermore, although the fatty acid metabolism pathway was not found to be statistically significantly associated with AMD in our analysis, the finding that all differentially expressed genes in this pathway were found exclusively in macular RPE/choroid underlines the geographical differences in gene expression patterns between macular and non-macular RPE/choroid regions, previously suggested by Whitmore et al. (2014) and Ashikawa et al. (2017). Specific examples of genes with a macular pattern of differential expression were Fatty Acid Desaturase 1 (*FADS1*) and Fatty Acid Desaturase 2 (*FADS2*), genes encoding delta-5 and delta-6 desaturases, implicated in drusen formation in a recent study (Ashikawa et al., 2017). Hence, fatty acid metabolism abnormalities may contribute to drusen formation, an area of interest following the suggestion of secretion by the RPE of the lipid component of soft drusen, a hallmark of AMD progression (Curcio, 2018a,b).

The PPI network analysis highlighted two central hub genes involved in the control of cell proliferation/differentiation processes, *HDAC1* and *CDK1*. *HDAC1* encodes an isoform of histone deacetylase that is ubiquitously expressed and has a role in transcriptional repression (Hassig et al., 1998). Modification of chromatin structure through histone deacetylation has been identified as a mechanism of epigenetic regulation associated with various neurodegenerative diseases (Anderson et al., 2015). HDAC family members are involved in multiple biological processes including angiogenesis, inflammation and cell cycle progression, all of which play an important role in the pathophysiology of AMD (Tang et al., 2013). Noteworthy in this respect are the findings from a comparative study of Alzheimer's disease and AMD donors that showed that *HDAC1, 2, 5,* and *6* expression decreased in the retina and frontal cortex of affected individuals (Noh et al., 2008). The other hub node identified, *CDK1* or cyclin-dependent kinase 1 plays an important role in the regulation of mitotic transition and phosphorylation of Bcl-2, Bcl-XL, and Mcl–1 proteins (Harley et al., 2010; Terrano et al., 2010). In the context of AMD, a retinal transcriptome analysis of senescence-accelerated OXYS rats revealed a possible role of CDK1 in the retinal extrinsic apoptotic processes associated with AMD. Specifically, the study associated the increased apoptotic activity with *CDK1*, which was identified as a hub gene for functional clusters associated with the MAPK and p53 signaling pathways in the interaction network constructed from the respective transcriptomic data (Telegina et al., 2015).

A limitation of this analysis is due to the paucity of samples representing the individual disease stage phenotypes and respective subgroup analyses of AMD (early, intermediate, advanced) in the original studies (**Supplementary Figure S3**)

resulting in reduced power and the ensuing application of pathway analyses on combined datasets of mixed disease stages. Thus the advanced AMD refers here to mixed advanced stages of AMD (both GA and NV AMD). Clearly, an increase in the clinical data available with post-mortem RPE/choroid samples used in omic technologies could enable more detailed studies into the pathophysiological processes particular to each stages of AMD highlighting key progression factors to target for further therapeutic intervention research (Handa et al., 2019).

In conclusion, integration of microarray data and RNAseq data allows transcriptomic analyses of increased power and identification of DE meta-genes in AMD RPE/choroid. Taking such an approach, this study identified two novel pathways characterized by significant enrichment of DE genes in AMD RPE/choroid, namely the neuroactive-ligand receptor interaction pathway and the ECM-receptor interaction pathway. In addition, the PPI network analysis highlighted two hub nodes that may link apoptotic and angiogenesis pathological processes in AMD. The integrated functional analysis of DE genes in AMD also revealed genes previously linked to other neurodegenerative disease such as Alzheimer's disease and Parkinson's disease. The approach used to integrate publicly available transcriptomic datasets obtained through different experimental platforms provided a novel insight and broadened the exploration of a larger number of potential genes and functional pathways with roles in AMD pathogenesis.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: "Newman, A. M., Gallo, N. B., Hancox, L. S., Miller, N. J., Radeke, C. M., Maloney, M. A., et al. (2012). Systems-level analysis of age-related macular degeneration reveals global biomarkers and phenotype-specific functional networks. Gene Expression Omnibus. GSE29801 and Orozco, L. D., Chen, H. H., Cox, C., Katschke, K. J., Arceo, R., et al. (2020). Integration of eQTL and a Single-Cell Atlas in the Human Eye Identifies Causal Genes for Age-Related Macular Degeneration. Gene Expression Omnibus. GSE135092."

AUTHOR CONTRIBUTIONS

DD, XL, and LP designed the study and wrote the manuscript. DD and XL performed the data analysis. All authors discussed the results, reviewed and approved the final version of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00808/full#supplementary-material

FIGURE S1 | PCA plot of macular AMD RPE/choroid vs. control RPE/choroid representing the datasets before and after applying ComBat algorithm.

FIGURE S2 | PCA plot of non-macular AMD RPE/choroid vs. control RPE/choroid representing the datasets before and after applying ComBat algorithm.

FIGURE S3 | Venn diagram showing the overlap of differentially expressed genes identified by subgroup analysis in macular locations of RPE/choroid in AMD.

TABLE S1 | Transcriptome studies of postmortem human AMD RPE/choroid.

TABLE S2 | Comparison of differential expressed genes in macular AMD RPE/choroid.

TABLE S3 | Comparison of differential expressed genes in non-macular AMD RPE/choroid.

TABLE S4 | Meta-gene list of differential expressed genes in macular AMD RPE/choroid.

TABLE S5 | Meta-gene list of differential expressed genes in non-macular AMD RPE/choroid.

TABLE S6 | Similar meta-genes between macular and non-macular AMD RPE/choroid.

TABLE S7 | Comparison of differential expressed genes in different subgroups of macular AMD RPE/choroid.

TABLE S8 | Meta-gene in macular AMD RPE/choroid ranking by random walk probability using Network Topology-based Analysis.

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REVIEW
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Flavins Act as a Critical Liaison Between Metabolic Homeostasis and Oxidative Stress in the Retina

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Derivatives of the vitamin riboflavin, FAD and FMN, are essential cofactors in a multitude of bio-energetic reactions, indispensable for lipid metabolism and also are requisites in mitigating oxidative stress. Given that a balance between all these processes contributes to the maintenance of retinal homeostasis, effective regulation of riboflavin levels in the retina is paramount. However, various genetic and dietary factors have brought to fore pathological conditions that co-occur with a suboptimal level of flavins in the retina. Our focus in this review is to, comprehensively summarize all the possible metabolic and oxidative reactions which have been implicated in various retinal pathologies and to highlight the contribution flavins may have played in these. Recent research has found a sensitive method of measuring flavins in both diseased and healthy retina, presence of a novel flavin binding protein exclusively expressed in the retina, and the presence of flavin specific transporters in both the inner and outer blood-retina barriers. In light of these exciting findings, it is even more imperative to shift our focus on how the retina regulates its flavin homeostasis and what happens when this is disrupted.

Keywords: riboflavin, retina metabolism, oxidative stress, redox potential, mitochondria, fatty acid oxidation, retbindin

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INTRODUCTION

The water-soluble vitamin, riboflavin (vitamin B2, aka lactochrome) was first isolated in 1879 from milk whey and purified as orange-yellow crystals. Subsequently, since Eijkman's Nobel Prize-winning work in 1929, vitamins and their biological implications have become a matter of great interest to both biochemists and clinicians alike. As a vitamin, riboflavin is especially essential for human health due to its vast involvement in the bioenergetics, metabolism, growth, and survival of all cells (Powers et al., 2012; Ashoori and Saedisomeolia, 2014; Barile et al., 2016; Saedisomeolia and Ashoori, 2018; Suwannasom et al., 2020). So, the association between low riboflavin levels and various neurodegenerative disorders, metabolic dysfunctions, diabetes mellitus and inborn errors of metabolism, like multiple acyl-CoA dehydrogenation deficiency (MADD) (Reddi, 1986; Barile et al., 2016; Marashly and Bohlega, 2017; Xin et al., 2017; Saedisomeolia and Ashoori, 2018; Chen et al., 2019, 2020) is least surprising.

All proteins associating with flavins are collectively known as the flavoproteome (Lienhart et al., 2013). Being such a vast and diverse set of proteins, the extent of the flavoproteome involvement in numerous metabolic pathologies is only now surfacing out (Barile et al., 2016; Davis et al., 2016; Olsen et al., 2016; Fan et al., 2018; Balasubramaniam et al., 2019; O'Callaghan et al., 2019; Ryder et al., 2019). One such example is highlighted by Petrovski et al. (2015), where the authors

describe the case of a 20-month-old female, who initially was being treated for a progressive neurological disease on the supposition that the illness was due to an autoimmune disease. But she was completely non-responsive to this treatment and only upon exome sequencing it was discovered that the child had a compound heterozygous genotype of two loss of function mutations in *SLC52A2*, a brain-specific riboflavin transporter. Following this discovery, she was immediately administered high-dose riboflavin therapy (10–70 mg/kg) and within 2–4 weeks of the treatment, most of her symptoms subsided (Petrovski et al., 2015). But recent discoveries are indicating that the effects of riboflavin deficiency are not limited to only neurological disorders in new-born babies (Olsen et al., 2016; Ryder et al., 2019). In another recent case report, a previously healthy 34-year old woman was suddenly presented with severe hearing and vision loss within 6 months and subsequently led to bilateral optic nerve atrophy, dysphagia, severe dyspnea, and quadriplegia (Camargos et al., 2018). Upon whole-exome sequencing, she was found to be carrying a novel homozygous insertion of 60 bp in *SLC52A3*, another riboflavin transporter (Camargos et al., 2018). High-dose riboflavin therapy (1,800 mg/day) for 6 months was able to improve her respiratory abilities and allowed her to walk with support but could not restore her neurosensory or visual loss (Camargos et al., 2018). Similarly, adult patients suffering from riboflavin deficiency due to malnutrition have been previously reported to have developed significant vision problems, including reduced rod and cone responses (Kruse et al., 1940). However, the majority of these studies date back a few decades and the renewed spotlight on vision loss due to riboflavin transporter mutation, calls for a comprehensive review of how diet or genetically induced riboflavin deficiency may affect the retina. The retina is a complex tissue lying at the back of the eye and formed of multiple layers of neuronal cells (**Figure 1**). Being metabolically active in both darkness and under light makes the retina (**Figure 1**) one of the most energy-consuming tissue as well as one of the most flavin enriched tissues. Thus, our focus in this review is to lay the foundation for future research on flavin homeostasis in the retina by highlighting the metabolic pathways flavins are intrinsically involved in and how dysregulation of these pathways is known to be associated with various debilitating retinal pathologies.

FLAVINS AND THEIR UNIQUE CHEMISTRY

The chemical structure of the tricyclic molecule riboflavin (aka 7,8-dimethylbenzo-pteridine-2,4-dione) is constituted of a ribitil side chain attached to an isoalloxazine ring, which is a benzene ring attached to a pteridine ring system (**Figure 2**; Massey, 2000; Powers, 2003). The presence of the pteridine ring gives it the name benzopteridine and reflects its relationship with another pteridine-based biochemical, i.e., folic acid (Thakur et al., 2017). For biological functioning, riboflavin is converted either to a phosphorylated (flavin mononucleotide, FMN) or an adenylated (flavin adenine dinucleotide, FAD) form of active redox coenzymes (Saedisomeolia and Ashoori, 2018). The conversion of riboflavin to FMN is catalyzed by the enzyme flavokinase or riboflavin kinase, which is an ATP dependent phosphotransferase (EC 2.7.1.26) (Reaction 1, as shown in **Figure 2**). Most FMN is then converted to FAD by FAD synthetase, which is an adenylyltransferase (EC 2.7.7.2) (Reaction 2, as shown in **Figure 2**; Muller, 1987; Powers, 2003). FMN is generated upon phosphorylation at the 5′-hydroxymethyl terminus of the ribityl side chain and that is converted to FAD upon addition of an adenylate group via pyrophosphate linkage (Shils and Shike, 2006). Even though multiple mutations in FAD synthetase have been reported to result in critical flavin deficient conditions, interestingly, none of these patients were found to have any structural or functional abnormalities in vision.

The importance of flavins is underlined by the chemistry of the compound (Massey, 2000). The biological activity of flavins is governed by the chemical versatility of the isoalloxazine ring. This is because it can exist in three different forms: oxidized, one-electron reduced, and the two-electron reduced state (**Figure 2**; Rivlin, 1970). It is important to note that these possible active states of all three flavins have mostly been detected in biological systems as protein-bound form and not in free form. This is of relevance as, compared to the free form in aqueous state, association with proteins markedly alters the stability of the one-electron reduced state (Joosten and van Berkel, 2007; McDonald et al., 2011). Though both non-covalent and covalent association can modulate flavin redox properties, but they act in a differential manner and is contingent upon the type of interaction (Massey, 1995; McDonald et al., 2011). Interestingly, most of the flavoenzymes have non-covalently than covalently bound FAD or FMN as cofactors (Joosten and van Berkel, 2007). But to further underline the importance of flavins in cellular respiration or metabolic biology, it is imperative to state that these are the foremost primary electron acceptors from soluble metabolites along with the nicotinamide coenzymes (NAD^+/NADH). Being tricyclic gives flavins the ability to efficiently function as a transformer between electron donors and electron acceptors while the central dihydropyrazine ring of dihydroflavins is highly reactive to molecular oxygen, thus acting as a cofactor for reduction of molecular oxygen to hydrogen peroxide and also for reductive activity of monooxygenation reactions (Giulian et al., 1989).

FLAVINS AS PHOTOSENSITIZERS IN THE RETINA

All three forms of flavins, i.e., riboflavin, FAD, and FMN, can act as photosensitizers (Oster et al., 1962). There are primarily two forms of photosensitization reactions they can take part in: (I) direct reaction between biomolecules and photosensitized flavins and (II) oxygen-dependent interaction between photosensitized flavins and biomolecules (Insinska-Rak and Sikorski, 2014; Fuentes-Lemus and Lopez-Alarcon, 2020; Fuentes-Lemus et al., 2020). However, both these oxidation reactions are undertaken when the isoalloxazine ring of flavins is excited upon exposure to blue light (Cardoso et al., 2012). As stated in the above section, both the one-electron and two-electron reduced forms of flavins

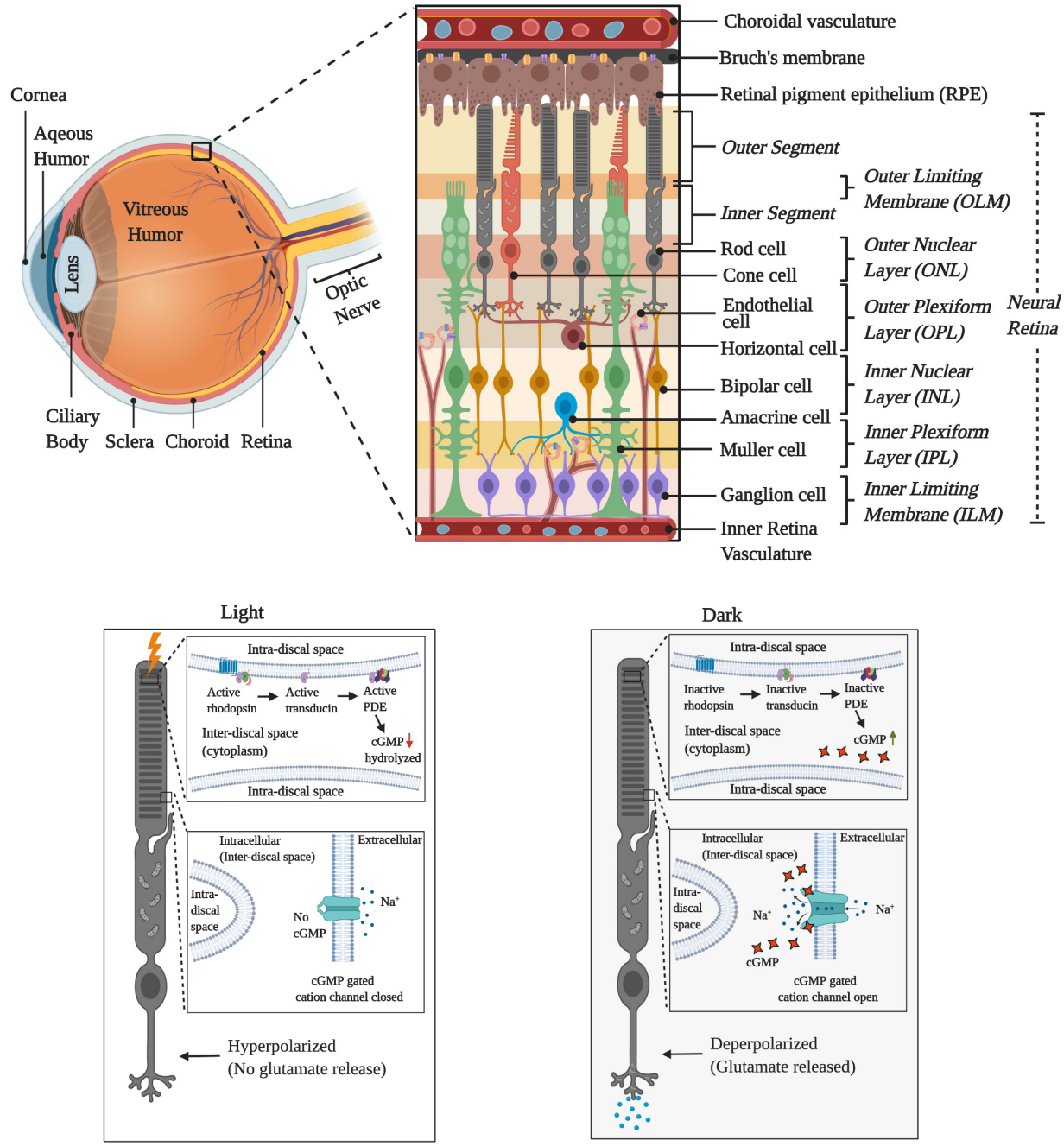

FIGURE 1 | Graphical illustration of the cellular layers of retina and the activity during the light and dark cycles.

(**Figure 2**) are highly reactive and thus leads to the formation of free radicals (Lopez-Alarcon et al., 2014). Due to this, flavins have been found to execute photosensitized oxidation of both lipid and protein biomolecules (Cardoso et al., 2013; Fuentes-Lemus and Lopez-Alarcon, 2020).

In type-I reactions, the two-electron reduced flavin (**Figure 2**) is quenched by the amino acid or lipid moieties most prone to be oxidized, resulting in the formation of a biomolecule radical cation and a flavin radical anion. The flavin radical anion can either react with oxygen to yield superoxide radical or accept a proton from the biomolecule radical cation or other donors to yield neutral free radicals. Subsequently, these neutral free radicals react with oxygen to form peroxyl radicals and eventually lead to the formation of hydroperoxides (Insinska-Rak and Sikorski, 2014; Fuentes-Lemus and Lopez-Alarcon, 2020). These species are further prone to decomposition in presence of redox active metal ions to yield alkoxyl radicals, which can add to the oxidative damage (Cardoso et al., 2012). Detailed

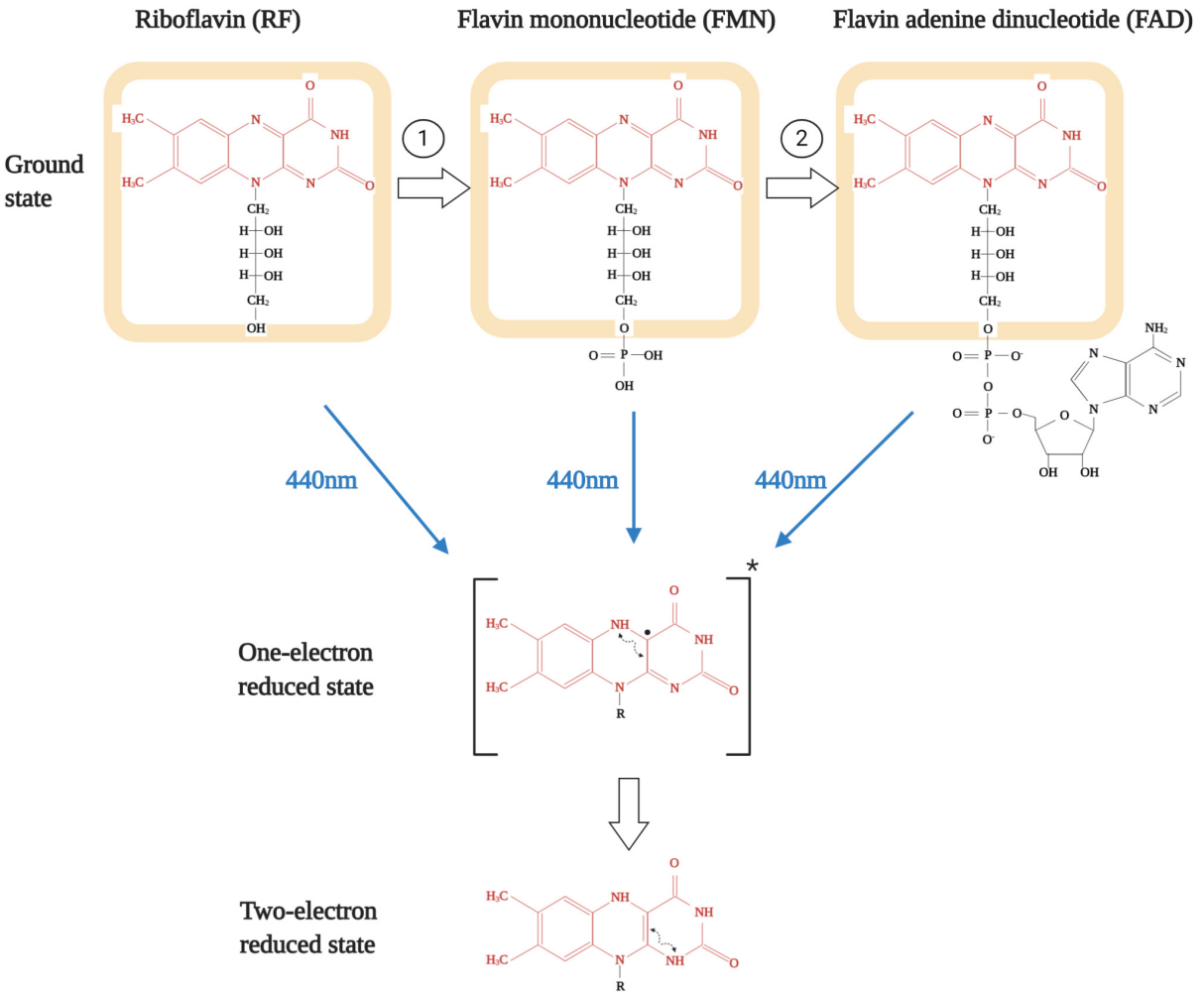

FIGURE 2 | Structures of various flavins and their subsequent excitation upon blue-light exposure. Isoalloxazine ring is shown in red while riboflavin structure is shown as highlighted in orange. Reaction 1 is catalyzed by riboflavin kinase and reaction 2 is catalyzed by FAD synthetase.

investigations have revealed that among all the amino acids, tryptophan is most susceptible to such flavin-sensitized photo-oxidation in an oxygen-independent mechanism (Bhatia et al., 1991; Silva et al., 1991, 1994; Garcia and Silva, 1997; Fuentes-Lemus and Lopez-Alarcon, 2020). Excited state riboflavin binds to tryptophan under a light-induced reaction and leads to cytotoxicities like axonal degeneration and further cascade of photo-adduct formation (Silva et al., 1991; Lucius et al., 1998; Lopez-Alarcon et al., 2014). Lipid peroxidation is also a common pathological marker of blue light mediated photo-toxicity in the retina (Wenzel et al., 2005). The retina is known to harbor a hypoxic environment and is frequently exposed to blue light for an extended period of time even in artificially lit conditions, thus making a hotspot for such oxygen independent flavin photosensitization reactions (Jaadane et al., 2015, 2020; Shang et al., 2017). In type-II reactions, the two-electron reduced flavin directly reacts with O_2 to convert it into the singlet state oxygen. This highly reactive form of oxygen can diffuse across a radius of 50–100 nm away from the site of formation and rapidly oxidize biomolecules like tryptophan, tyrosine, histidine, methionine, and cysteine amino acid residues since their kinetic rate constants are in the range of 10^6–10^7 M^{-1} s^{-1}. This is interesting since elevated methionine and cysteine oxidation and multiple protein oxidation markers are a common phenomenon in various age related retinal pathologies, especially age-related macular degeneration (Organisciak et al., 1998; Marc et al., 2008).

Since the eye is directly exposed to light, the cytotoxic effect of flavins acting as photosensitizers is even more common and specifically such photo-induced protein oxidation of retinal ganglion cells have been found to compromise mitochondrial efficiency (Silva et al., 1991; Lucius et al., 1998; Osborne et al., 2014). Such protein oxidation and lipid peroxidation can compromise protein function, enzymatic activity, and membrane integrity, as well as elevate reactive oxygen species (Huvaere et al., 2010; Remucal and McNeill, 2011; Lopez-Alarcon et al., 2014; Fuentes-Lemus and Lopez-Alarcon, 2020). Also increased fluorescence of the oxidized form of mitochondrial flavoproteins has come up as a new tool to diagnose oxidative

stress in retinal diseases, especially diabetic retinopathy and age-related macular degeneration (Spaide and Klancnik, 2005; Elner et al., 2008; Field et al., 2008, 2009; Litts et al., 2017; Andrade Romo et al., 2018). However, whether flavins as photosensitizers can affect the structure and function of photoreceptors or the retinal pigment epithelium (RPE) needs to be investigated. This is especially important given these two cells have the highest demand for riboflavin in the retina (Sinha et al., 2018). Blue light induced damage to the retina has been extensively investigated for decades and it is well established that the mechanism involves mitochondrial complexes as potential initiators of this phototoxic effect [reviews in Wenzel et al. (2005), Tao et al. (2019)]. Further mechanistic evaluation of blue light toxicity to the retina is outside the scope of this review. Here we focused on elucidating that flavins as photosensitizers can also be a major factor in blue light induced retinal damage and need to be considered in future mechanistic studies. Besides facilitating oxidation, exposure to light in acellular aqueous phase can lead to degradation of riboflavin itself, as has been previously reviewed (Sheraz et al., 2014). However, when we recently looked at the various conditions affecting riboflavin stability in the retina, we found protecting the retina from light (by dark adaptation) did not result in a change in retinal flavin levels (Sinha et al., 2018). Thus, it is likely that retinal riboflavin, similar to retinoids (Gonzalez-Fernandez et al., 2015), is somehow protected from degradation even though under constant exposure to light.

RETINAL FLAVIN HOMEOSTASIS AND OXIDATIVE BALANCE

The neural retina (NR) and the RPE together comprise the retina, which is one of the hotspots of highly reactive species in the whole body. The extremely high metabolic state of this tissue coupled with the high rate of oxygen consumption and the presence of multiple highly reactive phototransduction intermediates makes the retina vulnerable to various oxidative reactions. Thus, it is not surprising that to maintain homeostasis, the retina has developed an efficient system that counts on the ready availability of multiple electron acceptors and free radical scavengers. This is probably one of the reasons why both FAD and FMN are so highly enriched in the NR and the RPE (Sinha et al., 2018). The whole eye as an organ harbors arguably the highest level of riboflavin in the whole body (normalized to total protein content), and even though the cornea takes the major share of this, but it uses riboflavin mostly for structural purposes (Batey and Eckhert, 1990, 1991; Batey et al., 1992). The RPE and closely followed by the NR have the highest concentration of both the functional forms of riboflavin, FAD and FMN, and they are used critically as metabolic cofactors and free radical scavengers (Sinha et al., 2018, 2020b). It has been well elucidated that the glutathione based free radical scavenging system is highly dependent on flavins (Beutler, 1969). Glutathione peroxidase (GPx) reduces the intracellular H_2O_2 and toxic fatty acid hydroperoxides to water and in turn, GSH (reduced form) to GSSG (oxidized form). Glutathione reductase (GR), the enzyme that restores intracellular GSH (reduced form) levels by reducing GSSG (oxidized form) in an NADPH-mediated reaction, utilizes FAD as a cofactor (Higashi et al., 1978). Imbalance in the glutathione system has been shown to cause elevated retinal lipid peroxidation (Puertas et al., 1993). GSH depletion itself is a major cause of RPE ferroptosis and autophagy in a mitochondria independent manner (Sun et al., 2018). Absence of GSH downregulated RPE GPx (GPx4), a ferroptosis modulator, and increased LC3 expression, an autophagic marker (Sun et al., 2018). That riboflavin deficiency in experimental animals results in downregulation of GSH expression, reduced activity of GPx and increased lipid peroxidation in the eye, further raises the question if similar comorbidity happens in patients with retinal pathologies (Hirano et al., 1983; Horiuchi et al., 1984; Bates, 1991). If GSH cannot be recycled from GSSG due to reduced flavins, the RPE is unable to take up GSH exogenously and instead resorts to synthesizing it from secondary sources like glutamate, glycine, and cysteine (Davidson et al., 1994; Lu, 2013). This has a cascade effect on cellular metabolism as multiple metabolic resources now need to be repurposed to facilitate the adequate supply of these three amino acids. As an example, glucose is partially shunted away from glycolysis into the serine biosynthesis pathway, which is then converted to glycine (Sekhar et al., 2011; Lu, 2013; Panieri and Santoro, 2016; Sinha et al., 2020a,b). The RPE already harbors an efficient serine biosynthesis pathway in physiological conditions that may be upregulated to support enhanced GSH requirements. Interestingly, in the NR, even though the photoreceptors lack the repertoire for serine biosynthesis, Müller Cells (MC) has been shown to have the ability to synthesize serine and glycine for GSH production (Zhang et al., 2019).

Another close association between flavin deficiency and oxidative imbalance is via impaired mitochondrial redox balance, which is a major risk factor for ocular diseases like macular degeneration and diabetic retinopathy (Datta et al., 2017; Sinha et al., 2019). Patterson (Patterson and Bates, 1989) observed reduced oxygen consumption by the mitochondria in weanling rats fed riboflavin deficient diet associated with reduced weight gain per unit of food consumed (Tandler et al., 1969; Olpin and Bates, 1982; Patterson and Bates, 1989). Extremely hypoxic conditions can trigger reverse electron transfer and induce FMN to undergo reductive dissociation from complex-I of mitochondria, resulting in a robust decrease in complex-I function (Gostimskaya et al., 2007; Stepanova et al., 2017). Furthermore, significant accumulation of the reduced FMN can result in an equimolar amount of H_2O_2 in the mitochondrial matrix and can significantly contribute to oxidative stress (Massey, 1994; Kahl et al., 2018). Absence of flavins would also affect the β-oxidation of fatty acids in the RPE, which in turn would affect the flow of β-hydroxybutyrate to the retinal microenvironment, thus negatively impacting both the metabolic needs of the photoreceptors as well as the expression of oxidative stress resistance factors, as noted in other neurodegenerative disorders (Shimazu et al., 2013; Adijanto et al., 2014; Newman and Verdin, 2014). At the opposite extreme, Eckhert (Eckhert et al., 1993) reported that high levels of riboflavin can have harmful effects on the photoreceptors in a dose-dependent manner. He demonstrated

a reduction in the number of photoreceptors in rats fed excess riboflavin (30 mg/kg) versus the recommended level (6 mg/kg). However, this is the only report exhibiting toxicity from excess riboflavin in the eye. Interestingly the following work by the same group showed that rats fed excess riboflavin were unable to increase the residual amount of flavins in the retina (Batey et al., 1992). So, what contributed to the degeneration is still a mystery. Indeed, it was shown that 10-fold higher levels of FMN can potently inhibit GR activity in *in vitro* conditions (Schorah and Messenger, 1975). But in physiological conditions, excess riboflavin is rapidly cleared out from the body (Yang and McCormick, 1967). Thus, to speculate that excess riboflavin could be responsible for oxidative damage, it is important to first investigate what conditions can result in a buildup of excess riboflavin in the retina.

ROLE OF RETINAL FLAVIN HOMEOSTASIS AND GLUCOSE METABOLISM IN VISION

It has been shown that riboflavin plays a very prominent role in energy and glucose metabolism (Reddi et al., 1979). The retina is a metabolically active tissue with a high rate of energy demand and glucose consumption (Futterman and Kinoshita, 1959a,b). This is further validated by the highest activity of hexokinase in the inner segment of photoreceptors compared to the other cells of the NR as well as the brain (Burch et al., 1956; Lowry et al., 1961). This high activity is required for visual transduction as well as for the synthesis of new photoreceptor OS proteins, building new OS discs, and the shedding process. Using radioactive methionine, Young et al. showed that in rat, mouse, and frog, proteins synthesized in the photoreceptor IS are trafficked to OS in an ordered fashion, get accumulated in the lamellae in OS and subsequently are removed via shedding from the tip of OS in a light-dependent manner (Young, 1967). For this to effectively occur, a constant supply of energy and metabolites is required in the vicinity of the photoreceptors. To accommodate the high energy requirement, research on cattle and rabbit retinas demonstrated that high oxygen and glucose consumption occur via glycolysis, TCA cycle, and pentose phosphate pathway (Winkler, 1981).

Ames and colleagues showed that the retinal energy reserves are small, and withdrawal of glucose affects both the scotopic-a and b-wave of ERG (electroretinogram), which is an *in vivo* electrophysiological measurement of the retina (Ames et al., 1992). Surprisingly though, it had no immediate effect on oxygen consumption, indicating an alternate source of substrates for oxidative phosphorylation (Ames, 1992). This is in agreement with *ex vivo* results by Winkler (1981) showing that most of the glucose in the NR is converted to lactate and that inhibition of GAPDH (glycolytic enzyme) prevents the photoreceptors from having any extracellular potential, which is an *ex vivo* electrophysiological measurement of the photoreceptors and is similar to scotopic a-wave of the ERG. Oxygen withdrawal, on the other hand, leads to a Pasteur-effect with 2.7-fold increases in glycolysis and had a lower rate of decline in ATP production than in hypoxic conditions (Winkler, 1981; Ames et al., 1992). Thus, the authors demonstrated that retinal neurotransmission was heavily dependent on anaerobic glycolysis with it only contributing 18% of the total energy generated yet responsible for 80% of the total glucose consumed. Furthermore, they showed that phototransduction was dependent on oxidative metabolism with the dark current having the lion's share of 41% of oxygen consumption. The large Pasteur-effect was explained by the hypothesis that in hypoxic conditions, dark current was partly supported by glycolysis.

Following published work describing the utilization of non-oxidative metabolism of glucose by neuronal cells of the retina, Pellerin and colleagues showed that upon glutamate release at excitatory synapses, glucose utilization and lactate production were stimulated (Pellerin and Magistretti, 1994). Thus, glycolytic lactate production in the retina is tied with neurotransmission in the dark current (Pellerin and Magistretti, 1994). Poitry-Yamate et al. also argued that this lactate was observed to be a better substrate for photoreceptor oxidative metabolism, even though they do take up both lactate and glucose (Poitry-Yamate et al., 1995). This was demonstrated by showing that about 70% of radioactive lactate released by the MCs was taken up solely by photoreceptors. Winkler et al. (2003) also used glucose to study whether MCs are the primary producers of lactate in rat NR, aerobically serving as the principal fuel for the photoreceptor mitochondrial functioning. Acknowledging species difference, the authors used rat NR as the avascular model and guinea pig NR as the vascular model. Interestingly, their results showed that under aerobic conditions, photoreceptors tend to depend upon glucose as the principal energy substrate, as long as the supply is adequate (Winkler et al., 2003). To specifically delineate the metabolism of the outer retina, Wang et al. highlighted the importance of oxidative phosphorylation and aerobic glycolysis-based lactate formation under light and darkness (Wang et al., 1997). The authors showed that glucose is the most efficient substrate, the preferred metabolite for the bulk of the energy production in the outer retina and that about 80% of this glucose utilized is converted to lactate in aerobic conditions (Wang et al., 1997). In physiological conditions, the dark cycle has greater oxygen consumption than during the light cycle (Wangsa-Wirawan and Linsenmeier, 2003), which suggests that if the oxygen demand/supply goes <1 unit then a hypoxic condition will arise in the retina. Linking oxygen consumption to the bioenergetics, Okawa et al. further looked at the difference in ATP consumption in light versus dark by rod photoreceptors (Okawa et al., 2008). The authors found that the vertebrate rods consume about 10^8 ATP molecules per sec. The most dominant energy consumption is due to the ion fluxes associated with phototransduction and synaptic transmission. During daylight, the energy consumption drops by $>75\%$ due to perhaps inhibition of the dark response. The authors also showed that the cones are more energy consuming than rods (Okawa et al., 2008). Oxidative phosphorylation also seems to be the highest in photoreceptors compared to the rest of the NR since the highest cytochrome C activity (electron transport chain enzyme) is in the photoreceptors (Kageyama and Wong-Riley, 1984; Giulian et al., 1989) and the highest

content of mitochondria resides in the photoreceptors (Cohen, 1961; Hoang et al., 2002). Stone et al. (2008) showed that mitochondrial localization in the avascular retinas of mouse, rat, and humans to be primarily in the IS but also a minor pool at the axon terminals while in the avascular retinas of wallaby, and guinea-pig to be only in the IS. Working on the avascular retinas of zebrafish, Linton et al. (2010) proposed that the major energy production in photoreceptors occurs in IS-mitochondria and that this metabolic energy, in the form of phosphocreatine, is transmitted to the synaptic terminal in darkness and toward the OS in light. However, in the vascularized retina, the dependency is less on creatine kinase (Linton et al., 2010). Perkins et al. (2003) estimated that in primates there are 10 times more mitochondria in cones than in rods, while in mice, cones have twice the mitochondria of rods. Using ferret, cat, and monkey, Riley et al. showed similar evidence demonstrating that the IS of cones is more densely packed with mitochondria than that of rods (Kageyama and Wong-Riley, 1984). This was supportive of the previous evidence that the cones consume more energy (Scarpelli and Craig, 1963).

The high density of mitochondria also reflects higher flavin requirement by the photoreceptors as most of the mitochondrial enzymes are flavin-dependent (Ragan and Garland, 1969). Furthermore, the above also supports the notion that the inner segment of a photoreceptor is fueled by flavin based oxidative phosphorylation while the functioning of the outer segment could be fueled by aerobic glycolysis. Ames found that the sodium-potassium ATPase transporters consumed about half of all the energy used by the NR, i.e., 49% of oxidative energy and 58% of glycolytic energy. It is important to bear in mind that the authors could not account for the fate of about 49% of the energy generated by oxidative metabolism (Ames et al., 1992). Since flavins play an important role in oxidative phosphorylation and all the critical components of oxidative phosphorylation are concentrated in the inner segment, it is logical to assume that the inner segment must have a pool of riboflavin derivatives. It has been shown that the activity of some enzymes involved in oxidative phosphorylation is significantly lower in riboflavin deficient rats (Zaman and Verwilghen, 1975), thus indicating how an imbalance in flavin homeostasis can affect the retinal energy metabolism.

Powers et al. showed in various cell culture systems the importance of riboflavin for energy generation (Lee et al., 2013). In fact, in absence of riboflavin, the cells seem to be under considerable oxidative stress due to the increasing supply-demand gap of ATP. Cells deficient in riboflavin have lower ATP levels and as flavokinase activity is less sensitive to ATP levels due to a 20-fold lower Km than FAD synthetase, the levels of FAD drop further with diminishing levels of ATP (Lee et al., 2013). So even if excess riboflavin is provided at this point, until ATP levels reach the threshold in a flavin-independent mechanism, riboflavin would not be converted to FAD and oxidative phosphorylation cannot begin again. Thus, it is essential to maintain riboflavin homeostasis in the NR, such that glucose metabolism keeps functioning efficiently to meet the energy requirement of the photoreceptors.

It is evident that oxidative phosphorylation and glycolysis for both ATP production and biomolecular substrate generation in the NR have very unique dynamics. We know how important flavins are for all these processes. Thus, it is justified that to maintain the dynamicity, effective flavin transport and homeostasis are crucial to the retina.

FLAVIN HOMEOSTASIS AND LIPID METABOLISM IN THE RETINA

Unlike other cells where lipids constitute 1% of their membranes, the photoreceptor cell membrane is constituted of 15% lipids (Scott et al., 1988). This highlights the significance of lipid metabolism to the proper functionality of photoreceptors. Riboflavin deficient chicken embryos exhibit dysfunctional fatty acid metabolism whereby the significantly reduced activity of FAD-dependent medium acyl CoA dehydrogenases leads to the build-up of C10, C12, and C14 fatty acids (Abrams et al., 1995). The authors argue that the impairment of fatty acid oxidation drains out the carbohydrate reserves and in turn negatively impacts energy metabolism. The authors note that the only difference between the chicken and the adult humans and rats under riboflavin deficiency is that there is an increase of dicarboxylic acids fin both adult mammals but not for the chicken embryo (Abrams et al., 1995). There are several reports in the literature showing an impairment of β-oxidation of fatty acids as an effect of flavin deficient diet and the rationale behind this could be the depressed activity of the flavin-dependent dehydrogenases (Olpin and Bates, 1982; Liao and Huang, 1987; Parsons and Dias, 1991). It is noteworthy that these dehydrogenases include all three alternate dehydrogenases; short, medium and long-chain fatty acyl-coenzyme A dehydrogenase. All of these dehydrogenases are involved in the very first step of β-oxidation of fatty acids (Tandler et al., 1969; Olpin and Bates, 1982; Patterson and Bates, 1989). Tandler's work on isolated mitochondria from riboflavin deficient rat weanlings showed that the most drastic effect was on fatty acid oxidation, even though the oxidation of non-lipid substrates as succinate, pyruvate, glutamate, and α-ketoglutarate seemed to have a variable effect (Tandler et al., 1969). The rate-limiting step seemed to be the flavin-dependent acyl-CoA dehydrogenase activity (Tandler et al., 1969). The authors observed that the oxidation rates of both long-chain and intermediate chain fatty acid substrates dropped sharply as a result of ariboflavinosis (Tandler et al., 1969).

It is widely accepted that impaired β-oxidation of fatty acids can significantly contribute to vision loss and that it causes hypoglycemia (Taroni and Uziel, 1996; Kompare and Rizzo, 2008). Hypoketotic hypoglycemia, developed by patients having severely impaired β-oxidation of fatty acids (Taroni and Uziel, 1996) and 3-hydroxyacyl-CoA dehydrogenase deficiencies (Eaton et al., 2003; Tyni et al., 2004) have been reported to result in retinitis pigmentosa and peripheral neuropathy (Schrijver-Wieling et al., 1997; Tyni et al., 2004). Khan et al. (2011) showed that there is a close clinical effect of hypoglycemia on retinal function as detected by multifocal electroretinogram. This occurs in both normal subjects and those suffering from Type 1 diabetes,

whereby, the central retina is preferentially affected (Khan et al., 2011). In another study, Adijanto et al. (2014) referred to a novel metabolic coupling between the RPE and the photoreceptors, by which, the photoreceptor outer segment membrane components get recycled back into ketones, to be fed into the oxidative phosphorylation of the photoreceptors (Adijanto et al., 2014). The authors show that RPE cells produce a high amount of β-hydroxybutyrate by β-oxidation of fatty acids, and it is then shuttled to the photoreceptors via the monocarboxylate transporter 1 (MCT1) (Adijanto et al., 2014), which is present in both the apical side of RPE and the photoreceptor IS (Philp et al., 2003). The substrate for ketogenesis via β-oxidation of fatty acids may come from the vast pool of fatty acids shed as photoreceptor OS, which is constitutively taken up by the RPE cells (Boesze-Battaglia and Schimmel, 1997). It is also possible that β-hydroxybutyrate, besides helping in the metabolic needs of the photoreceptors, may act as a neuroprotective agent by suppressing oxidative stress in the retinal microenvironment (Shimazu et al., 2013; Newman and Verdin, 2014). Thus, when the photoreceptor layer gets parched for riboflavin its fatty acid oxidation can be adversely affected. This, in turn, can have a cascading effect on the lipid metabolism of the RPE. Also, if riboflavin moves from the inner retina to the RPE (Kubo et al., 2017), then a similar condition of hypoglycemia can begin in the RPE further affecting the RPE functioning, leading to vision problems.

RIBOFLAVIN ABSORPTION AND TRANSPORT

Since mammals have lost the ability to *de novo* synthesize riboflavin, it is acquired from the diet (Muller, 1987). Riboflavin absorption in the small intestine of rats and rabbits occurs across the brush border membrane in a specific carrier-mediated fashion, which is modulated by the level of riboflavin present in the vicinity (Said and Mohammadkhani, 1993; Subramanian et al., 2015). However, the body seems to get rid of excess plasma riboflavin within a span of a few hours, as has been reported for both animals (Yang and McCormick, 1967) and humans (Zempleni et al., 1996). In blood, riboflavin associates with plasma proteins like albumin (Wang et al., 2008) and reaches different parts of the body, enter various tissues either by diffusion or via specific transporters, and gets metabolically retained. In the last decade, it has been found that the brain has different transporters that are specific to riboflavin transport (Green et al., 2010; Haack et al., 2012; Johnson et al., 2012; Naik et al., 2014; Intoh et al., 2016). These are the same ones that have been identified earlier in other tissues. Recently, similar transporters were speculated to be present in the endothelial and epithelial cells of the inner and outer retina, respectively, as sh-RNA mediated knockdown and biochemical inhibition of these transporters resulted in decreased riboflavin uptake in TR-iBRB2, RPE-J and ARPE-19 cells (Said et al., 2005; Kubo et al., 2017, 2019). It was also shown that cultured RPE cells can take up riboflavin (Said et al., 2005), but this is yet to be validated *in vivo*.

At this juncture, it is important to state that most of the cellular riboflavin is known to be phosphorylated as in metabolic trapping to prevent its diffusion out of the cell (Gastaldi et al., 2000). The free form of riboflavin diffuses out of the cells into the plasma and is eventually excreted out in the urine (Aw et al., 1983; Chastain and McCormick, 1987). However, it is not clear what happens to the riboflavin of the extracellular matrix. Extracellular proteins, like riboflavin carrier proteins, may bind to riboflavin and prevent it from diffusing back to the plasma. That may explain why riboflavin carrier proteins have been reported in all those tissues where the concentration of riboflavin is more than that of blood plasma, making these proteins as major players in flavin homeostasis in these tissues (Prasad et al., 1992; Bhat et al., 1995). Examples of these proteins are the riboflavin binding protein (RBP) of the chicken egg (Rhodes et al., 1959) and retbindin (Rtbdn) of the retina (Kelley et al., 2015). Based on these studies, a schematic depicting possible routes of flavin transport through the inner and outer retina is shown in **Figure 3**.

FLAVIN CONCENTRATION IN DIFFERENT TISSUES

The concentration of total bound and free flavins (riboflavin, FAD, and FMN) in each tissue is determined by the metabolic demands of the tissue (Muller, 1987). Hepatic and plasma levels have been quantified linking them to various pathologies (Patterson and Bates, 1989). Besides liver and plasma, analyses of flavin levels in the brain have recently gained importance due to riboflavin transporter diseases receiving attention (Yoshimatsu et al., 2016). But despite the higher metabolic activity of the retina (Ames et al., 1992), analysis of retinal flavin levels have received little attention. Euler and Adler were perhaps the first to report that the retina has a high riboflavin content (Pirie, 1943). Batey et al. then reported that rat NR harbors 46.5 ± 2.8 pmol/mg protein FAD, 17.6 ± 0.7 pmol/mg protein FMN, and 4.8 ± 0.34 pmol/mg protein riboflavin (Batey and Eckhert, 1990). Subsequently, riboflavin content in fish and mammalian eyes were found to be high compared to other tissues (Pirie, 1943). Later, Batey et al. (1992) further reported that the NR contained the highest FAD and FMN levels of all the ocular tissues in rabbits fed with three different concentrations of diet (Batey and Eckhert, 1991). It was also shown that increasing riboflavin intake 3 mg/kg animal weight did not increase total flavin content in the rat NR (Batey et al., 1992), thus suggesting a tissue requirement specific transport mechanism. The mammalian cell does not have the machinery to retain excess riboflavin and hence it is excreted out in the urine within a short time (Zempleni et al.). The riboflavin absorption, distribution, and clearance in rats have long been extensively studied by Bessey et al. using radioactive compounds (Bessey et al., 1958).

THE FLAVOPROTEOME OF THE RETINA

The animal flavoproteome known so far can be widely divided into two types: One type is the coenzyme form of

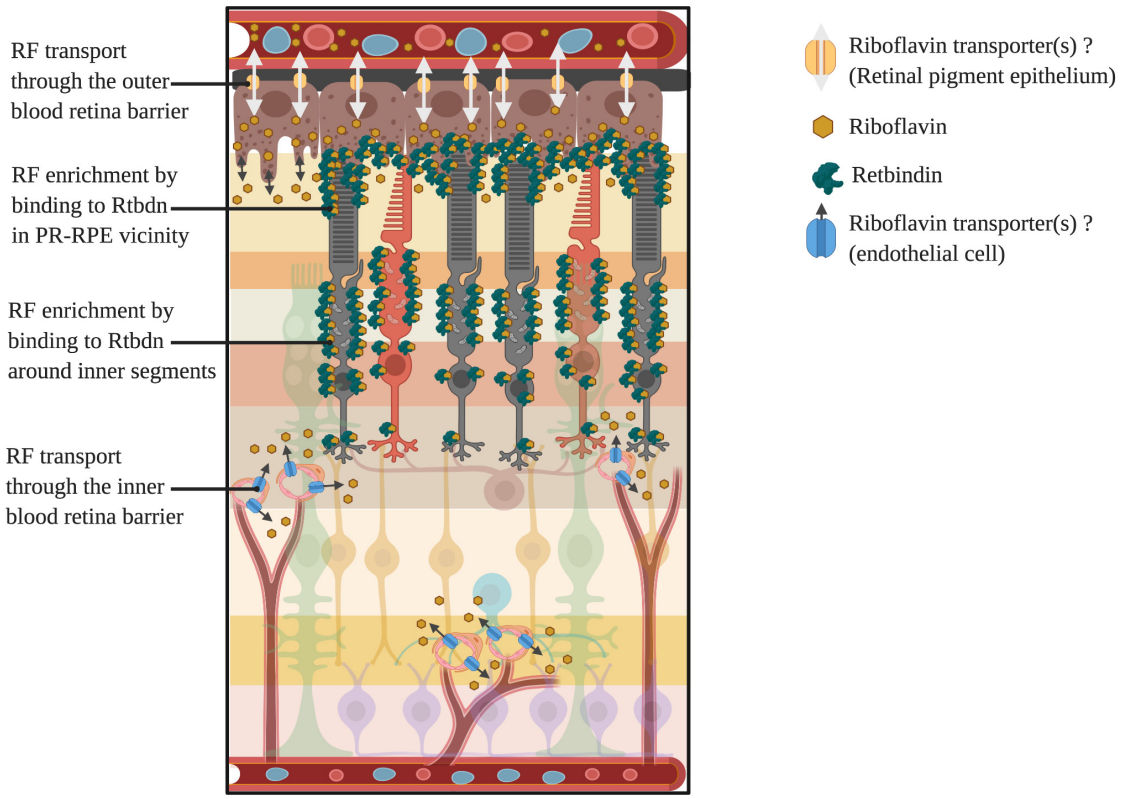

FIGURE 3 | Potential routes of flavin transport into and out of the retina and enrichment around photoreceptors. Here we have shown the two potential routes for inflow and outflow of riboflavin through yet to be identified transporters present in both the RPE (outer blood retina barrier) and endothelial cells (inner blood retina barrier). Also shown is the localization of the retinal riboflavin binding protein, retbindin, and its enrichment of bound flavins around the photoreceptor inner segments and RPE-outer segment junction.

flavin derivatives binding to apoproteins either by covalent or noncovalent bonds (Macheroux et al., 2011; Leys and Scrutton, 2016). Examples of this type would be acyl-CoA dehydrogenase (Lienhart et al., 2013), succinate dehydrogenase (Lienhart et al., 2013), and glycerol-3-phosphate dehydrogenase (Lienhart et al., 2013) among several others. The other type is proteins that associate with flavins and mostly act as flavin carriers or function to enrich flavins in specific tissues (Powers et al., 2012). Examples of this type would be RBP found in a chicken egg (Rhodes et al., 1959; Ostrowski et al., 1968), riboflavin-carrier protein in pregnant rats (Muniyappa and Adiga, 1980a,b) and Rtbdn of the mammalian retina (Kelley et al., 2015).

In a comprehensive review, Lienhart provides a detailed report on the human flavoproteome (Lienhart et al., 2013). The author mentions that about 60% of the members of the human flavoproteome are associated with clinical pathologies (Lienhart et al., 2013). This underlines the importance of flavins in the proper physiological functioning of mammalian proteins. It is also important to note that most of the dysfunctionalities in flavoprotein pathologies are related to the mitochondrial, endoplasmic reticulum, and peroxisomal dysfunctionalities (Lienhart et al., 2013). This is not surprising in the case of the mitochondrial dysfunctionalities since a good number of the flavoproteins are located in the mitochondria and play a role in energy metabolism (Chance et al., 1967; Ragan and Garland, 1969). Flavoproteins associated endoplasmic and peroxisomal dysfunctionalities, on the other hand, point to the role flavins play in the exclusive functions performed by both organelles to aid in lipid metabolism (Lienhart et al., 2013).

Among all the flavoproteins, the mammalian retinal Rtbdn is unique. Rtbdn has the highest sequence homology to RBP of the chicken egg (Kelley et al., 2015). In mammals, primarily rod photoreceptors express Rtbdn and it is the only known riboflavin binding protein to be present in the retina (Kelley et al., 2015). What is most interesting is that Rtbdn is a peripheral membrane protein present on the extracellular side and attached to the membrane via electrostatic interactions (Kelley et al., 2015). Probably this enables the protein to bind to riboflavin present in the extracellular matrix. Rtbdn localizes mainly in two pools: one at the outer segment-RPE interface and the other around the inner segment of the photoreceptors (Kelley et al., 2015). Since multiple nutrients are exchanged between the NR and the RPE at the outer segment-RPE junction, it makes sense for Rtbdn to be highly enriched at this location to facilitate riboflavin transport back and forth between the NR and RPE (**Figure 3**). The other localization of Rtbdn is consistent with the fact that photoreceptors' mitochondria are also present in highest concentration in the same region. Since

flavins are essential for mitochondrial functioning and that photoreceptors' mitochondria are highly active, it is possible that Rtbdn presence around the inner segment is chiefly to facilitate active flavin availability for oxidative phosphorylation. It would be worthwhile to validate this by investigating the rate of photoreceptor oxidative phosphorylation in absence of Rtbdn. But the importance of Rtbdn to a healthy retina is most obvious from the finding that in absence of Rtbdn, gradual degeneration is triggered (Kelley et al., 2017). Further, that this coincides with a decline in NR flavin levels, emphasizes how important Rtbdn is to maintain the retinal flavin demands. But mechanistic understanding behind this is lacking. Rtbdn may interact with other accessory membrane proteins which facilitate the internalization of flavins from Rtbdn itself. Also, since other flavoproteins are known to be unstable in absence of adequate flavins, whether the association of Rtbdn with the membrane is dependent on its binding to riboflavin is to be determined.

IMPORTANCE OF STUDYING THE ROLE OF RIBOFLAVIN AND RETBINDIN IN THE RETINA

Blindness is reportedly the disease that can be caused by the most diverse set of gene mutations than any other disease known (Punzo et al., 2012). Mutations in over 300 different genes or gene loci are known to be associated with inherited retinal diseases (IRDs) (RetNet, 2020, Accessed May 27th, 2020). Metabolic vulnerability and predisposition to oxidative stress have been touted as an underlying facilitator for such multi-genic retinal diseases (Leveillard et al., 2019). Unsurprisingly, therapeutic interventions targeted to ameliorate metabolic stress has shown that it is indeed a promising approach to treat such a wide spectrum of blinding diseases (Hurley and Chao, 2020; Wert et al., 2020). Given the importance of flavins in many metabolic pathways that are essential for retinal homeostasis, it is imperative to maintain optimum levels of flavins for a healthy retina. As reported by Amemiya (2007), the retina of rats fed with riboflavin deficient diet for 3 months showed clear signs of degeneration with edematous and disoriented MCs, disintegrating OS discs and RPE full of an abnormal number of lamellae. Interestingly, these seemed to be reversible since animals recovered when they were placed on a riboflavin enriched diet. In absence of literature presenting ultrastructural images of the effects of long term riboflavin deficiency, one can assume that the high number of lamellae in the RPE even after 7 h (shedding stops usually within few hours after the onset of the light cycle), is due to either slower rate of phagocytosis by the RPE or enhanced degenerating OS contributing to extended phagocytosis. That the tip of the OS seems to be affected may support the line of thought that the OS/RPE interface is affected, creating a stressful environment for the interphotoreceptor matrix in a state of ariboflavinosis.

The absence of Rtbdn also leads to a reduction in NR's flavin levels, which corresponds with retinal degeneration (Kelley et al., 2017). Most interesting is that even though rods specifically express Rtbdn, the cones are affected in Rtbdn's absence (Kelley et al., 2017). This is further supportive of previous evidence that rods express specific proteins that are essential for cone health (Chalmel et al., 2007). Further, a significant reduction in retinal flavin levels in only rod specific degeneration models indicated they are responsible for the majority of retinal flavins (Sinha et al., 2018). However, the fact that there still existed some amount of flavins is indicative that there must be another [likely a photoreceptor independent ("?" in **Figure 3**)] mechanism, which may be essential for flavin homeostasis of the inner retina. Thus, rod death during retinitis pigmentosa or other retinal pathologies could result in a local ariboflavinic environment around the photoreceptors, leading to a starving condition for the cones, triggering cone death that usually follows rod death as observed in RP patients and in models of IRDs (Punzo et al., 2012). Due to its role in retinal homeostasis, when Rtbdn was eliminated from a model of cone-rod dystrophy, the degenerative process was exacerbated (Genc et al., 2020). Expression of elevated levels of Rtbdn during retinal degeneration further indicated that the protein could be playing a protective role (Genc et al., 2020). It is possible that when confronted with a stressful condition as degeneration, the retina needs a higher level of energy, hence an increased need for flavins, to mitigate this insult and thus overexpresses Rtbdn. It is worth mentioning that the absence of Rtbdn triggered a compromise in retinal vasculature integrity and led to the formation of vascular tufts (Genc et al., 2020). It would be worthwhile to see if such a trend is mimicked in other models of retinal degeneration as well and whether there is a difference between the behaviors of models of cone dominant mutations versus those resulting from rod dominant mutation. A previous study (Venkataswamy, 1967) described the various ways ariboflavinosis can affect different parts of the eye. The authors cite Davson's (1949) chapter (Venkataswamy, 1967), highlighting the rich flavin content in the retina and its deficiency linked to night blindness. They also reported that patients suffering from ariboflavinosis showed resolution of their night blindness following a 10-days course of 10 mg riboflavin/day injections. The authors mention two previous reports by Pollak in 1945 and by Gordon in 1939, emphasizing the ability of riboflavin alone to improve dark adaptation (Pollak, 1945). However, supplementary evidence is lacking on these lines and needs to be validated by further research. Given the fact that pathology as riboflavin transporter disease improves with flavin-enriched diet (Timmerman and De Jonghe, 2014; Bashford et al., 2017), it is interesting to see if high riboflavin diet for an extended period can rescue RP animal models from photoreceptor degeneration.

FUTURE PERSPECTIVE

Putting all the research into perspective, it seems very important to look at both: (1) the role of flavin homeostasis in retinal physiology as well as (2) the role of flavin homeostasis in retinal pathologies, especially those where metabolic vulnerability and oxidative stress susceptibility is involved. One of the tools available to us to assess the role of flavins in retinal homeostasis is the Rtbdn knockout model (Kelley et al., 2015). The presence of a highly regulated barrier like the blood-retinal barrier, combined

with the high energy metabolism in the retina, such specialized proteins seems critical for retinal homeostasis. It is possible, that like RBP of the egg, Rtbdn, helps in the transport of riboflavin across the interphotoreceptor matrix and thus maintaining the high intracellular pool of riboflavin in the photoreceptors. It will also be beneficial to specifically identify the transporters that may be involved in riboflavin transport to the retina and investigate what happens if their levels are selectively altered, both in health and disease. Future research should also focus on identifying mutations in either Rtbdn or any of the riboflavin transporters that cause or modify retinal degenerative diseases. Moreover, since so little is known about any of retinal riboflavin carrier proteins, biochemical and biophysical characterization of both Rtbdn and riboflavin transporters would provide us with a greater understanding as to how such high flavin levels are maintained in the retina. Similarly, it will be worthwhile to investigate if flavin deficiency confounds retinal dystrophy in patients and whether maintaining optimum flavins provides better prognosis when the retina is under metabolic or oxidative stress. Thus, it seems important to do more work on the role of flavin homeostasis with respect to the structural and functional integrity of the retina and further our knowledge on the criticality of this underappreciated vitamin to the retina.

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TS, MN, and MA-U contributed to writing and editing the manuscript. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Cellular Changes in Retinas From Patients With *BEST1* Mutations

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Best disease (BD), also known as vitelliform macular dystrophy, is an inherited disease of the central retina caused by more than 300 pathogenic variants in the *BEST1* gene. The phenotype of BD is variable, and there are just a few reports on the histopathology of eyes from donors with BD. Here, we describe the histopathological comparison of donor's eyes from two patients with BD. Eyes obtained from 85-year-old (donor 1) and 65-year-old (donor 2) donors were fixed within 25 h postmortem. Perifoveal and peripheral retinal regions were processed for histology and immunocytochemistry using retinal-specific and retinal pigment epithelium (RPE)-specific antibodies. Three age-matched normal eyes were used as controls. DNA was obtained from donor blood samples. Sequence analysis of the entire *BEST1* coding region was performed and identified a c.886A > C (p.Asn296His) variant in donor 1 and a c.602T > C (p.Ile201Thr) variant in donor 2; both mutations were heterozygous. Fundus examination showed that donor 1 displayed a macular lesion with considerable scarring while donor 2 displayed close to normal macular morphology. Our studies of histology and molecular pathology in the perifovea and periphery of these two BD donor eyes revealed panretinal abnormalities in both photoreceptors and RPE cellular levels in the periphery; donor 1 also displayed macular lesion. Our findings confirm the phenotypic variability of BD associated with *BEST1* variants.

Keywords: best disease, *BEST1* gene, histopathology, retinal pigment epithelium, photoreceptors

INTRODUCTION

Best vitelliform macular dystrophy is an inherited disease of the central retina caused by pathogenic variants in the *VMD2* gene, now known as *BEST1* (Marquardt et al., 1998; Petrukhin et al., 1998). More than 300 disease-causing variants in the *BEST1* gene have been reported (Johnson et al., 2017; Nachtigal et al., 2020). Pathogenic variants in this gene are linked to at least three distinct retinopathies that can be distinguished by phenotype and mode of inheritance: the autosomal dominant Best vitelliform macular dystrophy or Best disease (BD), the autosomal dominant vitreoretinochoroidopathy (ADVIRC), as well as the autosomal recessive bestrophinopathy (ARB) (Nachtigal et al., 2020). The *BEST1* gene encodes Bestrophin-1, a regulator of intracellular Ca^{2+} localized at the basolateral membrane of the retinal pigment epithelial (RPE) cells (Marmorstein et al., 2000).

The morphological findings described in the eyes of BD patients evaluated with spectral domain optical coherence tomography (SD-OCT) are variable and, in sum, include (1) the accumulation of lipofuscin in the RPE and (2) photoreceptor degeneration over a morphologically intact RPE layer (Kay et al., 2012; Tsang and Sharma, 2018, Lima de Carvalho et al., 2019). A limited number of previous reports analyzed the histopathology of BD donor eyes (Frangieh et al., 1982; Weingeist et al., 1982, O'Gorman et al., 1988; Mullins et al., 2005, Bakall et al., 2007; Mullins et al., 2007). Here, we describe and compare the histology and molecular pathology in donor eyes from two patients with BD caused by c.886A > C (p.Asn296His) and c.602T > C (p.Ile201Thr) *BEST1* variants to provide insight into the pathophysiology of the disease. This is the first study of adult postmortem donor eyes from patients with BD due to these specific mutations.

MATERIALS AND METHODS

Donor Eye Acquisition, Imaging, and Genotyping

Postmortem eyes obtained from the Cole Eye Institute Eye Tissue Repository through the Foundation Fighting Blindness (FFB) Eye Donor Program (Columbia, MD). Eyes from BD donors (FFB# 928 and 458) were enucleated and fixed in 4% paraformaldehyde (PF) and 0.5% glutaraldehyde (GA) in D-PBS 12 and 25 h postmortem. Donors were an 85-year-old female and a 65-year-old male. Normal postmortem donor eyes from an anonymous 65- and 95-year-old woman and an 88-year-old male were fixed similarly within 4 and 18 h postmortem (FFB# 696, 784, and 789).

Eyes were cut through the ora serrata, transferred to a plexiglass chamber filled with D-PBS, and imaged by Spectral Domain-Optical Coherence Tomography (SD-OCT) and confocal scanning laser ophthalmoscopy (cSLO) as previously described (Bagheri et al., 2012). For the SD-OCT images, a single telecentric objective lens was employed to collect 5 × 5 mm and 10 × 10 mm FOV of the posterior pole using the following scan parameters: (1) 5-mm linear scan of the horizontal meridian through the optic nerve and fovea @ 1000 A-scans/B-scan, (2) 10-mm linear scan of the horizontal meridian through the optic nerve and fovea @ 1000 A-scans/B-scan, (3) 5-mm^2 volume scan of the posterior pole @ 500 B-scans/volume × 250 A-scans/B-scan, and (4) 10-mm^2 volume scan of the posterior pole @ 500 B-scans/volume × 250 A-scans/B-scan. SLO images were collected using a model HRA2 confocal scanning laser ophthalmoscope (Heidelberg Engineering, Inc.). The HRA2 was rotated 90^o so that the scan direction was perpendicular to the table surface. The system was operated in high-resolution mode, which provides an image pixel format of 1536 × 1536 when used with a 55° wide-field objective lens. SLO images of the posterior pole were collected using infrared reflectance (SLO-IR), infrared dark field (SLO-IRDF), autofluorescence (SLO-AF), and red-free dark field (SLO-RFDF) imaging modes at field of view (FOV) settings of 55°, 35°, and 25°.

Sequence analysis of the entire *BEST1* coding region was performed and reported by Dr. Edwin Stone (Lotery et al., 2000). DNA analysis of donor 1 detected a c.886A > C (p.Asn296His) *BEST1* pathogenic variant and a c.602T > C (p.Ile201Thr) *BEST1* pathogenic variant in donor 2; both mutations were heterozygous. The clinical evaluation of donor 1 was carried out at the University of Illinois with the approval of the Institutional Review Board (IRB) at the University of Illinois Medical Center.

Retina Histology

Fragments of retina–RPE–choroid were cut from the perifovea and periphery. Tissue fragments were further fixed by immersion in 2.5% GA in 0.1 M cacodylate buffer, post-fixed with 1% osmium tetroxide for 45 min on ice, sequentially dehydrated in ethanol, and embedded in Epon as previously described (Bonilha et al., 2015). Toluidine blue-stained sections were photographed with a Zeiss AxioImager. Z1 light microscope equipped with an MRc5 camera (Carl Zeiss AG, Oberkochen, Germany).

Immunohistopathology of Photoreceptors and RPE Layers

Another set of tissue fragments was fixed by immersion in 4% PF in D-PBS where they remained overnight at 4°C and then quenched with 50 mM NH_4Cl in D-PBS for 1 h at 4°C followed by changes to 10% (1 h) and 20% sucrose (overnight) made in the same buffer and finally a mix of 20% sucrose and Tissue-Tek "4583" (Miles Inc., Elkhart, IN). Finally, samples were transferred to a small cassette filled with the same sucrose and Tissue-Tek mix and frozen. Cryosections (10 μm) were collected on an HM 505E cryostat (Microm, Walldorf, Germany) equipped with a CryoJane Tape-Transfer system (Leica, St. Louis, MO). No perifoveal tissue from donor 2 was available for this analysis.

Autofluorescence of unlabeled cryosections was performed and analyzed using epifluorescence in the green channel (FITC filter: 490 nm excitation/519 nm emission) and red channel (TRITC filter: 550 nm excitation/570 nm emission). Autofluorescence was overlaid on differential interference contrast (DIC) images.

Cryosections were blocked in D-PBS supplemented with 2% BSA and 0.2% TX100 (D-PBS/BSA/TX) for 30 min and incubated with the following antibodies: GFAP (mouse, ab10062, 1:400, Abcam, Cambridge, MA), rhodopsin (mouse, ab5417, 1:1000, Abcam), MCT3 (rabbit, 1:100, a gift from Dr. N. Philp, Philadelphia University), EBP50 (rabbit, 1:200, Thermo Fisher Scientific, Waltham, MA), bestrophin-1 (NB300-164, mouse, E6-6,1:50, Novus Biologicals, Littleton, CO), and red/green opsin (AB5405, rabbit, 1:600, Millipore Sigma-Aldrich, Billerica, MA), in PBS/BSA/TX overnight at 4°C. Sections were then labeled with secondary antibodies conjugated with Alexa Fluor 488 and 594 (Molecular Probes, 1:1000) for 45 min at room temperature. Cell nuclei were labeled with TO-PRO®-3 iodide (Thermo Fisher). Sections were also labeled with PNA-Alexa488 (Thermo Fisher Scientific, 1:100) and WGA-Alexa594 (Thermo Fisher Scientific, 1:500). Images were acquired using a Leica laser scanning confocal microscope (TCS-SP8, Leica, Exton, PA) with a series of 0.33 μm xy (en face) optical sections. Microscopic panels were composed using Adobe Photoshop CC (Adobe, San Jose, CA). The perifovea of donor 2 was unavailable for analysis.

RESULTS

Clinical Findings

Donor 1 was last seen for a follow-up eye examination in September 1992 at 65 years of age, 20 years before her death. At that time, her visual acuity was correctable to 20/200 in both eyes. The lenses showed trace nuclear sclerosis. Ocular pressures were 18 mmHg in each eye. The fundus examination showed a hypertrophic scar in the right eye (**Supplementary Figure 1A**, OD), while the left eye showed areas of hypopigmentation within the fovea (**Supplementary Figure 1A**, OS). The patient reported a blurred vision in the right eye; this eye displayed a + 2 1/2 anterior cortical change. The left eye showed a + 1 anterior cortical opacity. Her visual field showed bilateral central scotomas. The donor was on blood pressure medication and a water pill. Her systemic health was negative for other major medical problems.

Due to the retrospective nature of this analysis, historical clinical records were unable to be obtained for donor 2.

Fundus Macroscopy and Histopathology of *BEST1* Mutations

Pathogenic variants in *BEST1* affect the function of bestrophin-1 and disrupt the ion transport by the RPE, resulting in the accumulation of fluid between the RPE and the photoreceptors (Singh et al., 2013, Marmorstein et al., 2015, Milenkovic et al., 2018; Nachtigal et al., 2020). This change in subretinal fluid likely results in separation of the neural retina from the RPE and the observation of the typical yellow yolk-like macular lesion upon fundus examination (MacDonald et al., 1993-2020). Images from donor 1 [c.886A > C (p.Asn296His) variant] showed an irregular whitish macular lesion with areas of hyperpigmentation that was more visible by visible light fundus macroscopy (**Figure 1A**) than either IRDF-cSLO or BAF-cSLO (**Supplementary Figure 2A**). Donor 2 [c.602T > C (p.Ile201Thr) variant] was absent of any obvious retinal lesions (**Figure 1A**). Histologically, an extensive fibrovascular scar was present in the perifovea of donor 1 (**Figure 1B**, star) via fundus microscopy. Immediately above the scar, the retina's outer nuclear layer was absent; this area also displayed thin RPE (**Figure 1B**) with no photoreceptors and contained some choroidal vessels. Adjacent to the scar, the retina's outer nuclear layer was reduced to a single discontinuous row of photoreceptor cell nuclei; the inner segments of surviving photoreceptor cells were shortened. In contrast, the perifovea of donor 2 had distinct ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL), and RPE (**Figure 1B**). In the periphery, both donor 1 and donor 2 displayed distinct GCL, INL, ONL, RPE, and choroid (Ch). Donor 1 also displayed edema of the interphotoreceptor matrix (**Figure 1B**, arrow).

The frequently observed vitelliform lesion in BD patients localizes to the macula's subretinal space and contains fluid and lipofuscin. Lipofuscin is a long-lived intracellular inclusion body, lipid- and bisretinoids-rich, and autofluorescent material that progressively accumulates in the RPE during aging and pathological conditions as BD (Ng et al., 2008; Sparrow et al., 2010, 2012). We compared the relative amount of autofluorescence in the RPE of control and BD eyes. RPE in the perifovea and periphery of donor 1 showed a substantial decrease of autofluorescence compared to RPE in the control eyes (**Figure 2**). Also, in the periphery of donor 2, the RPE showed a paucity of autofluorescence compared to the control RPE.

Photoreceptor Pathology of *BEST1* Mutations

The RPE–photoreceptor interface is an area of fundamental importance for supporting the proper retinal function. To gain insight, we carried out IHC evaluation of the BD retinas using a set of markers with known expression in the rod and cone outer segments. The distribution of rhodopsin was restricted to the outer segments of the control donor in both the perifovea and periphery (**Figure 3A**). In the perifovea of donor 1, rhodopsin labeling displayed a circular pattern close to the RPE surface, with a few cellular projections being observed in the outer plexiform layer. Redistribution of rhodopsin throughout the whole photoreceptor cell (**Figure 3A**, arrows) was observed in the periphery of donor 1. In the periphery of donor 2, rhodopsin labeling was overall decreased; outer segments were disorganized, and rhodopsin was distributed in the inner segments (**Figure 3A**).

We then investigated the distribution of cone photoreceptors in retinas harboring *BEST1* variants using red/green cone opsin labeling. In the control retina, red/green cone opsin was distributed along with the outer segments in the perifovea and periphery (**Figure 3B**, green). Red/green opsin-labeled cells were mostly absent in the retina adjacent to the fibrovascular scar of donor 1. Abnormal distribution of the red/green opsins throughout the entire cone cell body was observed in the periphery of donors 1 and 2 (**Figure 3B**, arrowheads). Moreover, Müller cells, labeled with GFAP antibodies, had undergone extensive activation throughout the retina. Their hypertrophic processes were observed in the periphery of donor 2 and perifovea of donor 1 (**Figure 3B**, red) when compared to the control retina. In the subretinal space, the interphotoreceptor matrix (IPM) surrounding the inner and outer segments of the cone (**Supplementary Figure 3**, green) and rod (**Supplementary Figure 3**, red) photoreceptors was analyzed in both control and BD donor retinas labeled with PNA and WGA lectins. In the control retina, PNA (green) bound to the extracellular matrix sheaths of the cone photoreceptor inner and outer segments while WGA (red) bound to the extracellular matrix sheaths of the rod photoreceptor inner and outer segments in both the perifovea and periphery. In the perifovea of donor 1, PNA and WGA labeling were observed dispersed through the photoreceptor inner and outer segments. In the periphery, PNA and WGA labeling colocalized in the outer segments. In the periphery of donor 1, PNA and WGA were also dispersed through the photoreceptor inner and outer segments; a visible decrease in PNA labeling was observed. PNA and WGA labeling evidenced edema of the IPM in both the perifovea and periphery of donor 1 (**Supplementary Figure 3**, arrows). In the periphery of donor 2, PNA and WGA labeling were visibly decreased, but restricted to the outer segments.

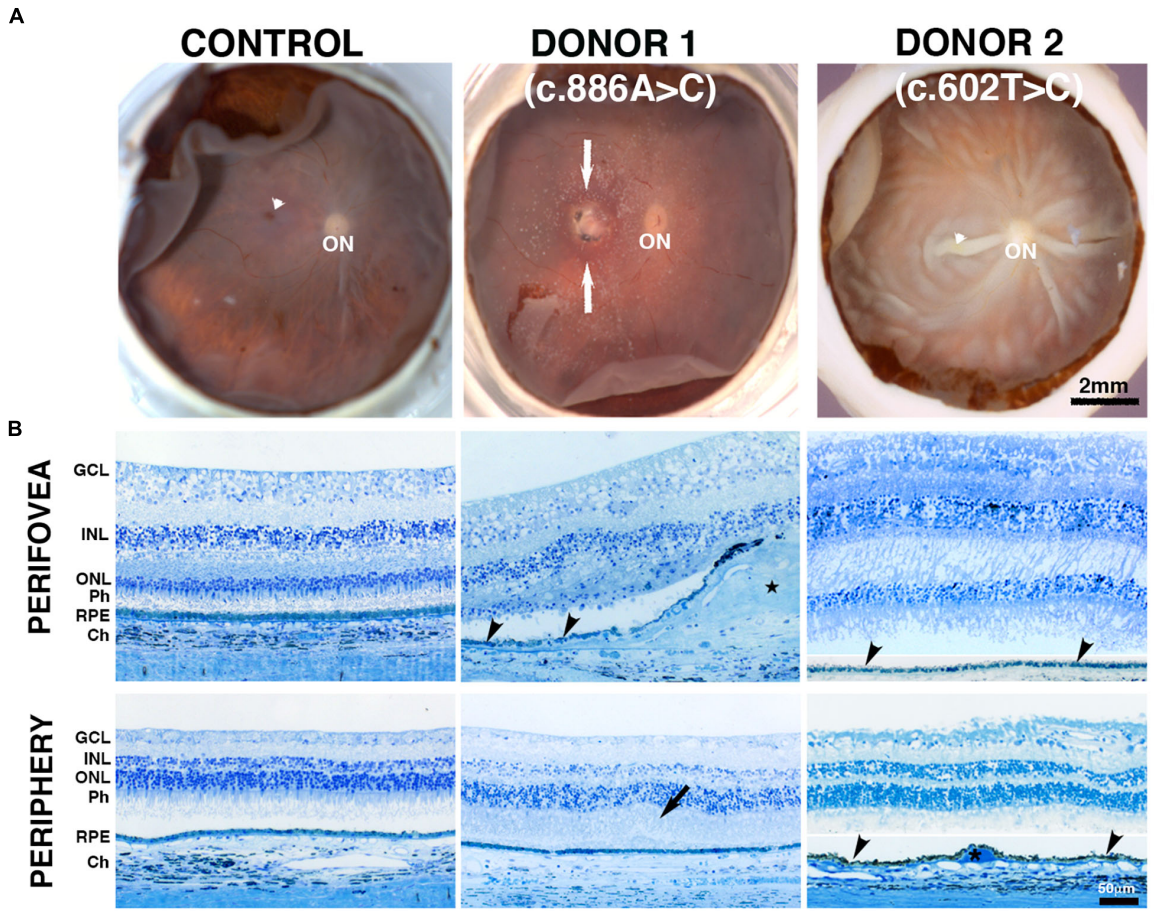

FIGURE 1 | Impact of *BEST1* pathogenic variants on gross pathology and retinal morphology compared to an unaffected, age-matched control eye. **(A)** The macroscopic fundus image shows a control eye free of any pathology. Donor 1 (c.886A > C, p.Asn296His, an 85-year-old donor) displayed a visible macular lesion (white arrows) while donor 2 (c.602T > C, p.Ile201Thr, a 65-year-old donor) did not display any obvious retinal lesion; however, this donor displayed significant postmortem fixation artifacts (areas of retinal detachment). Visible fovea is indicated by white arrowhead, optic nerve head = ON. **(B)** Histology of a control retina (a 95-year-old donor) in the perifovea and periphery displayed typical characteristics including structured lamina consisting of retinal cells. Donor 1 perifovea shows a fibrovascular scar present between the Bruch's membrane and the retina (star), accompanied by thin patchy RPE (black arrowhead) and inter photoreceptor matrix edema (black arrow); asterisk = drusen. GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer; POS = photoreceptor outer segments; RPE = retinal pigment epithelium, choroid (Ch). In the periphery, both donors 1 and 2 displayed a distinct GCL, INL, ONL, RPE, and Ch. Scale bar: A = 2 mm (all low-magnification images) and Scale bar B = 50 μm (all images).

RPE Pathology of *BEST1* Mutations

To gain insight into the effects of the *BEST1* variants on RPE morphology, we carried out IHC evaluation of the BD retinas using markers known to be expressed at either the RPE apical or basolateral surfaces. The distribution of ERM (ezrin, radixin, and moesin)-binding phosphoprotein of 50 kDa (EBP50), a protein that links apical transporters to ezrin and the actin cytoskeleton, was observed at the RPE apical microvilli in both the perifovea and periphery of the control retina; a minor presence was also observed in the basal surface of the cells as previously described (Bonilha and Rodriguez-Boulan, 2001; Nawrot et al., 2004). EBP50 also labeled Müller cell apical processes (**Figure 4A**, double arrowheads). In the perifovea of donor 1, EBP50 labeling significantly increased; the RPE apical microvilli were highly disorganized and seemed to form patchy structures of different lengths and thickness projecting into the photoreceptors. In the periphery of donor 1, similar but lessened EBP50 apical distribution was observed; a correspondent presence of basal punctate structures was observed (**Figure 4A**, arrows). In the periphery of donor 2, overall decreased EBP50 labeling of the apical RPE surface was observed. Deformed (domed shape) Müller cell apical processes were visible in all BD donor retinas due to the photoreceptor alterations.

We also investigated the distribution of monocarboxylate transporter 3 (MCT3), an RPE basolateral transporter (Philp et al., 2003). In both the perifovea and periphery of control retinas, MCT3 was localized to the RPE basolateral surface (**Figure 4B**). In the perifovea of donor 1, MCT3 was observed in all aspects of the RPE membrane (**Figure 4B**, arrowheads) with increased labeling density relative to control. In the periphery of donor 1, MCT3 was mostly confined to the basolateral surface; however, it was also observed in the RPE apical microvilli and

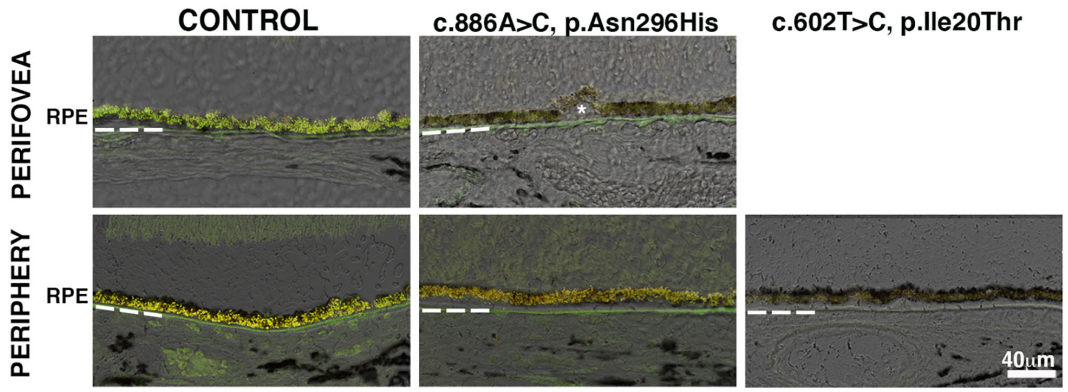

FIGURE 2 | Impact of *BEST1* pathogenic variants in RPE autofluorescent granules. Cryosections obtained from the BD donors and a 95-year-old control were observed using the green channel (FITC filter) and red channel (TRITC filter). Autofluorescence was overlaid on differential interference contrast (DIC) images. Bruch's membrane is indicated by hashed white line, asterisk = drusen. Scale bar = 40 μm (all images).

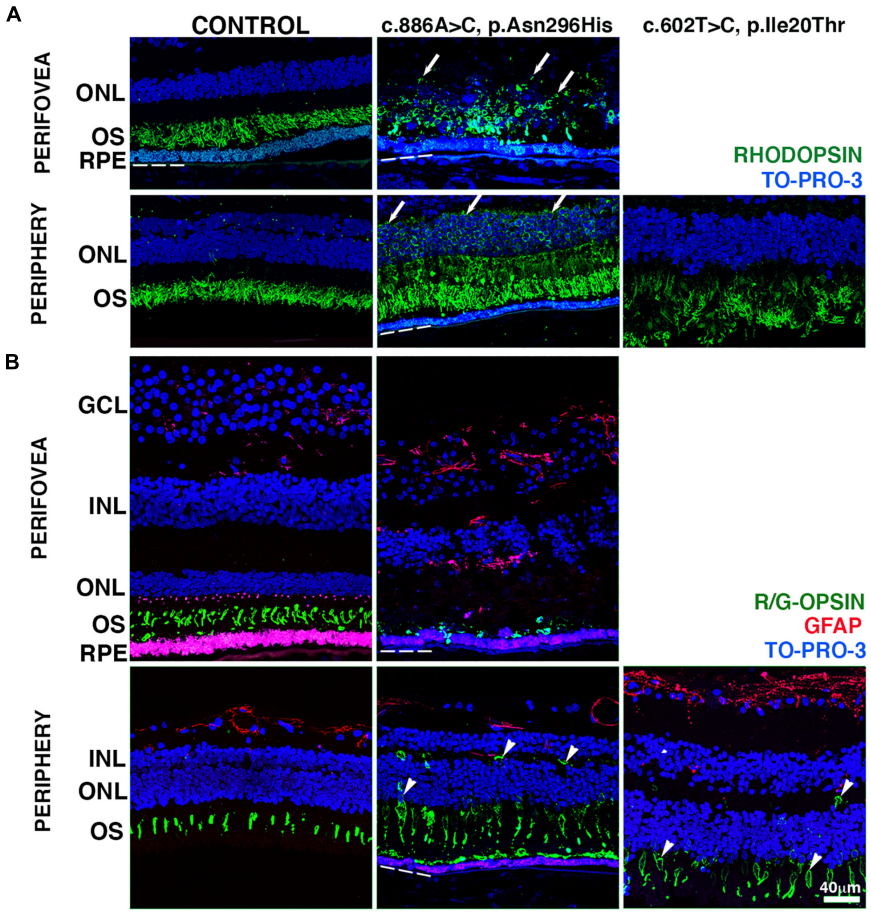

FIGURE 3 | Impact of *BEST1* pathogenic variants in photoreceptors. **(A)** Cryosections obtained from the BD donors and a 65-year-old control were labeled with antibodies specific to rhodopsin (green), while cell nuclei were labeled with TO-PRO-3 (blue). **(B)** Cryosections were also labeled with antibodies specific to red/green cone opsin (green) and GFAP (red), while cell nuclei have been labeled with TO-PRO-3 (blue). Arrow = abnormal distribution of rhodopsin into cell body; arrowheads = abnormal distribution of red/green cone opsin into the cell body. Bruch's membrane is indicated by the hashed white line. Scale bar = 40 μm (all images).

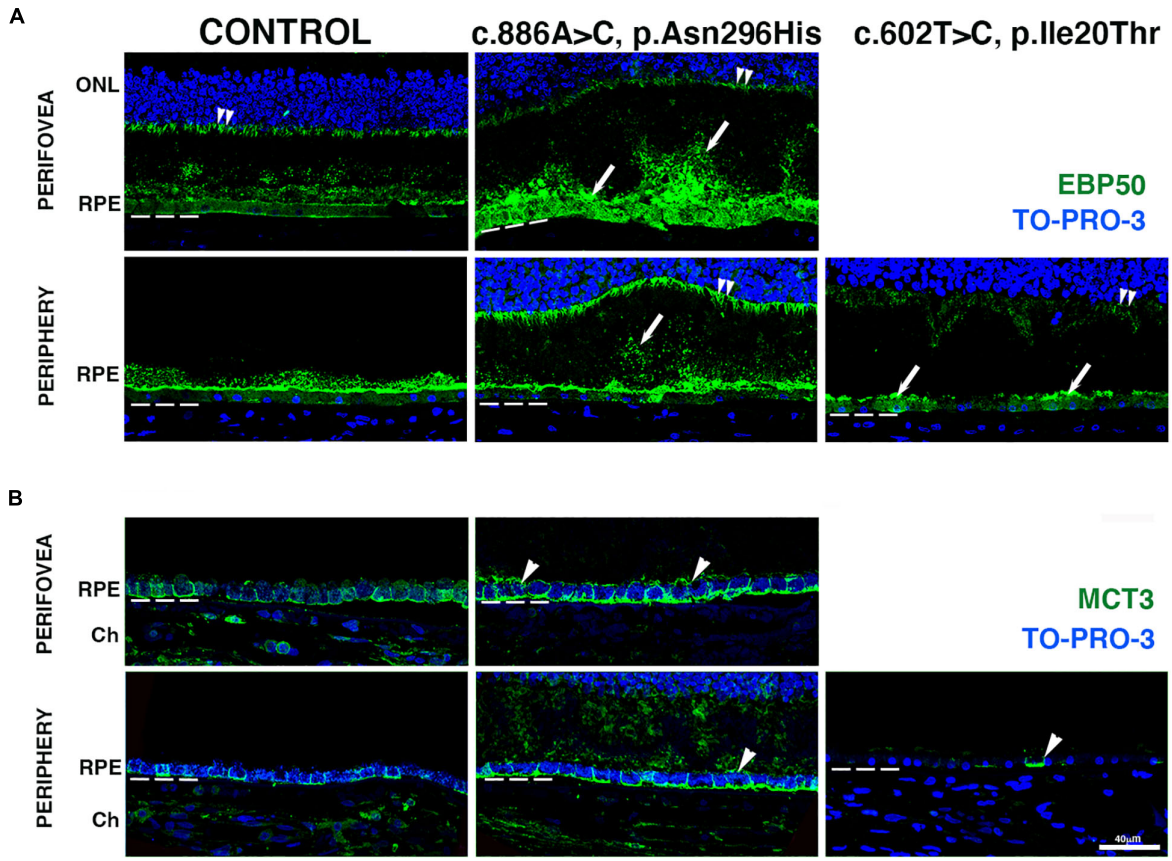

FIGURE 4 | Impact of *BEST1* pathogenic variants in gross pathology and RPE. **(A)** Cryosections obtained from the BD donors and an 88-year-old control were labeled with antibodies specific to EBP50 (green), while cell nuclei were labeled with TO-PRO-3 (blue). **(B)** Cryosections were also labeled with antibodies specific to MCT3 (green), while cell nuclei have been labeled with TO-PRO-3 (blue). Bruch's membrane is indicated by the hashed white line. Arrow = abnormal RPE apical microvilli; arrowheads = mislocalized apical RPE distribution of MCT3; double arrowheads = Muller cell apical processes. Scale bar = 40 μm (all images).

extended up to the photoreceptor nuclei (**Figure 4B**). In the periphery of donor 2, MCT3 was notably absent, with just a few cells labeled (**Figure 4B**, arrowhead).

Finally, we investigated bestrophin-1 distribution in control and BD eyes (**Figure 5**). Immunolabeled bestrophin-1 was more highly expressed in the periphery than in the perifovea of control samples, as previously reported (Mullins et al., 2007). In the perifovea of donor 1, decreased labeling of the RPE was observed; there were a few cytoplasmic punctated structures (**Figure 5**, arrows). In the periphery, labeling was present but diminished and still localized mostly to the basal surface and cytoplasm. In the periphery, of donor 2, overall bestrophin-1 labeling was also decreased, and the protein localized mostly to the apical surface.

DISCUSSION

BD is an inherited macular degeneration with variable penetrance and expressivity characterized by the loss of central vision, accompanied by the inability to perceive colors and resolve detail. A few previous studies reported the histopathology of donor eyes harboring known *BEST1* pathogenic variants p.Thr6Arg (Mullins et al., 2007), p.Trp93Cys (Bakall et al., 2007), and p.Tyr227Asn (Mullins et al., 2005, 2007). Here, we report the retinal findings from donors with a clinical diagnosis of BD harboring c.886A > C (p.Asn296His) (donor 1) or c.602T > C (p.Ile201Thr) (donor 2) variants in *BEST1* gene; the main findings previously described and in the present study are summarized in **Table 1**. These variants have been reported previously (Lotery et al., 2000), but to our knowledge, this is the first report on histopathological findings in the retinas from donors with these variants.

In our study, donor 1 [c.886A > C (p.Asn296His) *BEST1* variant] displayed a central macular lesion that was visible by fundus macroscopy. Immunohistological analysis of this tissue revealed that rod photoreceptors were less affected than cones. In the perifovea, rhodopsin labeling displayed a circular pattern close to the RPE surface, with a few cellular projections being observed in the outer plexiform layer. At the same time, red/green opsin-labeled cones were mostly absent. Although the RPE monolayer was morphologically intact, the distribution of plasma membrane proteins was significantly decreased in the periphery; the apical microvilli labeled by EBP50 could be observed to re-organize into patchy areas of variable

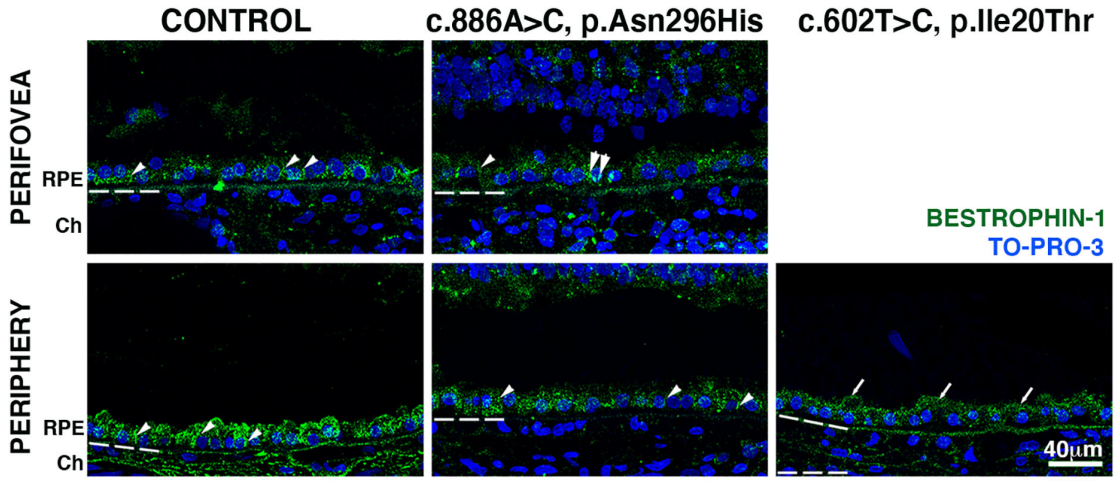

FIGURE 5 | Impact of *BEST1* pathogenic variants in bestrophin-1 RPE localization. Cryosections obtained from the BD donors and an 88-year-old control were labeled with antibodies specific to bestrophin-1 (green), while cell nuclei have been labeled with TO-PRO-3 (blue). Bruch's membrane is indicated by the hashed white line. Arrow = mislocalized apical RPE distribution of bestrophin-1; arrowheads = basolateral RPE distribution of bestrophin-1; double arrowheads = intracellular bestrophin-1. Scale bar = 40 μm (all images).

length with several short and enlarged microvilli and increased basolateral and cytoplasmic distribution while the transporter MCT3 was distributed in both the apical and basolateral membranes. Similar but lessened changes were observed in the periphery of this donor.

The fundus macroscopy of the retina of donor 2 [c.602T > C (p.Ile201Thr) *BEST1* variant] did not display any retinal lesion. However, it did display a substantial fixation artifact in the form of several small areas of retinal detachment. Immunohistological analysis of this tissue revealed that rhodopsin labeling was restricted to the rod outer segments but reduced in expression and substantially disorganized. While cone opsins were distributed throughout the whole cell in the periphery, the distribution of RPE plasma membrane proteins was significantly decreased in the periphery; EBP50 distribution was similar to that observed in control samples, but MCT3 was mostly absent from the RPE.

Müller cells upregulate the expression of the intermediate filament GFAP in response to retinal diseases and injuries (Bringmann et al., 2006). Older retinas frequently have only isolated glial cells overlying large blood vessels, whereas more extensive membranes are associated with disease (Edwards et al., 2016). Based on this previous report, we observed strong GFAP labeling associated with the control samples' blood vessels. Increased GFAP labeling in the periphery of donor 2 and perifovea of donor 1 was observed. A previous study has shown GFAP labeling outside the scar and in the interface between the scar and Bruch's membrane of eyes with BD donor possessing a p.Tyr227Asn mutation (Mullins et al., 2005).

Prior reports indicated that BD is characterized histopathologically by accumulating abnormal lipofuscin in the RPE (O'Gorman et al., 1988; Mullins et al., 2007). Although autofluorescent granules were detected in the RPE cytoplasm, their presence decreased compared to control samples. Our results agree with a previous study that reported a significant decrease in classical lipofuscin granules in the BD donor eyes harboring a p.Trp93Cys pathogenic variant in *BEST1* (Bakall et al., 2007). Our observations could be a direct result of the *BEST1* mutations and their consequences in the retinal physiology. Alternatively, our observations could be related to the disease stage or prolonged exposure to light during eye processing.

Because a primary defect in RPE causes BD, we analyzed the distribution of RPE markers. EBP50 is a PDZ-scaffold protein initially identified as an organizer and modulator of transporters and channels and links apical transporters such as the cystic fibrosis transmembrane conductance regulator (CFTR), the kidney proximal tubule Na + /H + exchanger (NHE3), and the β2-adrenergic receptor to ezrin and the actin cytoskeleton in epithelial microvilli. In the RPE apical microvilli, EBP50 binds to ezrin and the retinoid-binding protein CRALBP through different domains (Bonilha and Rodriguez-Boulan, 2001; Nawrot et al., 2004). In the present study, EBP50 labeling significantly increased in both the perifovea and periphery of donor 1, but it decreased in the apical RPE surface of the periphery of donor 2. EBP50 is upregulated during RPE aging (Gu et al., 2012) and cellular senescence (Althubiti et al., 2014). Moreover, EBP50 is upregulated in diverse cancers where its level of expression correlates with aggressive stage and poor prognosis (Vaquero et al., 2017).

The presence of MCT3, an RPE basolateral transporter (Philp et al., 2003) with an important role in regulating pH and lactate concentrations, was also analyzed. In the present study, MCT3 was observed in all aspects of the RPE membrane of donor 1; in the periphery, MCT3 was mostly confined to the basolateral surface; however, it was also observed in the RPE apical microvilli. In the periphery of donor 2, MCT3 was notably absent, with just a few cells labeled. Mutations in the MCT3

TABLE 1 | Main retinal features observed in retinas of BD donors with known bestrophin-1 mutations.

Manuscript	Histological findings (Macula-Perifovea)	Immunohistochemical findings (Macula-Perifovea)	Histological findings (Perimacula-Periphery)	Immunohistochemical findings (Perimacula-Periphery)
Mullins et al., 2005 (**Y227N** mutation)	– ONL attenuation and a region of severe photoreceptor degeneration resembling a scar and preservation of viable choriocapillaris	– negative GFAP labeling; – positive fibrinogen labeling; – positive BCIP/NBT kit (to detect vessels);	– remarkable degree of outer nuclear layer attenuation; – multiple drusen and areas of RPE detachment; – significant accumulation of basal laminar deposits; – occasional areas of RPE and photoreceptor atrophy	– typical GFAP labeling; – positive rhodopsin labeling; – bestrophin-1 labeling along the apical membrane, cytosol, and the basolateral membrane; – no obvious increase in the size, fluorescence intensity, or number of lipofuscin granules;
Mullins et al., 2007- (**T6R** mutation)	– disciform scarring (with RPE and ONL degeneration); – photoreceptor dropout and gliosis; – presence of "ghost" vessels;		– normal histology; – some peripheral drusen; – focal loss of inner and outer segments and ONL attenuation;	– increased GFAP labeling in areas of photoreceptor loss; – decreased lipofuscin accumulation;
Mullins et al., 2007- (**Y227N** mutation)	– disciform scarring; – photoreceptor dropout and gliosis; – RPE degeneration; – presence of "ghost" vessels;			
Bakall et al., 2007 (**W93C** mutation)	– localized regions of severe retinal degeneration with all retinal layers affected		– large serous retinal detachment; – focal loss of RPE cell; – well preserved retinal layering;	– bestrophin-1 staining localized throughout the RPE; – classical lipofuscin granules (the least dense fraction, in sucrose gradient) was either not present or significantly diminished, however, granules in fractions of higher density were increased;
Present study [**c.886A > C (p.Asn296His**) mutation]	– fibrovascular scar; – ONL was absent; – thin RPE;	– substantial decrease of autofluorescent granules; – rhodopsin labeling displayed a circular pattern close to the RPE surface, with a few cellular projections being observed in the ONL; – red/green opsin labeling significantly decreased; – increased GFAP labeling; – EBP50 labeling significantly increased (highly disorganized RPE apical microvilli seemed to form patchy structures of different lengths and thickness); – MCT3 labeling increased (in all aspects of the RPE membrane); – decreased bestrophin-1 labeling (cytoplasmic punctated); – PNA labeling dispersed through inner and outer segments; – WGA labeling dispersed through inner and outer segments);	– distinct GCL, INL, ONL, RPE and choroid; – edema of the interphotoreceptor matrix	– substantial decrease of autofluorescent granules; – rhodopsin throughout the whole photoreceptor cell; – red/green opsin labeling throughout the entire cone cell body; – EBP50 labeling increased (disorganized RPE apical microvilli seemed to form patchy structures of different lengths and thickness); – MCT3 labeling mostly confined to the basolateral surface (apical in a few cells); – decreased bestrophin-1 labeling (mostly basolateral and cytoplasmic); – visibly decreased PNA labeling (dispersed through inner and outer segments); – WGA labeling dispersed through inner and outer segments;
Present study [**c.602T > C (p.Ile201Thr**) mutation]	– distinct GCL, INL, ONL and RPE;	NOT ANALYZED	– distinct GCL, INL, ONL, RPE and choroid;	– paucity of autofluorescent granules; – overall rhodopsin labeling decreased; – red/green opsin labeling throughout the entire cone cell body; – increased GFAP labeling; – MCT3 labeling notably absent (a few cells labeled); – decreased bestrophin-1 labeling (mostly apical and cytoplasmic); – PNA labeling visibly decreased (outer segments); – WGA labeling visibly decreased (outer segments).

gene have not been linked to retinal disease; however, a previous report described that wounding of RPE monolayers resulted in the dedifferentiation of the cells at the edge of the wound in association with loss of MCT3 (Gallagher-Colombo et al., 2010). The RPE performs nursing functions that regulate and determine the health of the photoreceptors. All these functions rely on the presence of diverse plasma membrane transporters and receptors present either in the apical or in the basolateral membrane domains of RPE. Alterations in the expression or targeting of RPE proteins such as EBP50 and MCT3 would be expected to have a severe impact on the chemical composition of the subretinal space and on photoreceptors function and are thus related to the photoreceptor changes observed in our samples.

The gene responsible for BD is the *BEST1* gene, which encodes bestrophin-1, a transmembrane channel localized to the RPE basolateral plasma membrane. Bestrophin-1 has been extensively studied and described as a multifunctional protein implicated in mediating the flow of ions across the RPE, regulating calcium signaling and cell volume, and modulating the subretinal space milieu (Rosenthal et al., 2006; Hartzell et al., 2008, Neussert et al., 2010; Kane Dickson et al., 2014, Strauss et al., 2014; Yang et al., 2014, Milenkovic et al., 2015; Guziewicz et al., 2018). However, its multifaceted nature and complex interactions with photoreceptors in health and disease remain unsolved (Guziewicz et al., 2017). Here, we detected bestrophin-1 immunohistochemical labeling to the basal surface of the RPE in the periphery of the eye from donor 1 [c.886A > C (p.Asn296His) *BEST1* variant]. In contrast, it was mostly present in the RPE apical surface in the periphery of the eye from donor 2 [c.602T > C (p.Ile201Thr) *BEST1* variant]. The c.704T > C; p.(V235A) *BEST1* mutation was previously reported to be mislocalized at least in part to the apical surface of hiPSC-RPEs from an autosomal dominant vitreoretinochoroidopathy patient (Carter et al., 2016). Our observations suggest that proper bestrophin-1 localization is mutation-dependent. Moreover, our data suggest that BD results from bestrophin-1 dysfunction and its consequences in the RPE function.

Significant insight into the BD pathological mechanisms has been obtained from recent studies employing stem cells for disease modeling since RPE can be readily produced and purified (Singh et al., 2013; Domingo-Prim et al., 2019, Nachtigal et al., 2020). Specifically, human iPS cell (hiPSC)-RPE who harbored p.Ala146Lys or p.Asn296His mutations in *BEST1* reported appropriately polarized distribution of plasma membrane proteins and displayed typical RPE features including apical microvilli, intracellular pigment granules, and uniformly expressed tight junction protein ZO-1 in tight-junctional complexes but displayed decreased net fluid transport and delayed degradation of photoreceptor outer segments associated with increased oxidative stress (Singh et al., 2013). Remarkably, the localization and distribution of bestrophin-1 were similar in the control and BD hiPSC-RPE cells, suggesting that BD most likely resulted from bestrophin-1 dysfunction. Significantly higher autoflourescence levels were detected in BD hiPSC-RPE.

The data presented here provide new insights into the pathology and disease manifestation caused by c.886A > C (p.Asn296His) and c.602T > C (p.Ile201Thr) *BEST1* pathogenic variants. Although the sample size is limited, these two examples suggest that different variants in the *BEST1* gene can result in substantially different diagnostic imaging phenotypes. The different RPE phenotypes observed in the donor eyes and in the BD hiPSC-RPE may result from how the individual variants affect bestrophin-1 structure and function, and how these consequently modulate the subretinal space and photoreceptors. Alternatively, the presence and interaction of one or more modifier genes with *BEST1* may affect the expressivity of the mutation and their manifestation into BD, as previously proposed (Mullins et al., 2007).

Presently, there is no treatment available to treat BD; thus, a better understanding of *BEST1*-related pathogenesis may help to define therapeutic targets. Our results suggest that although BD etiology remains poorly understood, further efforts to understand the unique pathogenesis of each BEST1 mutation are warranted. Only with the full understanding of the cellular and tissue effects on the pathologies is a targeted and efficient therapeutic approach plausible and promises to be successful in the long term. The lower level of bestrophin-1 protein found in both RPE cells does suggest that increasing the levels of this protein, through gene augmentation therapy or by rescuing mutant Best1 from proteasomal degradation, may be a viable means of preventing vision loss in BD. This study resulted in tangible improvements in our understanding of BD pathology. However, it is a snapshot of the BD pathology caused by the BEST variants. Moreover, our study was limited by the lack of clinical data and by the unavailability of perifoveal tissue from donor 2 to be analyzed by immunohistochemistry.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All procedures in this study adhered to the tenets of the Declaration of Helsinki regarding research involving human tissue and were approved by the Institutional Review Boards of the Cleveland Clinic (IRB14-057).

AUTHOR CONTRIBUTIONS

VLB and JGH performed the conceptualization. GAF performed clinical input. VLB, BAB, and MJD performed experimental input. VLB, GAF, SAH, and JGH provided resources. All authors have read, reviewed, edited, and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.573330/full#supplementary-material

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REVIEW
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Not All Stressors Are Equal: Mechanism of Stressors on RPE Cell Degeneration

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Age-related macular degeneration (AMD) is a major cause of irreversible blindness among the elderly population. Dysfunction and degeneration of the retinal pigment epithelial (RPE) layer in the retina underscore the pathogenesis of both dry and wet AMD. Advanced age, cigarette smoke and genetic factors have been found to be the prominent risk factors for AMD, which point to an important role for oxidative stress and aging in AMD pathogenesis. However, the mechanisms whereby oxidative stress and aging lead to RPE cell degeneration are still unclear. As cell senescence and cell death are the major outcomes from oxidative stress and aging, here we review the mechanisms of RPE cell senescence and different kinds of cell death, including apoptosis, necroptosis, pyroptosis, ferroptosis, with an aim to clarify how RPE cell degeneration could occur in response to AMD-related stresses, including H_2O_2, 4-Hydroxynonenal (4-HNE), N-retinylidene-N-retinyl-ethanolamine (A2E), Alu RNA and amyloid β (Aβ). Besides those, sodium iodate ($NaIO_3$) induced RPE cell degeneration is also discussed in this review. Although $NaIO_3$ itself is not related to AMD, this line of study would help understand the mechanism of RPE degeneration.

Keywords: AMD, RPE, oxidative stress, senescence, cell death

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AGE-RELATED MACULAR DEGENERATION

Age-related macular degeneration (AMD) is a major cause of irreversible blindness among the elderly population. The prevalence of AMD is projected to reach 288 million in 2040 due to the increase of the aging population, which could lead to low life quality of the elderly and represent a significant economic burden to the society (Pascolini and Mariotti, 2012; Wong et al., 2014). Macula is the central region of the retina which is critical for the central vision. Early AMD is characterized by drusen deposit underneath the Bruch membrane and disordered pigmentation in the choroid/retinal pigment epithelium (RPE) layers in the macula (de Jong, 2006; Jager et al., 2008). Late AMD has both "dry" and "wet" forms. Geographic atrophy (GA), the advanced form of dry AMD, is featured by the irreversible loss of the RPE, photoreceptors (PRs) and choriocapillaris, which eventually lead to vision loss. Choroidal neovascularization (CNV), as shown by the abnormal growth of new and leaky blood vessels from the choroid into the retina, is a hallmark of wet AMD. Dry and wet AMD accounts for 80–90% and 10–20% of AMD cases, respectively (Bressler, 2002). Currently, antibodies to vascular endothelial growth factor (VEGF) have been approved by FDA to treat wet AMD. Although some clinical studies have shown that antioxidant vitamins and zinc supplements help to slow AMD disease progression

(Age-Related Eye Disease Study Research, 2001; Age-Related Eye Disease Study 2 Research, 2013), there is no available cure for dry AMD. The pathogenesis of AMD is still unclear, genetic, environmental factors as well as advanced age, each contributes to the disease progression (Klein et al., 1998; Tuo et al., 2004; Jonasson et al., 2011). Genome-wide association study has identified a list of AMD-associated genetic variations, including Complement factor family members (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005), Apolipoprotein E (APOE) (Ngai et al., 2011), Fibroblast growth factor 2 (FGF2) (Brion et al., 2011), DNA excision repair protein (ERCC6) (Baas et al., 2010) and Age-related maculopathy susceptibility protein 2 (ARMS2) (Micklisch et al., 2017). However, the No. 1 risk factor for AMD is advanced age, with one third of adults over 75 are affected by AMD (Jonasson et al., 2011). Persons over 85 years old have 10 folds higher prevalence of late AMD than persons who are 70–74 years old (Jonasson et al., 2011). Cigarette smoking, which induces systemic oxidative stress, is the second most consistent and modifiable risk factor for AMD development, associated with 2 to 3 folds increased risk for AMD (Klein et al., 1993).

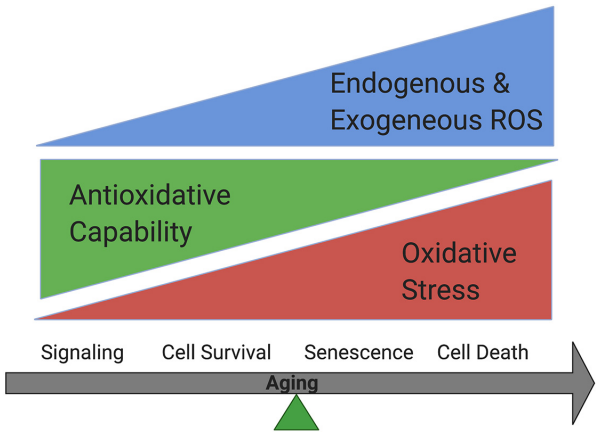

FIGURE 1 | Oxidative stress accumulation increases while antioxidative capability decreases in cells during aging. Cells could survive when oxidative stress is balanced by antioxidative systems while senescence could occur under mild oxidative stress and cell death could happen under more severe oxidative stress.

REACTIVE OXYGEN SPECIES, OXIDATIVE STRESS AND AMD

Free radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS). They are produced during normal metabolism as well as in some pathological conditions (Phaniendra et al., 2015). ROS regulate cellular homeostasis and could contribute to disease pathophysiology, including AMD (Beatty et al., 2000). In normal cells, ROS are produced during metabolic process by enzymes including nicotinamide adenine dinucleotide phosphate [NADPH] oxidases (Noxes), other oxidases and lipoxygenases, and serve as active regulators of cellular signaling. They are balanced by powerful antioxidative systems. Excessive ROS production could occur when cells are exposed to exogenous oxidative stressors, including UV light, ionizing radiation, diet and cigarette smoking. Oxidative stress accumulation due to the increased endogenous and exogenous ROS, and/or decreased antioxidative capability could lead to oxidative modification to major cellular macromolecules, which lead to features of aging including metabolic dysfunction, cell senescence or cell death (Beckman and Ames, 1998; Droge, 2002; Valko et al., 2007; Rajendran et al., 2014; Davalli et al., 2016; Pizzino et al., 2017; **Figure 1**).

Reactive oxygen species consist of Superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), and singlet oxygen ($^1O_2$). Molecular oxygen (O_2) undergo single electron reduction and form superoxide anion ($O_2^{\cdot -}$). Once formed, $O_2^{\cdot -}$ is involved in a reaction that in turn generates H_2O_2 ($2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$). Hydroxyl radical ($OH^{\cdot}$) is generated by reaction of $O_2^{\cdot -}$ with H_2O_2 through Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$) (Winkler et al., 1999). Peroxynitrite ($ONOO^-$) is also a physiological product generated by the interaction of superoxide ($O_2^{\cdot -}$) and nitric oxide (NO) which can generate ROS and induce cell death (Szabo et al., 2007). High intracellular ROS level could modify and damage carbohydrates, membrane lipids, proteins, and nucleic acids, with pathological consequences. ROS can easily react with membrane lipids and cause the lipid peroxidation. Polyunsaturated fatty acids (PUFAs) are particularly susceptible to free radical damage which generates products including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) which show higher levels in AMD retina (Gardner, 1989; Halliwell and Chirico, 1993; Spiteller et al., 2001). Oxidation of docosahexaenoate (DHA)-containing lipids produces carboxyethylpyrrole (CEP), which is also abundant in AMD retina (Crabb et al., 2002). Oxidation in proteins can cause fragmentation, cross-linking, aggregation of proteins, and enhanced proteolysis (Negre-Salvayre et al., 2008). For example, reactive carbonyl compounds formed during lipid peroxidation, such as MDA and 4-HNE, form adducts and cross-links with proteins, which causes protein damage and functional deficiency (Stadtman and Levine, 2000; Negre-Salvayre et al., 2008). ROS-induced nuclear and mitochondrial genomes damage, such as DNA strand breaks, base modifications and DNA-protein cross linkages, are associated with aging and age-related diseases (Bohr et al., 1998, 2007).

Antioxidative systems, include enzymatic and non-enzymatic systems, have evolved to protect against ROS. Enzymatic antioxidants include superoxide dismutase (SOD) and glutathione peroxidase (GPX) et al. SOD catalyze superoxide converse to H_2O_2 and O_2 and reduce ROS levels (Halliwell and Gutteridge, 1986). GPX reduces H_2O_2 and lipid peroxides to water and lipid alcohols via the expense of reduced glutathione (GSH) (Arthur, 2000). Thus, GSH is also considered as a kind of non-enzymatic antioxidant. Other non-enzymatic antioxidants include tocopherol homologs, carotenoids, flavonoids, etc. When accumulated free radicals cannot be eliminated by antioxidant systems, damage to DNA, proteins, and lipids happens, which can subsequently cause cell death and diseases (McCord, 2000; Therond, 2006; Birben et al., 2012).

Reactive oxygen species and oxidative stress both have been implicated in AMD. As mentioned above, cigarette smoking is the #2 risk factor for AMD. Retina is a tissue which continually exposes to light, contains high levels of PUFAs and consumes oxygen at a high rate which all increase ROS production in the cells (Beatty et al., 2000; Khandhadia and Lotery, 2010). It has been reported that increased oxidative DNA damage, as well as the accumulation of CEP, 4-HNE, and MDA, is found with aging in retina tissue (Jarrett and Boulton, 2012). These oxidative products have been shown to induce inflammatory response and retinal phenotype in animal models of AMD (Suzuki et al., 2007; Hollyfield et al., 2008). For more information of oxidative stress and AMD, refer to reviews of Jarrett and Boulton (2012) and Mettu et al. (2012).

RPE BIOLOGICAL FUNCTIONS

The RPE monolayer of the retina functions as the outer blood-retina barrier and help to transport nutrients and waste between PRs and choroid. RPE cells in the adults are post-mitotic and polarized with proteins/organelles distributed and/or secreted asymmetrically in apical or basolateral domains of the cells (Burke, 2008). The functions of RPE cells include: (1) Maintaining essential function of the retina. Melanins are synthesized and stored in the melanosome of RPE cells which help to absorb light that pass through the PR layer and also absorb reflected light that may degrade the visual image (Weiter et al., 1986). Melanin synthesis decreases with age (Simon et al., 2008). Some transporters on the membrane of RPE help to provide a stable environment for RPE and nearby cells. For example, sodium/potassium adenosine triphosphatase (Na^+/K^+-ATPase) is located apically in RPE cells and helps to maintain the volume, ion concentrations and chemical composition of the subretinal space. These are essential for the functions of neural retina and RPE (Wimmers et al., 2007). (2) Maintaining PRs function. Microvilli of RPE cells envelop and interact with the outer segments (OS) of both rod and cone PRs. PRs regeneration of the PR outer segment (POS) occurs every 7–12 days through phagocytosis function of RPE (Young and Droz, 1968), which protects PRs from chronic oxidative stress exposure (Bok, 1985). (3) Participating in the visual cycle. Visual cycle is the process that cycles retinoids between the rod OS and the RPE. Light isomerizes 11-*cis* retinal into all-*trans* retinal, which is released from the visual pigment opsins, causing visual pigment activation. The photoproducts then enter the RPE, where 11-*cis* retinal is regenerated before returning to PRs (Bernstein et al., 1987). (4) Regulating retinal immune response. RPE cells secret cytokines such as IL-1α, IL-1β, IL-7, TNF-α, IFN-γ, TGF-β. Cytokines secreted by RPE play an important role in the homeostasis of the retina, as well as in inflammatory responses by activation of resident cells and attraction and activation of inflammatory cells (Holtkamp et al., 2001). Overall, RPE cells are critical for metabolism and homeostasis of retina, especially PRs. Due to their exposure to high light and oxygen, oxidized POS and PUFAs, RPE cells are exposed to high oxidative stress conditions, and vulnerable to degeneration if the antioxidative defense mechanism is compromised. Several AMD-related risk factors can affect RPE structure and function. Aging leads to RPE structural changes, such as loss of melanin granules, accumulation of residual bodies, drusen formation, thickening of Bruch's membrane, RPE microvilli atrophy and et al. (Bonilha, 2008). Also, factors such as cigarette smoking, high fat diet and genetic factors are believed to lead to oxidative stress and inflammation which are related to RPE degeneration (Datta et al., 2017). For more information regarding RPE function, refer to review of Sparrow et al. (2010).

MODES OF CELL DEGENERATION AND DEATH

Retinal pigment epithelial degeneration in AMD involves RPE dysfunction, senescence and cell death. This review will focus on RPE senescence and cell death. An overview of cellular senescence and cell death mechanisms will first be introduced (**Figure 2** Overview of cell death pathways).

Apoptosis

Apoptosis is a classic mode of programmed cell death. It plays important roles in both physiological and pathological processes. Classic features of apoptosis include membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin fragmentation and the formation of apoptotic bodies (Kerr et al., 1972). Apoptosis can be initiated by either intrinsic or extrinsic pathways. The intrinsic apoptosis pathway is promoted by cellular stresses include DNA damage, oxidative stress and irradiation. The extrinsic pathway relies on signaling through transmembrane receptors of the tumor necrosis factor (TNF) receptor family (Igney and Krammer, 2002). Apoptosis is regulated by the caspase family of proteins. Caspases are synthesized as inactive proenzymes containing a N-terminal peptide or pro-domain, and two subunits. Cleavage of caspases occurs at specific asparagine (Asn) residues located after the pro-domain and between the large and small subunits, forming active heterotetramers. Caspase-8/10 act as initiator caspases which are activated by extrinsic signal. Caspase-9 also functions as initiator caspase but is activated by intrinsic signal. Endoplasmic reticulum (ER) stress could induce the activation of Caspase-4 (Hitomi et al., 2004). These signals then activate downstream caspases 3/6/7 and subsequent apoptosis (Cohen, 1997). Caspases-3/6/7 are considered as executioner caspases due to their similar short pro-domains. Caspase-3 is needed for efficient cell death and also could block ROS production, but activation of Caspase-6 alone cannot cause apoptosis (Gray et al., 2010). Caspase-7 is responsible for ROS production and aids in cell detachment during apoptosis (Brentnall et al., 2013). Usually, active Caspase-3 is detected in most apoptotic cells. A pan-caspase inhibitor z-VAD-FMK can be used to inhibit apoptosis. For details about apoptosis, refer to review of Elmore (2007).

Necrosis

Necrosis was considered to be a passive and unregulated cell death in responsive to infections, toxins or trauma. Recent

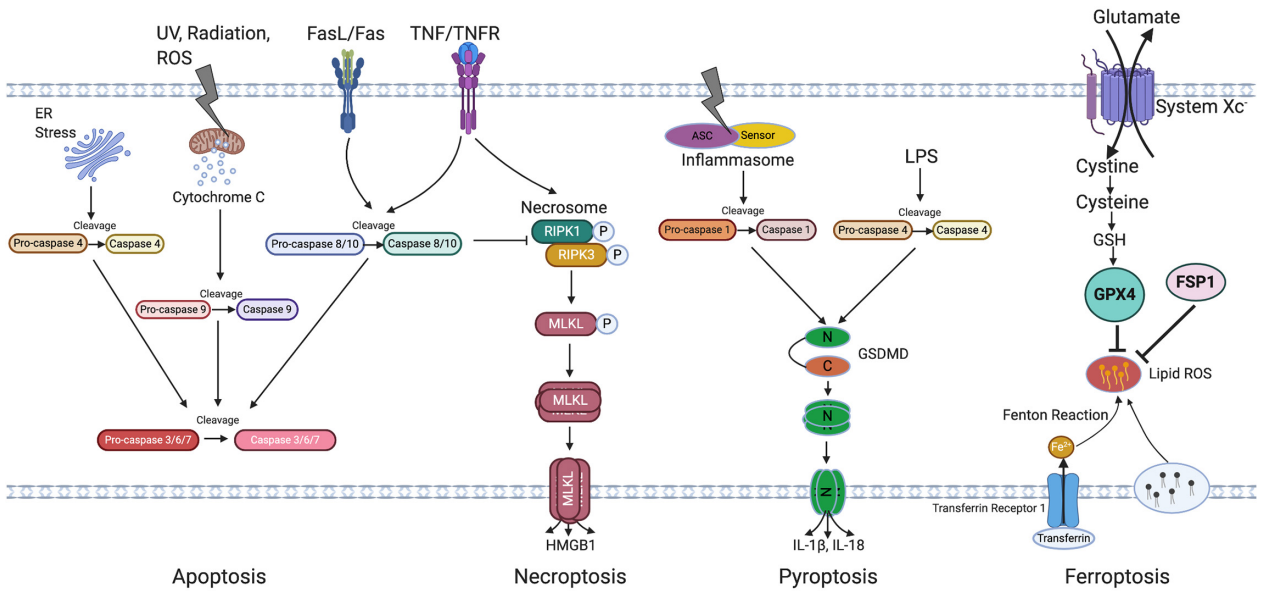

FIGURE 2 | Overview of cell death pathways. Apoptosis is triggered by either intrinsic (by UV, radiation, exogenous ROS, or ER stress) or extrinsic signals (through FasL/Fas or TNF/TNFR) and is regulated by the caspase family of proteins. Caspase 4, 9, and 8/10 serve as initiator caspase, while Caspase 3/6/7 serve as executor caspase. Necroptosis is initiated by activation of TNFR and subsequent activation of necrosome containing phosphorylated RIPK1 and RIPK3, phosphorylation and oligomerization of MLKL then result in cell rupture, death and HMGB1 release. Pyroptosis is induced through both Caspase-1 and Caspase-4 activation which induce GSDMD cleavage, followed by oligomerization of N terminal of GSDMD which then leads to cell rupture and death. Caspase 1 cleaves IL-1β and IL-18, leading to its activation and release from the cells. Ferroptosis is induced by the inhibition of system Xc and is featured by accumulation of lipid ROS which can be inhibited by GPX4 and FSP1. Irons are transferred through transferrin and its receptor and are involved in the Fenton reaction leading to lipid peroxidation. ER, Endoplasmic reticulum; UV, Ultraviolet light; TNF/TNFR, Tumor necrosis factor/Tumor necrosis factor receptor; RIPK3, Receptor-interacting protein kinase 3; MLKL, Mixed Lineage Kinase Domain Like Pseudokinase; HMGB1, High mobility group box 1; ASC, Apoptosis-associated speck-like protein containing a caspase recruitment domain; LPS, Lipopolysaccharide; N-GSDMD, N terminal of gasdermin D; C-GSDMD, C terminal of gasdermin D; IL-1β, Interleukin-1β; IL-18, Interleukin-18; GSH, Glutathione; GPX4, Glutathione Peroxidase 4; FSP1, Ferroptosis suppressor protein 1; ROS, Reactive oxygen species.

studies showed that some necrosis could be regulated. Regulated necrosis includes but is not limited to necroptosis, pyroptosis and ferroptosis.

Necroptosis

Necroptosis is morphologically characterized by cells swelling and bursting, with releasing their intracellular contents. It can be initiated by activation of TNF receptor (TNFR) and subsequent activation of two members of the receptor interacting protein kinase (RIPK) family (RIPK1 and RIPK3), when Caspase-8 is not activated (Fritsch et al., 2019). These kinases then form "necrosomes" via specialized domains termed RIP homotypic interaction motifs (RHIM). Reciprocal interactions between RIPK1 and RIPK3 lead to phosphorylation of a pseudokinase called Mixed lineage kinase domain like pseudokinase (MLKL) (Rodriguez et al., 2016). Once phosphorylated, MLKL translocates to the cell membrane and form tetramers, leading to osmotic cell membrane rupture by disrupting cellular ion homeostasis and the release of inflammatory cytokines such as high-mobility group box-1 (HMGB1) (Sun et al., 2012; Dondelinger et al., 2014; Gong et al., 2017). Several inhibitors can be used to block necroptosis, include Necrostatin-1 (Nec-1), a direct RIPK1 inhibitor; Necrostatin-5 (Nec-5), an indirect RIPK1 inhibitor; Necrostatin-7 (Nec-7) that targets RIPK1-independent necrosis; GSK'872, a specific RIPK3 inhibitor and Necrosulfonamide (NSA), a MLKL inhibitor. For details about necroptosis, refer to review of Weinlich et al. (2017).

Pyroptosis

Pyroptosis is featured by plasma membrane rupture and release of proinflammatory intracellular contents, include Interleukin-1 beta (IL-1β) and Interleukin-18 (IL-18) (He et al., 2015). Pyroptosis can be induced through both canonical and non-canonical inflammasome pathways (Liu and Lieberman, 2017; Xu et al., 2018). In canonical pyroptosis, inflammasomes include absent in melanoma 2 (AIM2), Pyrin, or the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family (NLRP1, NLRP3, and NLRC4) are activated by pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) (Wang et al., 2019). Inflammasomes then recruit Caspase-1 via the CARD-domain containing adaptor protein (ASC, also called PYCARD), which cleave pro-Caspase-1 to its active form. The activated Caspase-1 subsequently induces the maturation and secretion of IL-1β and IL-18 and cleaves gasdermin D (GSDMD) into N-terminal and C-terminal domains. The N-terminal fragments then oligomerize, translocate to the cell membrane and form membrane pores which leads to cell swelling, membrane rupture, release of inflammatory factors and cell death (Ding et al., 2016;

Liu et al., 2016). In the non-canonical pathway, Caspase-4/5 (Caspase-11 in mice) recognize cytosolic lipopolysaccharide (LPS) via CARD domain and subsequent GSDMD cleavage which then leads to cell death (Kayagaki et al., 2011; Shi et al., 2014; Kovacs and Miao, 2017). Inhibitors of pyroptosis include Ac-YVAD-CMK, a caspase-1 inhibitor; MCC950, a NLRP3 inhibitor and so on. For details about pyroptosis, refer to review of Bergsbaken et al. (2009).

Ferroptosis

Ferroptosis is a regulated cell death defined in 2012 (Dixon et al., 2012) and is characterized by lipid peroxidation and iron involvement, but its molecular pathway is yet to be clearly defined. Ferroptotic cells do not show the typical morphological characteristics of necrosis, such as cell swelling and cell membrane rupture, but mainly display mitochondria shrinkage, increased mitochondria membrane density and mitochondrial cristae reduction (Yagoda et al., 2007; Yang and Stockwell, 2008). It can be induced by the inhibition of system X^{c-}, a glutamate/cystine antiporter on the cell membrane. System X^{c-} helps cells to take up cysteine, which stimulates the synthesis of GSH. This promotes the activity of glutathione peroxidase 4 (GPX4), an antioxidative enzyme which reduces lipid hydroperoxides and lipid ROS production in cells (Brigelius-Flohe and Maiorino, 2013). Inhibition of System X^{c-} or GPX4 activity leads to lipid ROS accumulation. Extreme accumulation of lipid ROS is toxic to the cells and results in ferroptosis. It's been reported that FSP1/AIFM2 functions to suppress ferroptosis, representing a new pathway to regulate ferroptosis (Bersuker et al., 2019; Doll et al., 2019). Lipid ROS scavenger, Liproxststatin-1, Ferrostatin-1, Vitamin E can block ferroptosis. Iron chelator DFO can be used to inhibit ferroptosis as well. For details about ferroptosis, refer to review of Li et al. (2020).

Cellular Senescence

Cellular senescence was first identified as a stable cell cycle exit from cell culture (Hayflick, 1965). It is now considered as a protective stress response, which includes metabolic reprogramming, chromatin rearrangement and autophagy modulation (Kuilman et al., 2010). Senescent cell accumulation could drive aging and age-related diseases (van Deursen, 2014; Childs et al., 2015). Senescent cells show enlarged cell size, arrested growth, increased ROS levels, persistent DNA damage response, apoptosis resistance, changes in chromatin organization and gene expression (Ogryzko et al., 1996; Chen et al., 2000; Hampel et al., 2004; Herbig et al., 2004). Various biomolecules also can be released by senescent cells to exert changes to neighboring cells, including chemokines, cytokines, proteases, growth factors, which is called senescence-associated secretory phenotype (SASP) (Nelson et al., 2012). Senescence associated (SA)-β-gal can be detected in most senescent cells and acts a marker for senescence (Dimri et al., 1995; Lee et al., 2006). Other upregulated markers for senescence include cell cycle regulators $p16^{INK4a}$, p21, and p53 (Collado and Serrano, 2010). Rapamycin and related mTORC1 inhibitors, ruxolitinib, glucocorticoids and metformin can be used to inhibit senescence. For details about senescence, refer to review of Khosla et al. (2020).

Generally, apoptosis and regulated necrosis are different both morphologically and molecularly. However, cross talk exists among those pathways. For example, inflammasomes mainly mediate pyroptosis, but also can activate Caspase-8 and induce apoptosis (Hitomi et al., 2004; Liu and Lieberman, 2017). Also, NLRP3 inflammasome can be activated by RIPK3 and MLKL which then leads to IL-1β inflammatory responses (Kang et al., 2013). In mouse erythroid precursors, GPX4 which is a key antioxidant in ferroptosis pathway was found to also prevent necroptosis (Canli et al., 2016). In some conditions, cells could go through alternative pathway. Once TNFR is activated, apoptosis happens when Caspase-8 exists but necroptosis could be induced in the absence of Caspase-8 in cells (Fritsch et al., 2019). Ferroptosis and necroptosis were also found to be alternative pathways since deletion of MLKL block necroptosis and cells will go through ferroptosis (Muller et al., 2017). Different modes of cell death have been reported in RPE cells, depending on the type, dosage and duration of stresses. This creates some controversy regarding how RPE cells die response to different stresses in AMD. A clear answer to this question could facilitate therapeutic development for dry AMD, especially GA. Thus, we review here the recent understanding of RPE cell senescence and death mechanism in response to AMD-relevant stresses, including H_2O_2, 4-HNE, N-retinylidene-N-retinyl-ethanolamine (A2E), Alu RNA and Amyloid beta (Aβ). Besides those, sodium iodate ($NaIO_3$) induced RPE cell degeneration is also discussed in this review. Although $NaIO_3$ itself is not related to AMD, this line of study would help understand the mechanism of RPE degeneration.

DIFFERENT STRESSES IN RPE SENESCENCE AND/OR CELL DEATH

Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide functions during both normal metabolism and under oxidative stress conditions (Sies and Chance, 1970). The sources of H_2O_2 include one or two-electron reduction reactions catalyzed by Noxes and other oxidases (Bedard and Krause, 2007), as well as the complexes in the mitochondrial respiratory chain (Brand, 2016). When not being metabolized, H_2O_2 can convert to $OH^{\cdot}$ via the Fenton reaction which increases oxidative damage to the cell. It's been reported that prolong treatment of RPE cells with low concentration H_2O_2 can lead to RPE senescence. Marazita et al. (2016) found that 80% of the ARPE-19 cells exposed to 150 uM H_2O_2 and cultured in maintenance medium for 10 days exhibited SA-β-Gal positivity and increased $p16^{INK4a}$ and p21 expression. Higher concentration H_2O_2 can lead to cell death and the mode of cell death induced by H_2O_2 appears to be dependent on its concentration. Many studies reported apoptosis is involved in H_2O_2 induced RPE cell death. In Barak's study, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays combine propidium Iodide (PI)/Annexin V staining were used to detect RPE apoptosis/necrosis after exposure to H_2O_2

(0.5–2.5 mM) for 16–24 h. They concluded that H_2O_2 at 1 mM induced mostly apoptosis and at 2.5 mM induces mostly necrosis in ARPE-19 cells (Barak et al., 2001). Alge et al. (2002) found increased Caspase-3 activity in H_2O_2 treated human RPE cells, as determined by the chromophore p-nitroaniline (pNA) release after its cleavage by activated Caspase-3 from the labeled caspase-specific substrate. Zhao et al. (2019) reported increased Caspase-3 cleavage in H_2O_2 treated ARPE-19 cells by Western blotting. There are also many other studies showed apoptosis in H_2O_2-treated RPE cells while some chemicals or proteins can protect cells from apoptosis, such as taxifolin (Xie et al., 2017), kinsenoside (Luo et al., 2018), kaempferol (Sreekumar et al., 2005), and genipin (Zhao et al., 2019). Kim et al. (2003) proposed that H_2O_2 induced both apoptosis and necrosis in RPE. H_2O_2 at 400 uM was shown to induce early apoptosis in ARPE-19 cells with condensed and fragmented nuclei. Higher H_2O_2 concentrations lead to late apoptotic and necrotic RPE cell death, while concentrations above 700 uM mainly caused necrotic RPE cell death. Chromatin condensation and marginalization were shown in ARPE-19 cells treated with 500 uM H_2O_2 under transmission electron microscope, while 600 uM H_2O_2 induces organelle swelling and membrane rupture in the cells. However, in the study of Li et al. (2010), H_2O_2 at 400 uM was able to induce ARPE-19 cell death and the cells showed cell swelling, cell membrane rupture, and nuclei condensation which are typical features of necrosis. Our laboratory also studies the nature of oxidative stress induced RPE cell death. In our study, typical necrotic characteristics like PI membrane permeability, RIPK3 activation and HMGB1 release from the nucleus were shown in ARPE-19 cells treated by 300 uM H_2O_2. Caspase inhibitor z-VAD could not reduce H_2O_2 induced ARPE-19 cell death. However, RIPK1 inhibition and RIPK3 knockdown significantly rescued ARPE-19 cells from H_2O_2 treatment (Hanus et al., 2013). One of the major differences between apoptosis and necrosis is the level of cellular ATP. ATP is required for several processes in apoptosis, including caspase activation, enzymatic hydrolysis of macromolecules, chromatin condensation, bleb formation and apoptotic body formation (Richter et al., 1996). Usually, intracellular ATP levels remain unchanged during the whole apoptotic process while ATP depletion happens in necrosis (Eguchi et al., 1997). We found about 90 and 97% ATP depletion in ARPE-19 cells induced by 300 and 500 uM H_2O_2 treatment, respectively (Hanus et al., 2013). DNA fragmentation or Caspase-3 cleavage was not seen in H_2O_2 treated RPE cells. Some other studies also reported ATP depletion in H_2O_2 treated RPE cells (Giddabasappa et al., 2010; Du et al., 2016). It has been found that in human RPE cells, Caspase-8 mRNA and protein levels were low compared with other cell types. Low Caspase-8 levels may protect RPE cells from apoptosis (Yang et al., 2007). Thus, the role of apoptosis in RPE cell death still needs to be verified. So far, there is no report about H_2O_2-induced ferroptosis in RPE cells, but a similar stress tert-butyl hydroperoxide (tBH)-induced ferroptosis in ARPE-19 cells has been reported (Marazita et al., 2016). More experiments are still needed to clarify the mechanism of RPE cell death induced by H_2O_2. The choice of RPE cells [ARPE-19, primary RPE cells or induced pluripotent stem (IPS)-derived RPE cells] and culture conditions (including cell density, differentiation status) should be carefully controlled.

4-Hydroxynonenal (4-HNE)

Lipid peroxidation is the process that oxidants such as free radicals attack lipids containing carbon-carbon double bonds on the membranes of cells and/or subcellular organelles (Yin et al., 2011). 4-HNE is one of the end products of lipid peroxidation. Under physiologic conditions, 4-HNE usually presents at very low concentration in plasma (0.28–0.68 μM) and a bit higher (≤5 μM) in cells under physiologic conditions (Niki, 2009; Schaur et al., 2015). However, its concentration can be increased by 100 times in response to oxidative stress (Esterbauer et al., 1991). 4-HNE concentrations also increase during aging. It was reported in one study that plasma 4-HNE concentration was 68.9 ± 15.0 nmol/L in the young group (up to 30 yr old) which increased to 107.4 ± 27.3 nmol/L in the elderly group (older than 70 yr) (Gil et al., 2006). 4-HNE has protective functions as a signaling molecule at the physiological level but has cytotoxic effect at abnormally high levels (Ayala et al., 2014). 4-HNE accumulation is associated with cell cycle arrest, cell differentiation and cell death (Esterbauer et al., 1991; Niki, 2009; Shoeb et al., 2014). It can also modify histidine, cysteine, and lysine residues of proteins and form HNE-protein adducts. 4-HNE accumulation has been reported to be involved in the pathology of many age-related diseases including Alzheimer's disease (Skoumalova and Hort, 2012), Parkinson's disease (Kilinc et al., 1988), and cancer (Nair et al., 2007). 4-HNE has also been shown to be significantly increased in the retina of AMD eyes as well as in patient plasma (Schutt et al., 2003; Ethen et al., 2007). Ethen et al identified nineteen proteins in AMD retina which are involved in energy production and stress response, were consistently modified by 4-HNE regardless of stage of AMD or retinal region (Ethen et al., 2007). Sharma et al reported that 4-HNE induces activation, phosphorylation, and increased nuclear accumulation of p53 in human RPE and ARPE-19 cells. Signaling components involved in p53-mediated apoptosis were activated as well. JNK and Caspase-3 as markers of apoptosis pathway are both activated by 4-HNE as well (Sharma et al., 2008). It's been found that increased p21 expression (as a senescence marker) was induced by 4-HNE in neuroblastoma cells (Laurora et al., 2005) and colon cancer cells (Cerbone et al., 2007). However, whether 4-HNE could induce senescence in RPE cells hasn't been reported so far. Based on the definition of ferroptosis, lipid peroxidation is believed to be involved in ferroptosis process. As one of the end products of lipid peroxidation, 4-HNE accumulation was shown in ferroptotic murine heart and kidney tissues (Martin-Sanchez et al., 2017; Fang et al., 2019). It would be interesting to know whether 4-HNE is involved in ferroptosis in RPE cells.

N-Retinylidene-N-Retinyl-Ethanolamine (A2E)

N-retinylidene-N-retinyl-ethanolamine is a by-product of visual cycle which is formed by the reaction of two *trans*-retinal molecules with phosphatidylethanolamine (Sparrow et al., 2003a). It is a major fluorophore identified in lipofuscin

from aged human eyes and is accumulated in RPE with age (Sparrow and Boulton, 2005). A2E undergoes photooxidation and produces oxygen adducts in the presence of blue light and oxygen (Wielgus et al., 2010), which subsequently induces increased oxidative stress and proteins and DNA damage in RPE cells (Ferrington et al., 2016; Sparrow et al., 2003b). Apoptosis has been implicated in A2E accumulated RPE cells in several studies. Shaban et al. (2001) reported A2E induced apoptosis in RPE cells. The cells showed declined mitochondrial activity and release of cytochrome c and apoptosis-inducing factor. They then reported A2E leads to more severe apoptotic cell death in cultured human RPE cells in the light compared to those in the dark (shown by PI/Annexin-V staining). Also, A2E induces increased H_2O_2 level and decreased GSH level (Shaban et al., 2002). Sparrow and Cai (2001) observed Caspase-3 activation (using a Caspase-3 fluorescence probe) in ARPE-19 treated with A2E and blue light while an apoptosis inhibitor Z-DEVD-fmk decreased the numbers of apoptotic cells. Alaimo et al. (2019) reported blue light and A2E co-treatment induces ROS generation and increased pro-caspase-3 expression level in ARPE-19 cells. Early and late apoptotic ARPE-19 cells were observed after the treatment. Anderson et al found that A2E induces upregulated IL-1β production and ASC cluster formation in ARPE-19 cells. NLRP3 knock down and Caspase-1 inhibitor Z-WEHD-FMK both can inhibit A2E induced IL-1β production (Anderson et al., 2013). These are makers of pyroptosis which may indicate the involvement of pyroptosis in the process. Wang et al reported that A2E triggers telomere dysfunction and accelerates cellular senescence in ARPE-19 cells (Wang et al., 2018). They found SA-β-gal positivity and SASP in A2E treated ARPE-19 cells, while telomerase overexpression suppressed A2E mediated RPE cell senescence. More studies are needed to clarify the involvement of apoptosis and pyroptosis in A2E-induced RPE degeneration.

Alu RNA

Alu RNAs are non-coding transcripts belonging to the Alu family of retrotransposons (Cordaux and Batzer, 2009). Free Alu transcripts are expressed at very low levels in physiological conditions, approximately only 10^2–10^3 molecules per cell; while the levels increases under various stresses, such as heat shock (Pandey et al., 2011), hyperglycemia (Wang et al., 2016) and viral infection (Panning and Smiley, 1994). It's been reported that Alu RNA accumulation induces ROS production and impedes SOD2 expression in cells while higher oxidative stress leads to more severe Alu RNA accumulation (Wang et al., 2016; Hwang et al., 2019). Kaneko et al. (2011) found that deficiency of *Dicer1*, an RNase III involved in microRNA biogenesis (Bernstein et al., 2001), leads to cytotoxic Alu RNA accumulation in human RPE cells and RPE degeneration in mice shown by Fundus examination and RPE/choroid flat mount. They showed a more than 40 folds increase in the Alu RNA levels in the RPE of human eyes with GA but not in the neural retina. Also, Dicer1 knockdown in human RPE cells leads to increased Alu RNA accumulation. Subretinal injection of Alu RNA induced RPE degeneration in wild-type mice. Caspase-3 activation was shown in the RPE cells of *Dicer1*$^{-/-}$ mice and Alu RNA-treated human RPE cells. These suggest Dicer1 dysregulation induces Alu RNA accumulation and may cause apoptotic RPE cell death in GA. Tarallo et al. (2012) from the same group showed that Alu RNA exposure induces mitochondria ROS production, activates NLRP3 inflammasome and triggers IL-18 secretion in RPE cells. Inhibition of NLRP3, PYCARD, Caspase-1, or IL-18 alleviates RPE degeneration induced by Dicer1 deletion or Alu RNA exposure. They also observed elevated NLRP3, PYCARD, IL-18 levels and increased Caspase-1 activation in the RPE of human eyes with GA. They showed Alu RNA led to Caspase-1 activation in human RPE cells using both Western blot and a fluorescent reporter of substrate cleavage. Intravitreal delivery of the Caspase-1 inhibitor Z-WEHD-FMK, blocked IL-18 maturation and Alu RNA induced RPE degeneration in wild type mice. Similarly, *Caspase-1*$^{-/-}$ mice treated with Alu RNA did not exhibit RPE degeneration. In human RPE cells transfected with fluorescent tagged PYCARD (GFP-PYCARD), Alu RNA treatment induced NLRP3 inflammasome activation. Alu RNA didn't induce RPE degeneration in either *Nlrp3*$^{-/-}$ or *Pycard*$^{-/-}$ mice, demonstrating the critical importance of the inflammasome in Alu RNA cytotoxicity. Pyroptosis can also proceed independent of IL-18. However, they found that IL-18 induced RPE degeneration in *Caspase-1*$^{-/-}$ mice which couldn't be rescued by a pyroptosis lysis inhibitor glycine. Therefore, they claimed that Alu RNA induced RPE degeneration does not occur via pyroptosis. In a later paper (Kim et al., 2014), they reported an increased total Caspase-8 protein level in the RPE of human eyes with GA compared with healthy, age-matched eyes. Since Caspase-8 can function either upstream or downstream of inflammasome activation, so they tested whether Caspase-8 is required for AluRNA induced RPE cell death and cytokine production. They observed Caspase-8 activation in primary human RPE cells treated with Alu RNA. RPE specific knock out of Dicer1 as well as Alu RNA subretinal injected wild type mice exhibited significantly greater Caspase-8 activation in RPE/choroid tissues while blockage of Caspase-8 protects RPE from Alu RNA toxicity. They then found that IL-18 induced RPE degeneration can be inhibited by knockout of Caspase-8. Also, subretinal injection of Alu RNA lead to elevated Fas ligand (FasL) and Fas receptor expression, which are required for the cleavage of pro-caspase 8 in RPE/choroid tissues; while *Fas* or *FasL* knock out inhibited Alu RNA induced RPE degeneration. Caspase-8 inhibitor failed to reduce Caspase-1 activation in Alu RNA treated human RPE cells which indicated that Caspase-8 acted downstream of Caspase-1. Previously, they reported Alu RNA induced activation of Caspase-3 in human RPE cells (Kaneko et al., 2011), a critical executioner in apoptotic cell death. In this paper, they showed Caspase-3 activation was induced by AluRNA in the RPE of wild type mice which could be inhibited by a Caspase-8 inhibitor. Taken together, they claimed that Caspase-8 functions between Caspase-1 and Caspase-3 in Alu RNA-induced RPE cell death process. In addition, they found that Nec-1, a necroptosis inhibitor, did not protect against Alu RNA-induced RPE degeneration. Overall, they suggest that Alu RNA/IL-18 induced Caspase-8 mediated RPE apoptosis in GA through Fas and FasL signaling in a non-cell autonomous manner. Yamada et al. reported that in human RPE cells, transfected with 2.5 nM Alu RNA for 96 h showed increasing expression of $P16^{INK4a}$ and SA-β-Gal positivity (senescence markers) as well as expression of

IL-18 and IL-1β (Yamada et al., 2020). However, more studies related to Alu RNA induced RPE cell senescence still need to be done to elucidate the mechanisms. The probability of pyroptosis in Alu RNA induced RPE death could be further examined besides the pyroptosis lysis inhibitor experiment. In addition, how Alu RNA leads to inflammasome activation could be further studied.

Some other RNA species, such as double strand RNA (dsRNA) analog poly(I : C), has been shown to induce necroptosis in RPE cells. RIPK3-dependent release of HMGB1 to the vitreous and TNF-α and IL-6 production were observed. In $Rip3^{-/-}$ mice, both necrosis and inflammation were prevented. In RIPK3-deficient RPE cells, poly(I : C)-induced necrosis was inhibited which subsequently suppressed HMGB1 release and TNF-α and IL-6 induction. Cleavage of caspase-3 was observed in mice retina 2 days after poly(I : C) injection. However, Z-VAD did not show any additional significant protective effect on RPE or PRs in WT or $Rip3^{-/-}$ mice after poly(I : C) injection. Therefore, necroptosis is more crucial in dsRNA-induced RPE degeneration (Murakami et al., 2014).

Amyloid Beta (Aβ)

Amyloid beta peptide (Aβ) is a 37 to 49 amino acid residue peptide cleaved from amyloid precursor protein (APP) (Nunan and Small, 2000). The C-terminal fragment of APP (C99) is firstly generated by β-secretase cleavage, then γ-secretase cut C99 at multiple sites to produce fragments with different lengths that are further cleaved to the final Aβ forms. 40-amino-acid (Aβ1-40) and the 42-amino-acid (Aβ1-42) are two of the most abundant Aβ forms (Takami et al., 2009; Olsson et al., 2014). Aβ1-42 has a comparatively higher propensity to form prefibrillar aggregates and has been reported to be more toxic than Aβ1-40 (Dahlgren et al., 2002). Aβ is the main component of Amyloid plagues which are most commonly found in the neocortex in the brain of Alzheimer's disease patients (Chen et al., 2017). Aβ1-42 is believed to induce oxidative stress during Alzheimer's disease pathogenesis (Butterfield et al., 2013). Elevated Aβ levels have also been found in aged retina, and Aβ is also believed to play an role in the progression of AMD (Ohno-Matsui, 2011). Ye et al. (2018) detected apoptosis in RPE cells treated with 60 μM Aβ25–35 (a highly toxic Aβ peptides), shown as a significant increase in PI-negative and Annexin V-positive cells. The level of activated Caspase-3 protein, measured by western blotting, was markedly increased in cells treated with 60 μM Aβ25–35 for 36 h. Liu et al. (2014) demonstrated that Aβ1−40 stimulates chronic inflammation via NF-κB activation and plays an role in AMD pathology. They also found that Aβ1−40 induces inflammasome activation which in turn upregulates IL-6, TNF-α, IL-1β, IL-18, Caspase-1, and NLRP3 in RPE, choroid and neuroretina (Liu et al., 2013). Masuda et al. found Aβ increases the level of pigment epithelium-derived factor (PEDF) at a low concentration and thus inhibits the apoptosis of RPE cells. At a high concentration, Aβ induces Caspase-9 cascade in RPE cells and in turn leads to cell death. It also enhances VEGF-A transcription in RPE cells which may lead to the occurrence of CNV (Masuda et al., 2019). Gao et al. (2018) found that Aβ induces inflammasome activation and activates both pyroptosis and apoptosis RPE cells. Caspase-1 immunoreactivity was enhanced by 77% in the RPE layer of Aβ-injected rat eyes. IL-18 was elevated in the vitreous, showed a more than six folds higher immunoreactivity and a 58% increased band intensity in protein lysates in the RPE layer of Aβ-injected eyes, compared to the control eyes. RPE area was significantly increased in Aβ injected eyes, presumably due to swelling of the RPE cells. The cleaved N-terminal fragment of GSDMD (N-GSDMD) was increased while the uncleaved full-length GSDMD was decreased in the RPE/choroid tissue of Aβ-injected eyes. A higher immunoreactivity level of active Caspase-3 was shown in PR inner segments and RPE of the Aβ-injected eyes. X-chromosome-linked inhibitor of apoptosis (XIAP), a classic anti-apoptosis factor, was downregulated at both mRNA and protein levels in Aβ-injected eyes. Liu et al. (2015) showed increased $p16^{INK4a}$ expression in Aβ1−42 subretinal injected mice RPE on day 7 post-injection which indicated the involvement of senescence. Based on these studies, apoptosis, pyroptosis and senescence could be all related to Aβ induced RPE degeneration. More experiments are needed to see the upstream and downstream of the Aβ induced metabolic changes and could give a hint about which cell death pathway is prominent. In a clinical trial, intravenous amyloid β inhibition with GSK933776 did not slow the rate of GA enlargement compared with placebo, and no meaningful differences relative to placebo were observed in visual function testing over 18 months (Rosenfeld et al., 2018). The potential of Aβ as therapeutic target for AMD should be further clarified.

Sodium Iodate ($NaIO_3$)

Sodium iodate injection-induced retinal degeneration displays features similar to AMD and has been used as a RPE dystrophy and GA model (Tang et al., 2013). $NaIO_3$ induces ROS production and RPE damage and cell death. The affected RPE cells could cause secondary effects on PRs and the choriocapillaris (Noell, 1953; Korte et al., 1984). Additionally, $NaIO_3$ could lead to the inhibition of enzyme activity in RPE cells and the disruption of the blood-retina barrier (Ashburn et al., 1980; Baich and Ziegler, 1992; Konda et al., 1994). In Mao et al. (2018) study, Annexin-V positive ARPE-19 cells increased from 1.7 to 8.8% during 48 h treatment with 1 mg/ml $NaIO_3$. Increased expression and activity of Caspase-3 and -7 (shown by Western blotting) was also induced in $NaIO_3$ treated ARPE-19 cells. They also showed upregulated expression of NLRP3, Caspase-1 and IL-1β, key molecules in pyroptosis, in $NaIO_3$ treated ARPE-19 cells. However, co-culture with mesenchymal stem cells could suppress these effects. Moriguchi et al. (2018) using TUNEL staining showed that $NaIO_3$ initially damage the RPE cells and then the neighboring PRs, which is consistent with the results of the Chen's study (Chen et al., 2014). However, TUNEL assay identifies DNA break, not just apoptotic DNA ladder formation. Our lab has reported that 10 mM $NaIO_3$ treatment could induce necrosome formation in ARPE-19 cells by transfecting the cells with a RIPK3-GFP-expressing plasmid. Under normal condition, RIPK3 was evenly distributed in the cytoplasm. With 2 h of 10 mM $NaIO_3$ treatment, RIPK3 formed punctuates in the periphery region of cells which indicates necrosome formation. We also observed the release of HMGB1 to the cytoplasm and fragmented and clustered mitochondrial network within 4 h of

$NaIO_3$ treatment. Nec-1, Nec-5 and GSK'872 inhibited ARPE-19 cell death induced by $NaIO_3$, while Nec-7 had no protective effect. Moreover, the pan-caspase inhibitor Z-VAD could not protect ARPE-19 cells from $NaIO_3$ treatment. We didn't see formation of inflammasomes in $NaIO_3$-treated RPE cells. In addition, Caspase-1 inhibitor Ac-YVAD could not rescue RPE cells from $NaIO_3$ treatment. Retro-orbital injection of $NaIO_3$ at low dose (20 mg/kg) was also performed to mouse retina to test RPE cell death *in vivo*. RPE appeared swollen 72 h after the injection. Retro-orbital PI injection followed by retinal flat mount was then used to detect RPE necrosis in this model. PI-positive RPE cells began to appear at 24 h after $NaIO_3$ injection. TUNEL-positive RPE cells started to show up at 24 h, increased at 48 h, then decreased at 72 h. Active Caspase-3 was detected only in PR layer but not in RPE cells by staining. RIPK3 aggregation was observed at 24 and 48 h after retro-orbital injection with $NaIO_3$ in a transgenic mouse line expressing human RIPK3 in RPE cells. Also, Nec-1 inhibits RPE cell death *in vivo*. Based on our results, we propose that $NaIO_3$ could induce RIPK1- and RIPK3-dependent necroptosis in RPE cells both *in vitro* and *in vivo* (Hanus et al., 2016). Ma et al. (2020) found that inhibition of thyroid hormone signaling protects RPE from $NaIO_3$ induced necroptosis *in vivo*. Although NaIO3-induced RPE cell death cannot be completely interpreted as the mechanism of GA, the mechanism of NaIO3-induced RPE cell death still need to be clarified which could provide clues to understand how RPE could die and how RPE-damage mediates PRs and choriocapillaris damage in the context of GA.

CONCLUSION MARKS AND FUTURE DIRECTIONS

Retinal pigment epithelial cells are critical for metabolism and homeostasis of retina. However, they are vulnerable to oxidative stress and other relevant stresses due to high metabolism, high exposure to light, oxidized POS and PUFAs. Together with aging, this could lead to RPE dysfunction, degeneration and AMD pathogenesis. Although apoptosis was initially suggested as the major mechanism of RPE cell death, RPE senescence and different modes of cell death has been recently studied in RPE cells under different AMD-relevant stress conditions. Different modes of cell death have been reported in RPE cells in response to H_2O_2, 4-HNE, $NaIO_3$, A2E, Alu RNA, and Aβ, depending on the experimental conditions. Although apoptosis has been reported in RPE cells by all the stressors listed above, necrosis was also reported from recent studies, making the problem murky. For example, H_2O_2, $NaIO_3$, and dsRNA could induce necroptosis, 4-HNE could induce ferroptosis, while Alu RNA, A2E, and Amyloid-β could induce pyroptosis. Moreover, all these stressors could induce RPE senescence. The actual fate of RPE cells in response to stresses likely depends on the type, dosage and duration of the stressors. In order to develop targeted therapy for AMD, it is important to identify the types of RPE cell death in response to stresses, since the implications from different cell deaths are different. For example, necrosis is inflammatory, but apoptosis is not. Further experiments are needed to ascertain the RPE cell fate and mechanism of RPE senescence and death *in vivo* and in AMD patients. Rounding, dissociation and sloughing of RPE cells have been observed in human atrophic AMD samples (Sarks et al., 1988; Curcio et al., 2017). The nature of RPE cell degeneration and death in AMD needs further study, although TUNEL positive "apoptotic" RPE cells were observed at the edge of atrophic areas in AMD (Dunaief et al., 2002). TUNEL assay has been used to identify apoptosis but it only detects DNA break that could happen in other types of cell death. A battery of molecular markers is needed to unequivocally determine the mechanism of RPE cell senescence and death in AMD. Also, oxidative stress has an interrelated relationship with inflammation. A bunch of inflammatory factors and cytokines could be activated in the context of oxidative stress which then leads to severer oxidants produce and subsequent cell death or senescence (Chatterjee, 2016). Thus, in future studies, it is also worthful to clarify the relationship between different stresses/cell death pathways and inflammation. Moreover, some of the *in vitro* and *in vivo* studies could be revisited with the more complete toolsets to clarify different modes of cell death. Regarding therapeutic development, caution should be taken whether alternative cell death pathway could be triggered if one type of cell death is inhibited. Cell-type specific mechanism of cell death should also be considered when performing mechanistic and therapeutic studies. For example, post-mitotic cells, like RPE cells that have limited regeneration potential, could be more resistant to apoptosis *in vivo* (Annis et al., 2016).

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Both authors contributed in writing the review.

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Cone Photoreceptor Degeneration and Neuroinflammation in the Zebrafish Bardet-Biedl Syndrome 2 (*bbs2*) Mutant Does Not Lead to Retinal Regeneration

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Bardet-Biedl syndrome (BBS) is a heterogeneous and pleiotropic autosomal recessive disorder characterized by obesity, retinal degeneration, polydactyly, renal dysfunction, and mental retardation. BBS results from defects in primary and sensory cilia. Mutations in 21 genes have been linked to BBS and proteins encoded by 8 of these genes form a multiprotein complex termed the BBSome. Mutations in *BBS2*, a component of the BBSome, result in BBS as well as non-syndromic retinal degeneration in humans and rod degeneration in mice, but the role of BBS2 in cone photoreceptor survival is not clear. We used zebrafish *bbs2*$^{-/-}$ mutants to better understand how loss of *bbs2* leads to photoreceptor degeneration. Zebrafish *bbs2*$^{-/-}$ mutants exhibited impaired visual function as larvae and adult zebrafish underwent progressive cone photoreceptor degeneration. Cone degeneration was accompanied by increased numbers of activated microglia, indicating an inflammatory response. Zebrafish exhibit a robust ability to regenerate lost photoreceptors following retinal damage, yet cone degeneration and inflammation was insufficient to trigger robust Müller cell proliferation. In contrast, high intensity light damage stimulated Müller cell proliferation and photoreceptor regeneration in both wild-type and *bbs2*$^{-/-}$ mutants, although the *bbs2*$^{-/-}$ mutants could only restore cones to pre-damaged densities. In summary, these findings suggest that cone degeneration leads to an inflammatory response in the retina and that BBS2 is necessary for cone survival. The zebrafish *bbs2* mutant also represents an ideal model to identify mechanisms that will enhance retinal regeneration in degenerating diseases.

Keywords: cilia, BBSome, regeneration, zebrafish, Bbs2, Müller cell

INTRODUCTION

Bardet-Biedl Syndrome (BBS) is a pleiotropic, autosomal recessive disorder that is genetically and clinically heterogeneous (Beales et al., 1999; Weihbrecht et al., 2017). BBS is a ciliopathy and the primary features of BBS include obesity, retinal degeneration, cognitive impairment, postaxial polydactyly, renal abnormalities, and hypogonadism.

Mutations in more than 20 different genes cause BBS (Weihbrecht et al., 2017). A subset of the proteins encoded by *BBS* genes (Bbs1, Bbs2, Bbs4, Bbs5, Bbs7, Bbs8, Bbs9, and Bbs18) assemble into an octomeric protein complex called the BBSome (Nachury et al., 2007; Nachury, 2018). The BBSome facilitates cargo movement through cilia through multiple mechanisms. By interacting with the Intraflagellar Transport (IFT) complex, the BBSome serves as an adaptor to expand the pool of possible cargos (Liu and Lechtreck, 2018) and to facilitate exit of G-protein coupled receptors (GPCRs) from the ciliary membrane (Datta et al., 2015; Ye et al., 2018).

Retinal dystrophy is associated with greater than 95% of all patients and BBS proteins play a critical role in photoreceptor morphogenesis and survival (Hsu et al., 2017; Weihbrecht et al., 2017). Humans and mice lacking Bbs function exhibit retinal degeneration and photoreceptor loss (Fulton et al., 1993; Abd-El-Barr et al., 2007; Davis et al., 2007; Zhang et al., 2011, 2013; Dilan et al., 2018). In photoreceptors, protein trafficking is essential for the elaboration and growth of the outer segment (OS). The outer segment is a modified sensory cilium and utilizes the same mechanisms of protein trafficking as primary cilia (Insinna and Besharse, 2008). The outer segment of photoreceptors contains hundreds of tightly stacked disk membranes containing the proteins required for phototransduction. These disks are shed from the OS tips after approximately 10 days as new disks are formed at the base of the outer segment (Young, 1967). Proper disk formation and outer segment growth requires ciliary trafficking and BBSome activity (Hsu et al., 2017; Dilan et al., 2018). The primary GPCR in rod photoreceptors is rhodopsin and the cone opsins are GPCRs for the cone photoreceptors. Unlike in primary cilia, however, a role for the BBSome in GPCR trafficking in photoreceptors is questionable (Dilan et al., 2018). Rhodopsin and cone opsins do not migrate between the OS and the plasma membrane and there is little evidence that disruption of the BBSome results in a significant block in rhodopsin trafficking (Seo and Datta, 2017). While the majority of studies have focused on the effects of BBSome disruption to rod photoreceptors, less is known about the role of the BBSome in cones. Furthermore, evidence from mouse has suggested that photoreceptor function and morphology can be rescued if BBSome function is restored within an early window (Hsu et al., 2017). A prior characterization of *Bbs2*-null mice documented a slow retinal degeneration and other defects that resemble BBS in humans (Nishimura et al., 2004). While rhodopsin mislocalization was reported, the effects on cones was not investigated. It is important, therefore, to consider how photoreceptors, particularly cones, are impacted by loss of Bbs2 and how cones may be rescued or restored through regeneration.

We now report on a zebrafish model of *bbs2*. Zebrafish are cone-dominant, freshwater fish that have the ability to regenerate lost photoreceptors following acute injury (Goldman, 2014), but few studies have examined regeneration in the context of a progressive disease model in zebrafish. We report that zebrafish lacking *bbs2* exhibit early signs of visual dysfunction and undergo a slow, progressive degeneration of cones. Degeneration is accompanied by an inflammatory response from the microglia but not reactive gliosis by Müller cells. Importantly, acute injury stimulates Müller glia proliferation and modest regeneration of cones.

MATERIALS AND METHODS

Animal Maintenance

Adult zebrafish were maintained and housed in 1.5, 3.0, and 10 L tanks in an Aquatic Habitats recirculating system (Pentair; Apopka, FL, United States) as previously described (Lessieur et al., 2019).

Ethics Statement

All animal procedures were done with approval by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic. Experiments were conducted according to relevant guidelines and regulations.

CRISPR/Cas9 Gene Editing

Potential CRISPR target sites were identified in exon 4 of the zebrafish *bbs2* gene using ZiFiT[1] (Sander et al., 2010) and gRNA synthesis was performed according to the protocol by Talbot and Amacher (Talbot and Amacher, 2014). Briefly, oligonucleotides for gRNA synthesis (5′ TAGGAGGAAACTGTGCTCTTCA 3′ and 5′ AAACTGAAGAGCACAGTTTCCT 3′) were annealed and ligated into plasmid pDR274 (Addgene, Watertown, MA, a gift from Keith Joung). Purified plasmid DNA was digested with *Dra*I (New England BioLabs, Beverly, MA) and used for *in vitro* transcription reaction to generate gRNA. Zebrafish embryos were injected at the 1-cell stage with a 1 nL solution of *bbs2* gRNA (200 ng/μL) and Cas9 protein (10 μM; New England BioLabs, Beverly, MA). Mutagenesis was confirmed by High Resolution Melt Analysis (HRMA) in injected F_0 animals at 3 days post fertilization (dpf). Remaining F_0 injected animals were raised to adulthood and outcrossed to wild-type fish and HRMA was performed on DNA from F_1 progeny to screen for mutations. F_0 founders producing a high degree of mutant F_1 progeny were kept and individual *bbs2* alleles were identified by sequencing.

Optokinetic response (OKR) Behavior Assay

OKR assays were performed on larval zebrafish as previously described (Lessieur et al., 2019). Briefly, 5–6 dpf larvae were immobilized in 35-mm petri dishes containing 4% methylcellulose and oriented using a dissecting needle. Dishes were placed inside the VisioTracker (VisioTracker 302060 Series, TSE Systems, GmbH Bad Homburg, Germany) and assayed for both contrast sensitivity and spatial frequency response functions (Rinner et al., 2005). All tests were conducted between 12 and 6 p.m. to avoid circadian variation (Emran et al., 2010). The genotype of each animal was verified by PCR following the OKR tests.

[1] http://zifit.partners.org/ZiFiT/

Histology

Light-adapted larvae were prepared for histology as previously described (Lessieur et al., 2019). Briefly, larvae were anesthetized and bisected through the swim bladder. The heads were prepared for electron microscopy and the tails were used for genotyping. Fixation, plastic embedding, and electron microscopy were done as previously described (Lessieur et al., 2019).

Immunohistochemistry

Zebrafish larvae were euthanized on ice and subsequently fixed in 4% paraformaldehyde, equilibrated in PBS + 30% sucrose for 2 h. After washing with PBS, larvae were embedded in Tissue Freezing Medium (Electron Microscopy Sciences, Hatfield, PA). Adult zebrafish were deeply anesthetized with tricaine methanesulfonate (0.4 mg/mL) and decapitated with a razor blade. Eyes were enucleated and fixed for 2 h in 4% paraformaldehyde. Eyes were washed in PBS and equilibrated in 5% sucrose/PBS for 3 h at room temperature and then in 30% sucrose overnight at 4°C. Eyes were washed in a 1:1 solution of 30% sucrose:Tissue Freezing Medium overnight at 4°C and subsequently embedded for cryosectioning.

Transverse cryosections sections (10 μm) were cut on Leica CM1950 cryostat and mounted on Superfrost Plus slides and dried at room temperature overnight. Slides were washed 3 × 10 min in PBS and then incubated in blocking solution (PBS + 2% BSA, 5% goat serum, 0.1% Tween-20, 0.1% DMSO) for 1 h. The following primary antibodies were used: mouse monoclonal zpr1 (1:100, Zebrafish International Resource Center (ZIRC), Eugene, OR, United States), mouse monoclonal zpr3 (1:100, ZIRC), mouse monoclonal 4C4 (1:1,000, a gift from Dr. Peter Hitchcock, University of Michigan), rabbit polyclonal L-plastin (1:1,000, GeneTex, Irvine, CA, United States, GTX124420), mouse monoclonal PCNA (1:100, Sigma, St. Louis, MO, United States, clone PC-10), rabbit polyclonal syntaxin3 (1:100; Synaptic Systems, Atlanta, GA, United States), Peanut agglutinin (PNA)-lectin conjugated to Alexa-568 (1:100, Thermo Fisher Scientific, Waltham, MA, United States). EdU labeling was detected with the Click-iTEdu Alexa Fluor-555 Imaging Kit (Thermo Fisher Scientific). Alexa-conjugated secondary antibodies were used at 1:500 in blocking buffer and incubated for at least 1 h. Slides were counterstained with 4, 5-diamidino-2-phenylendole (DAPI) to stain nuclei. Sections were imaged on a Zeiss Imager Z.2 equipped with an ApoTome using Zen2 software and post-processed in ImageJ. All analysis was performed only on sections that included or were immediately adjacent to the optic nerve.

Light Damage

Light damage experiments were performed using a protocol adapted from Thomas and Thummel (Thomas and Thummel, 2013). Adult zebrafish were first dark adapted for 36–42 h. Up to 5 animals were placed in a 250 mL glass beaker with system water that was seated inside a 1L glass beaker with Milli-Q water and exposed to high-intensity light from a 120W X-CITE series 120Q metal halide lamp (Excelitas) for 30 min and then exposed to 14,000 lux light from a illumination cage for 4 h. Animals were allowed to recover in system water for 3 or 30 days. To label proliferating cells during early stages of regeneration, animals were injected intraperitoneally with 20 μL of a 20 mM EdU solution (PBS) at 2 days post injury and eyes were enucleated 24 h later (3 days post injury). To assess regeneration, animals were allowed to recover for 30 days post injury prior to enucleation. Retinas were processed for immunohistochemistry as described above.

Statistics and Data Analysis

All data was analyzed and graphed using GraphPad Prism (v8). OKR data was analyzed by two-way ANOVA with Sidak corrections for multiple comparisons. Between 9 and 13 animals were tested by OKR. Cone outer segment density was quantified by counting PNA + outer segments and measuring the curvilinear distance of retina in ImageJ. Outer segment counts were analyzed in Prism8 by using one-way Brown-Forsythe and Welch ANOVA tests with Dunnett T3 corrections for multiple comparisons. Sample sizes are provided for each experiment.

RESULTS

Generation of a Zebrafish *bbs2* Mutant

To assess the role of *BBS2* in photoreceptor function, we utilized the CRISPR/Cas9 gene editing system to target exon 4 of the zebrafish *bbs2* gene. One mutant line (*lri82*) was recovered that resulted in the insertion of a 15-base pair fragment combined with a 4-bp deletion, thereby yielding an 11-bp insertion and a frameshift with a premature stop codon (**Figure 1A**). The truncated protein is predicted to contain the first 30% of the WD40 domain and then terminate after an additional frameshifted 58 amino acids (**Figure 1B**). Founder (F_0) animals were outcrossed to wild-type animals to generate *bbs2* heterozygote ($bbs2^{+/-}$) zebrafish. Genotyping of clutches from $bbs2^{+/-}$ intercrosses revealed that homozygous offspring ($bbs2^{-/-}$) appeared normal and were present in Mendelian ratios at 5 days post-fertilization (dpf) (**Figure 1C**). $bbs2^{-/-}$ mutants survived to at least 1 year of age, but developed spinal curvatures and were smaller than $bbs2^{+/-}$ and wild-type siblings (**Figure 1D**). These phenotypes resemble those of other zebrafish ciliopathy mutants (Lessieur et al., 2019).

Zebrafish *bbs2* Mutants Have Impaired Visual Function at 5 dpf

Visual function was assessed in $bbs2^{-/-}$ mutant larvae and control siblings (+/+ and +/−) at 5–6 dpf using the optokinetic response (OKR). Larvae were presented with moving stimuli that vary by either contrast or spatial frequency (Rinner et al., 2005). The OKR gain of control (n = 13) and $bbs2^{-/-}$ mutants (n = 9) was assessed as previously described (Emran et al., 2010; Daniele et al., 2016; Lessieur et al., 2017). As previously noted (Rinner et al., 2005), the OKR gain is a linear function of the log of contrast in control larvae (**Figure 2A**; black circles). Relative to control animals, the OKR gain was reduced in $bbs2^{-/-}$ mutants presented with varying contrast stimuli (**Figure 2A**; 2-way ANOVA, $p < 0.0001$). When presented with stimuli that

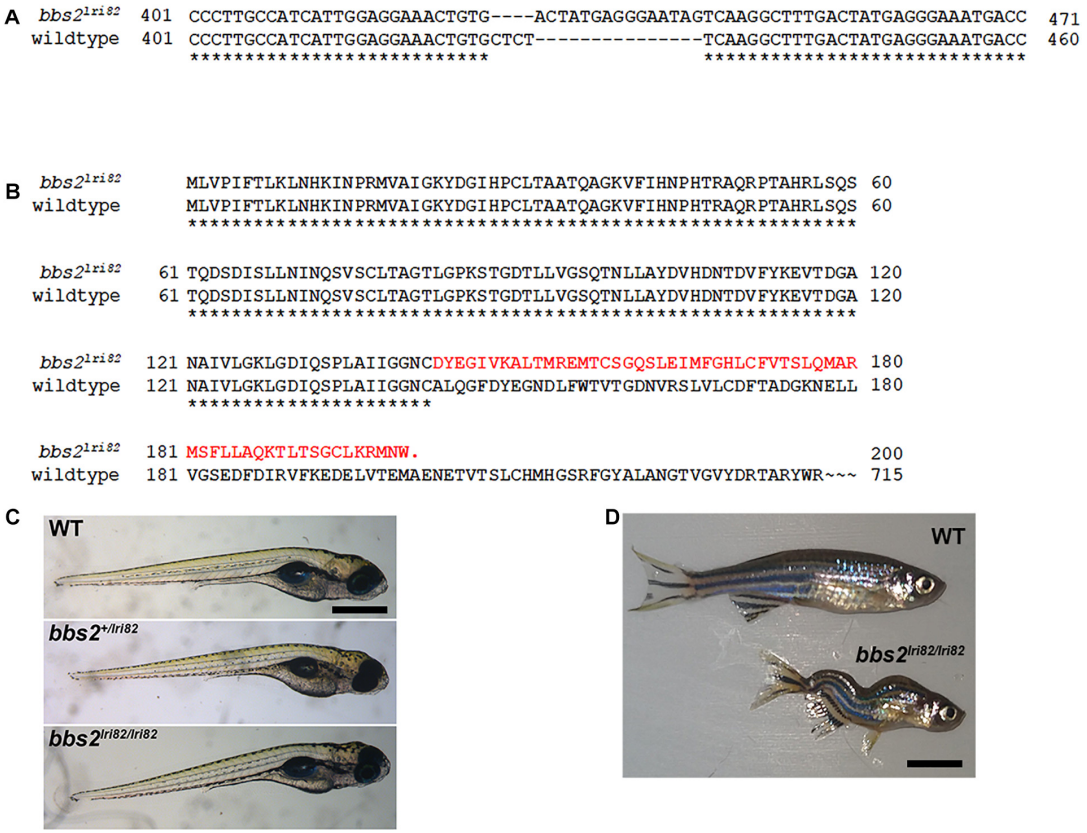

FIGURE 1 | Mutation in zebrafish *bbs2* exon 4 causes a frame shift and premature truncation. **(A)** Coding sequence alignment of the wild-type and *bbs2*^{lri82} alleles. A 15 bp fragment replaces "CTCT" from the wild-type allele, yielding a net 11-bp insertion and a frame shift. Numbering is relative to the start codon. **(B)** Amino acid sequences the of the wild-type and *bbs2*^{lri82} peptides. The *bbs2*^{lri82} mutation results in a frame shift that terminates after 58 codons (red). **(C)** Lateral view of 5 dpf wild-type (top), *bbs2*$^{+/lri82}$ heterozygotes (middle) and *bbs2*$^{lri82/lri82}$ homozygotes (bottom) that were verified by genotyping. No differences could be observed. **(D)** Lateral view of a 10 month old wild-type (top) and *bbs2*$^{-/-}$ homozygous mutant (bottom). Mutants are smaller and exhibit spinal curvature as adults. Scale bar: **(C)** 400 μm; **(D)** 7 mm.

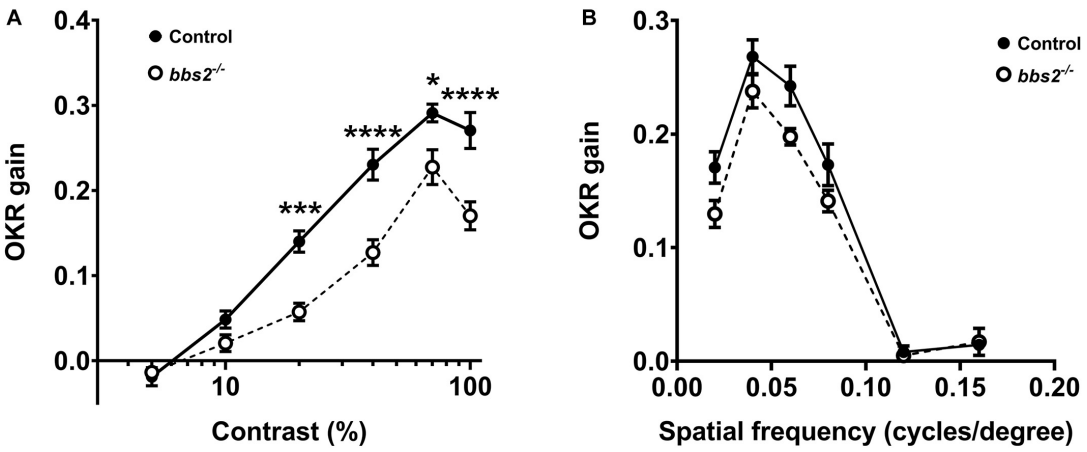

FIGURE 2 | *bbs2* $^{-/-}$ mutant zebrafish have visual function deficits at 5 dpf. **(A)** Contrast response function and **(B)** spatial frequency function from 5 dpf control (closed circles, $n = 13$) and *bbs2*$^{-/-}$ mutants (open circles, $n = 9$) plotted as the OKR gain vs. the log contrast. OKR data are from 13 control and 9 mutant larvae. Error bars indicate SEM. Significant differences at individual points were discerned from Sidak's multiple comparisons tests and indicated with asterisks. Significance levels are as follows: *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

varied by spatial frequency, the *bbs2*$^{-/-}$ mutants again showed an overall deficit in visual function (**Figure 2B**; 2-way ANOVA, $p < 0.005$). These results demonstrate that *bbs2*$^{-/-}$ larvae exhibit deficits in both general visual function and reduced ability to discriminate spatial differences.

bbs2$^{-/-}$ Mutants Have Shorter and Disorganized Photoreceptor Outer Segments

To determine if the reduced visual function was associated with abnormal retinal anatomy, light microscopy on semi-thin plastic sections and transmission electron microscopy (TEM) was used to assess retinal histology. At 5 dpf, the *bbs2*$^{-/-}$ mutants had similar eye size and normal retinal lamination when compared to a wild-type sibling control (**Figures 3A,B**). Within the photoreceptor layer the outer nuclear layer (ONL) was similar to controls. Within the zebrafish cone mosaic, cones are tiered with red/green double cones residing apically compared to blue-sensitive and UV-sensitive cones (Ramsey and Perkins, 2013). In both semi-thin sections and by transmission electron microscopy (TEM) the cone outer segments (COS) were noticeably shorter in the *bbs2*$^{-/-}$ mutants (**Figures 3C–F**, white brackets). Both wild-type siblings and *bbs2*$^{-/-}$ mutants exhibited normal basal body positioning, cilia architecture, and tightly stacked disc membranes within the outer segments (**Figures 3G,H**). Thus, loss of Bbs2 resulted in shorter photoreceptor outer segments in larval zebrafish.

Photoreceptors Degenerate in Adult *bbs2*$^{-/-}$ Mutants

We next asked whether the photoreceptor disorganization observed in 5 dpf *bbs2*$^{-/-}$ would progress to photoreceptor loss. We previously found obvious rod dysfunction and between 40 and 50% cone loss by 6–7 mpf in *cep290*$^{-/-}$ mutants (Lessieur et al., 2019). We therefore examined adult *bbs2*$^{-/-}$ mutants at 7 mpf by immunohistochemistry for rod and cone markers and for evidence of Müller glia proliferation. In *bbs2*$^{-/-}$ mutants, rhodopsin was mislocalized to the rod inner segments. Although rhodopsin was mislocalized, the size of the rod outer segments did not appear to differ between wild-type and mutant animals (**Figures 4A,B**). This phenotype resembled that of the zebrafish *cep290*$^{-/-}$ mutant, which also showed signs of degeneration but no net loss of rods (Lessieur et al., 2019). Acute retinal injury and cell death triggers the reprogramming and proliferation of Müller glia within the inner nuclear layer (INL) (Goldman, 2014; Wan and Goldman, 2016). In contrast, limited death of rod photoreceptors stimulates proliferation of unipotent rod precursors that reside in the ONL and subsequently differentiate into rod photoreceptors (Stenkamp, 2011). Retinal sections were stained with antibodies against proliferating cell nuclear antigen (PCNA) to identify proliferating cells. In wild-type retinas, only 1–2 cells that stained positive for PCNA were found in the ONL. These likely represent rod precursors, which can also differentiate into new rods as the eye slowly grows in size (Stenkamp, 2011). In contrast, *bbs2*$^{-/-}$ zebrafish had numerous PCNA + cells in the ONL and a modest increase in PCNA + cells in the

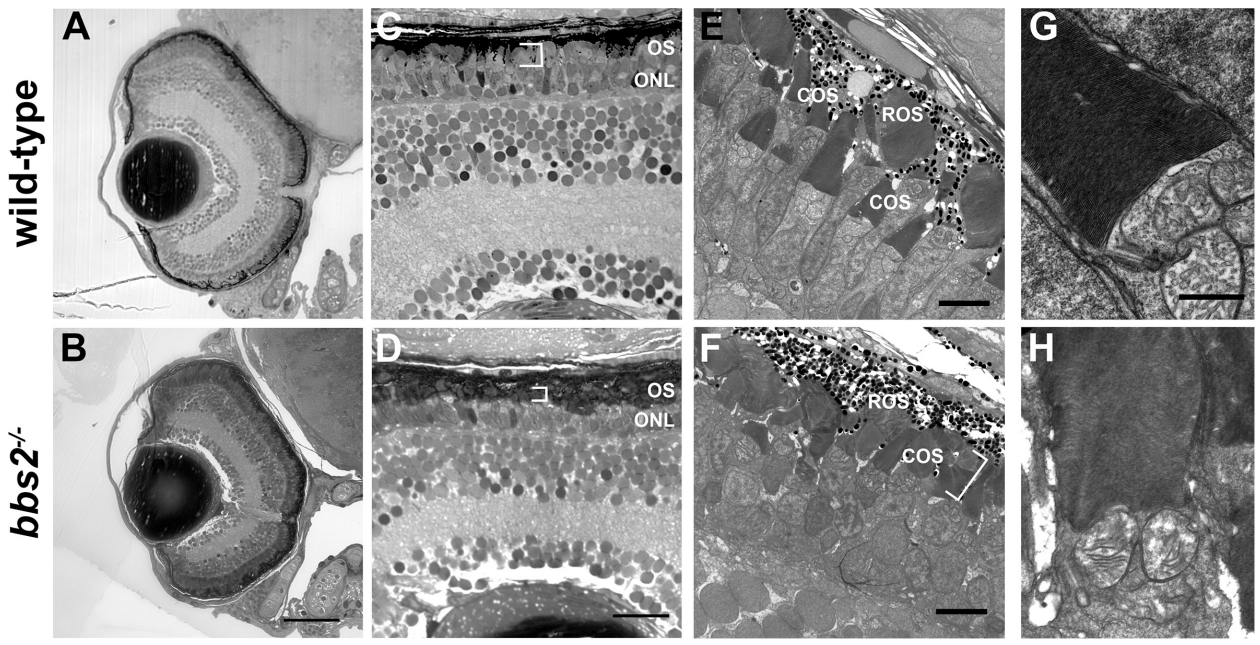

FIGURE 3 | *bbs2* $^{-/-}$ mutant zebrafish have shortened and disorganized photoreceptor outer segments at 5 dpf. Representative images of wild-type and *bbs2*$^{-/-}$ mutants at 5 dpf. **(A,B)** Semi-thin histological sections show that *bbs2*$^{-/-}$ mutants have normal retinal lamination. **(C,D)** Higher magnification images of the dorsal retina show that *bbs2*$^{-/-}$ mutants have shorter photoreceptor outer segments (OS; white brackets) but gross anatomical structure remains normal. **(E–H)** Transmission electron microscopy shows shorter and disorganized cone outer segments (COS) in *bbs2*$^{-/-}$ mutants (white bracket). Cilia architecture appears normal. Scale bar: 50 μm **(A,B)**; 10 μm **(C,D)**; 5 μm **(E,F)**; 1 μm **(G,H)**. COS, outer segments; ROS, rod outer segments.

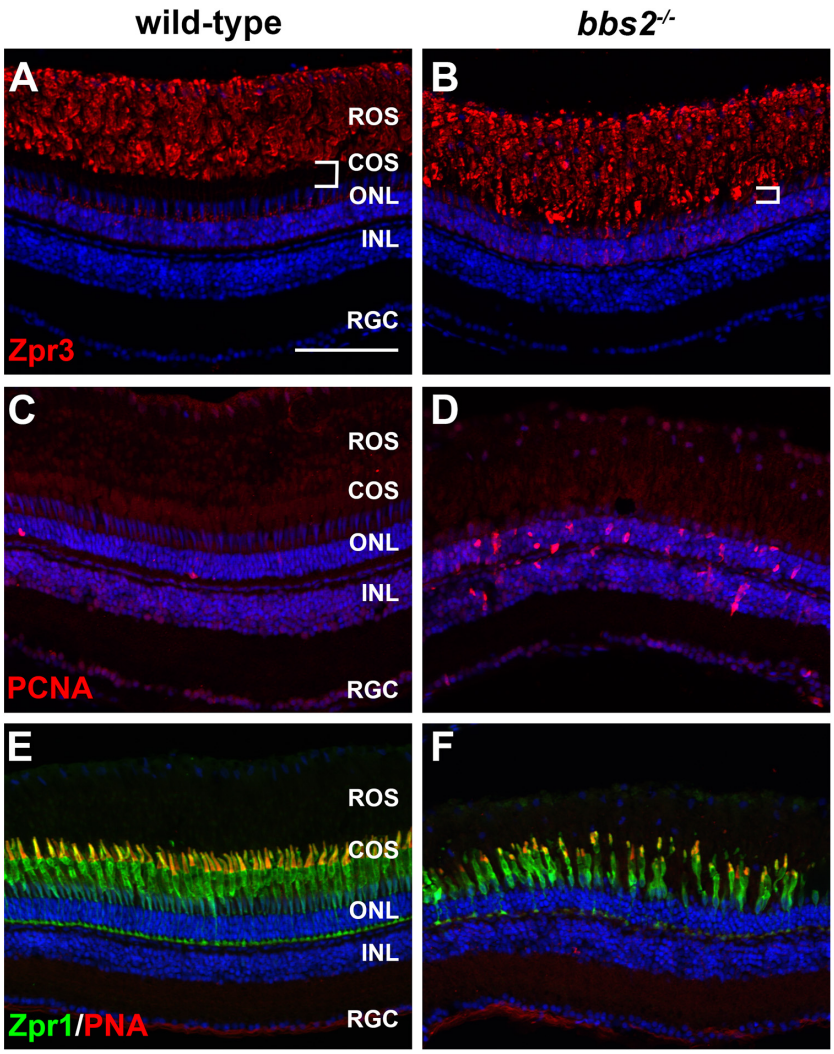

FIGURE 4 | Photoreceptor degeneration in zebrafish retina lacking Bbs2. **(A,B)** Transverse cryosections of 7 mpf wild-type sibling and *bbs2*$^{-/-}$ mutant retina stained with Zpr3 (red) to label rhodopsin. The region containing cone outer segments (white brackets) is smaller in *bbs2*$^{-/-}$ mutants. **(C,D)** Cryosections of wild-type and *bbs2*$^{-/-}$ mutants stained with PCNA (red) to label proliferating cells. **(E,F)** Cryosections of wild-type and *bbs2*$^{-/-}$ mutants stained with PNA (red) and Zpr1 (green) to label cone outer segments and cone inner segments, respectively. Sections were counterstained with DAPI (blue). Scale bar: 100 μm. RGC, retinal ganglion cells; INL, inner nuclear layer; ONL, outer nuclear layer; COS, cone outer segment layer; ROS, rod outer segment layer.

INL (**Figures 4C,D**). Increased proliferation of unipotent rod precursors in the ONL is consistent with regeneration of dying rod photoreceptors (Morris et al., 2005; Montgomery et al., 2010). Retina cryosections were also co-stained with the zpr-1 antibody, which recognizes arrestin 3a on the red-green double cones (Ile et al., 2010), and with peanut agglutinin (PNA), which labels the extracellular matrix surrounding cone outer segments (Blanks and Johnson, 1984). Compared to wild-type retinas, the *bbs2*$^{-/-}$ mutants had fewer cones and the outer segments appeared shorter and more disorganized, suggesting ongoing cone degeneration (**Figures 4E,F**). The loss of cones also correlated to the reduced distance between the base of the rod outer segments and the ONL (**Figures 4A,B**, white brackets). Taken together, the data indicate that both rod and cone photoreceptors degenerate; however, rods likely regenerate from the proliferating unipotent rod precursors in the ONL while cones continue to die. The proliferating cells in the INL likely represent those Müller glia that produce rod precursors as a distinct lineage from the multipotent neurogenic progenitors that regenerate cones following acute damage (Stenkamp, 2011).

Accumulation of Syntaxin-3 in Photoreceptor Outer Segments in Zebrafish Lacking Bbs2

Initially discovered in the *Lztfl1/Bbs17* mouse mutant, a number of studies of *Bbs* mutant mice have showed that the SNARE protein syntaxin-3 (Stx3) accumulates in the photoreceptor outer segments (Datta et al., 2015; Hsu et al., 2017; Dilan et al., 2018) Like many other SNARE proteins, Stx3 normally localizes

throughout the inner segments and the synaptic terminals (Kwok et al., 2008). The BBSome is believed to function as a coat adaptor complex between Intraflagellar Transport (IFT) molecules and ciliary cargo, with current evidence suggesting the BBSome ensures exit of ciliary cargo (Liu and Lechtreck, 2018; Nachury, 2018). Thus, the loss of BBSome components prevents proper binding between retrograde IFT trains and ciliary cargo, such as Stx3. The subsequent accumulation of Stx3 in the outer segment may be responsible for photoreceptor degeneration observed in *Bbs* mice (Datta et al., 2015). We chose to examine Stx3 localization in *bbs2*$^{-/-}$ mutants at 12 months of age, a time when cone photoreceptor degeneration is significant but rod photoreceptor outer segments remain. The specificity of the polyclonal Stx3 antibodies was validated by *stx3a* morpholino knockdown (**Supplementary Figure 1**). In wild-type photoreceptors, Stx3 staining was primarily observed in the plexiform layers (**Figure 5A** and **Supplementary Figure 1**). In *bbs2*$^{-/-}$ mutants, Stx3 accumulated in the rod outer segments and signal from the inner segments and outer plexiform layer was reduced (**Figure 5B**). Similarly, in wild-type photoreceptors Stx3 did not exhibit strong localization with peanut agglutinin (PNA), a marker for cone outer segments (**Figure 5C**). Stx3 accumulated in the area of rod outer segments and did not appear enriched in cone outer segments of *bbs2*$^{-/-}$ mutants (**Figure 5D**). These results are consistent with prior studies in mouse and suggest that BBSome function is conserved between zebrafish and mammals.

Photoreceptor Degeneration Triggers an Inflammatory Response in *bbs2*$^{-/-}$ Mutants

Acute retinal damage triggers a significant inflammatory response characterized by activated immune cells and reactive Müller glia (White et al., 2017; Mitchell et al., 2018, 2019). To determine if ongoing retinal degeneration in *bbs2*$^{-/-}$ mutants was associated with elevated inflammation, retinas from 7 mpf animals were stained with the monoclonal antibody 4C4, which is specific for zebrafish microglia (Tsarouchas et al., 2018; Mazzolini et al., 2020), and L-plastin, a pan-leukocyte marker (Herbomel et al., 1999; Le Guyader et al., 2008). In wild-type adult zebrafish, the 4C4 + microglia maintain a ramified morphology and reside on the basal and apical surfaces of the INL, within the retinal ganglion cells (RGC), and in the outer plexiform layer (**Figure 6A**). Microglia are also seen in the RPE/choroid. The majority of 4C4 + microglia in the neural retina also stained with L-plastin. Significantly more 4C4 + microglia were observed in the RPE/choroid and the region containing rod and cone outer segments (i.e., outer retina) of 7 mpf *bbs2*$^{-/-}$ mutants (**Figures 6B,C**). Activated microglia appeared amoeboid in shape and largely localized to the outer retina. L-plastin + macrophages also accumulated in the vitreous, suggesting these were infiltrating macrophages responding to degeneration. No morphological changes and no significant numerical increase was observed in the microglia population in the plexiform layers or the INL or GCL (**Figure 6C**). These data indicate that zebrafish exhibit a robust immune response in response to chronic degeneration. Surprisingly, activated microglia accumulate near outer segments and not in the ONL as is seen in mouse models of retinitis pigmentosa (Zhao et al., 2015).

Müller Cells of *bbs2*$^{-/-}$ Mutants Become Proliferative in Response to Acute Injury

In zebrafish, photoreceptor death caused by light damage, mechanical injury, or cytotoxic lesion triggers a robust regeneration response that restores lost photoreceptors (Hyde and Reh, 2014; Wan and Goldman, 2016). As *bbs2*$^{-/-}$ mutants undergo progressive degeneration, we wondered whether *bbs2*$^{-/-}$ mutants had lost capability to regenerate cones. To address this question, high intensity light was used to cause

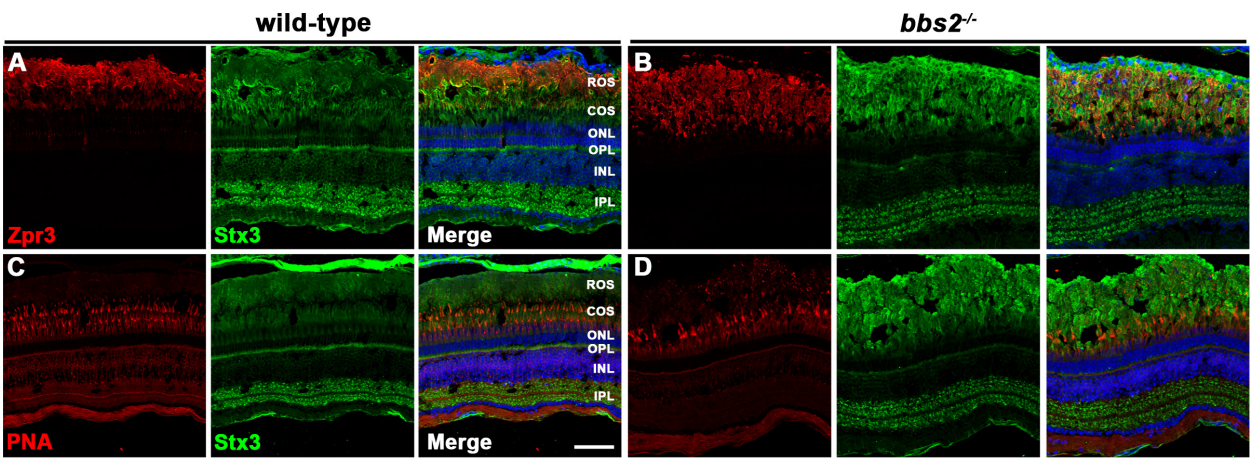

FIGURE 5 | Immunolocalization of syntaxin-3 and cone markers in zebrafish retina lacking Bbs2. **(A,B)** Transverse cryosections of 12 mpf wild-type sibling and *bbs2*$^{-/-}$ mutant retina stained with Zpr3 (red) and syntaxin-3 (Stx3; green). **(C,D)** Cryosections of wild-type and *bbs2*$^{-/-}$ mutants stained with PNA (red) to label cone outer segments or syntaxin-3 (Stx3; green). Sections were counterstained with DAPI (blue). Scale bar: 50 μm. IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; COS, cone outer segment layer; ROS, rod outer segment layer.

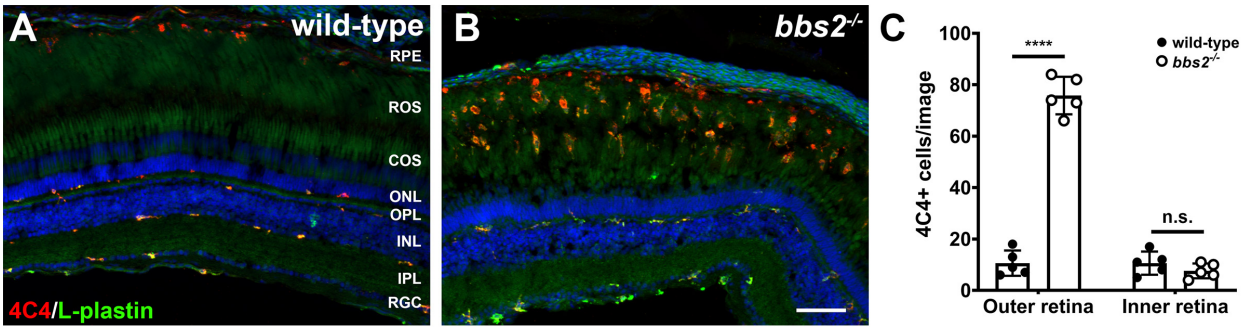

FIGURE 6 | Immunolocalization of immune cells in zebrafish retina lacking Bbs2. **(A,B)** Transverse cryosections of 7 mpf wild-type sibling and *bbs2*$^{-/-}$ mutant retina stained with 4C4 (red) and L-plastin (green). Sections were counterstained with DAPI (blue). **(C)** Quantification of 4C4 + microglia per image (1,388 × 1,040 pixels). Each data point represents quantification from individual fish ($n = 5$). ****$p < 0.0001$. Scale bar: 50 μm. RGC, retinal ganglion cells; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; COS, cone outer segment layer; ROS, rod outer segment layer; RPE, retinal pigment epithelium.

acute photoreceptor damage (Vihtelic and Hyde, 2000; Thomas and Thummel, 2013). The first steps in regeneration are the de-differentiation and reprogramming of Müller glia and the subsequent proliferation of Müller glia derived progenitor cells. Müller cell proliferation was assessed by EdU incorporation at 3 days post lesion (see section "Materials and Methods"). In undamaged wild-type animals at 5 mpf, little to no proliferation in the ONL or INL was observed (**Figure 7A**), while some EdU + cells were observed in the ONL and INL of undamaged *bbs2*$^{-/-}$ mutants (**Figure 7B**). At 3 days post lesion (dpl), cone photoreceptors were lost and neurogenic clusters of EdU + cells were observed in the INL of both wild-type and *bbs2*$^{-/-}$ mutants (**Figures 7C,D**). From the position of these proliferating clusters in the INL, it was inferred that these are Müller cell derived retinal progenitors. From these data, we concluded that, like wild-type animals, *bbs2*$^{-/-}$ mutants respond to acute injury by stimulating Müller cell proliferation and neurogenesis.

Photoreceptors Regenerate to Pre-lesion Densities in *bbs2*$^{-/-}$ Mutants

To determine if neurogenesis in light damaged *bbs2*$^{-/-}$ mutants could result in regeneration of cone photoreceptors, animals were allowed to recover for 1 month after light damage. We first tested regeneration in older animals (e.g., 16 mpf) that had lost the majority of cones and where even modest regeneration could be more readily observed. Cone density was quantified in 16 mpf control (wild-type + heterozygous siblings) and *bbs2*$^{-/-}$ mutants after 1 month of recovery (**Figure 8**). In undamaged animals, the number of photoreceptors was significantly reduced in the *bbs2*$^{-/-}$ mutants due to ongoing degeneration (**Figures 8A,B**). Previous groups have demonstrated that exposure to high intensity light results in photoreceptor death and subsequent regeneration primarily in the dorsal retina (Vihtelic et al., 2006; Thomas et al., 2012). Consistent with these observations, we noted that photoreceptor loss 3 days after light damage was limited to the dorsal retina in both control and *bbs2*$^{-/-}$ mutants (data not shown). Significant EdU incorporation in the INL and ONL was observed in the dorsal retina at 1 month of recovery (**Figures 8C,E**). Areas of the same retinas that did not undergo light damage (e.g., "surround") had few EdU + cells (**Figures 8D,F**), suggesting limited regeneration. These regions served as internal controls for photoreceptor density and evidence of Müller cell proliferation. To determine if regeneration restored cone density in the lesioned areas of the dorsal retina, the cone outer segment density was quantified in the area containing EdU + cells (e.g., "lesion") and then compared to the density of cone outer segments in the surround. Following 1 month of recovery, the cones in the lesioned areas of control retinas had regenerated to pre-lesioned levels (**Figures 8C,D,K**). Cones in the *bbs2*$^{-/-}$ retinas also regenerated to pre-lesion densities. However, there were significantly fewer cones both before and after regeneration in *bbs2*$^{-/-}$ mutants as compared to control retinas that received light damage (**Figures 8E,F,K**). Regeneration was also tested in younger adult animals (e.g., 5 mpf) and similar results were observed. EdU + cells were seen throughout the light-damaged region of both control and *bbs2*$^{-/-}$ retinas (**Figures 8G,I**) but not in the undamaged surrounding retina (**Figures 8H,J**). While the initial density of cones was higher in 5 mpf animals, regeneration restored cones only to pre-lesion levels in the *bbs2*$^{-/-}$ mutants (**Figure 8L**). These data indicate that *bbs2*$^{-/-}$ mutants could regenerate damaged cone photoreceptors following acute injury. This ability appears limited, however, to restoring only those cones present at the time damage was incurred, regardless of age. Thus, this partial regeneration was insufficient to restore the *bbs2*$^{-/-}$ retina to wild-type cone densities.

DISCUSSION

The evidence reported here contribute to the broader understanding of BBS pathogenesis across multiple species. As in humans (Shevach et al., 2015) and mice (Nishimura et al., 2004), zebrafish with mutations in *bbs2* undergo a progressive photoreceptor degeneration. Whereas mice lacking other BBSome components, such as Bbs8, show significant degeneration of photoreceptors within a few

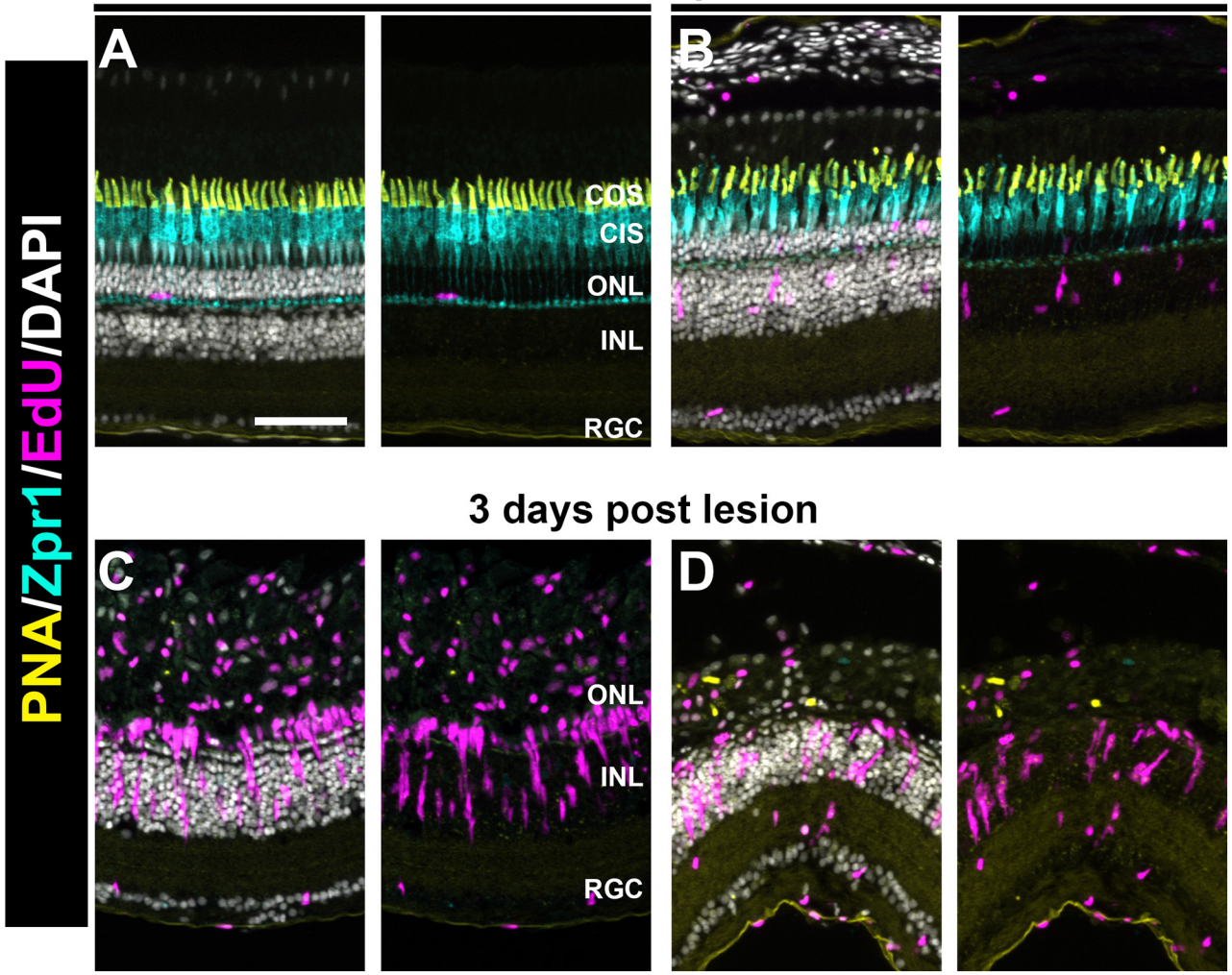

FIGURE 7 | Light damage triggers proliferation of Müller glia in wild-type and *bbs2*$^{-/-}$ mutants. Transverse cryosections of undamaged retinas from control **(A)** and *bbs2*$^{-/-}$ mutant **(B)** adult (5 mpf). Cryosections were stained with PNA to label cone outer segments (yellow), Zpr1 to stain cone inner segments (cyan), EdU to identify cells that underwent proliferation (magenta) and DAPI (grays). Right panels omit DAPI channel to permit better visualization of EdU labeling. Cone photoreceptors are disorganized and a small number of proliferating cells are seen in the ONL and INL of *bbs2*$^{-/-}$ mutants. **(C,D)** Images of transverse cryosections of light damaged control animals **(C)** and *bbs2*$^{-/-}$ mutants **(D)** at 3 days post light lesion. Light damage has largely eliminated photoreceptors and neurogenic clusters of EdU + cells are seen in both groups. Scale bar: 50 μm. RGC, retinal ganglion cells; INL, inner nuclear layer; ONL, outer nuclear layer; CIS, Cone inner segments; COS, cone outer segment layer.

weeks (Hsu et al., 2017; Dilan et al., 2018), the loss of photoreceptors occurs over months (Nishimura et al., 2004) and humans with *BBS2* mutations may only exhibit mild visual impairment. Our findings that zebrafish *bbs2*$^{-/-}$ mutants exhibit progressive degeneration over months is consistent with the rate of degeneration seen in mice and humans. Thus, the affected gene and the nature of the mutations should be considered when evaluating the potential time course of retinal dystrophy in BBS.

Numerous studies of mouse models lacking individual components of the BBSome have established a requirement for the BBSome in photoreceptor morphogenesis and survival (Mykytyn et al., 2004; Abd-El-Barr et al., 2007; Zhang et al., 2014; Datta et al., 2015; Murphy et al., 2015; Dilan et al., 2018). The observed pathology of *bbs2*$^{-/-}$ mutants illustrates that BBSome function is conserved in zebrafish. We show that visual function is compromised in larval zebrafish, similar to the ERG defects reported in mice lacking *Bbs4* and *Bbs8* (Abd-El-Barr et al., 2007; Dilan et al., 2018). These visual function defects are associated with abnormalities in rod and cone outer segment morphogenesis in the zebrafish *bbs2*$^{-/-}$ mutants. It should be noted, however, that zebrafish vision depends exclusively on cone function during larval stages (Brockerhoff et al., 1995; Bilotta et al., 2001). Thus, the OKR defects in *bbs2*$^{-/-}$ zebrafish indicate compromised cone function. This differs from an earlier study that found normal cone function

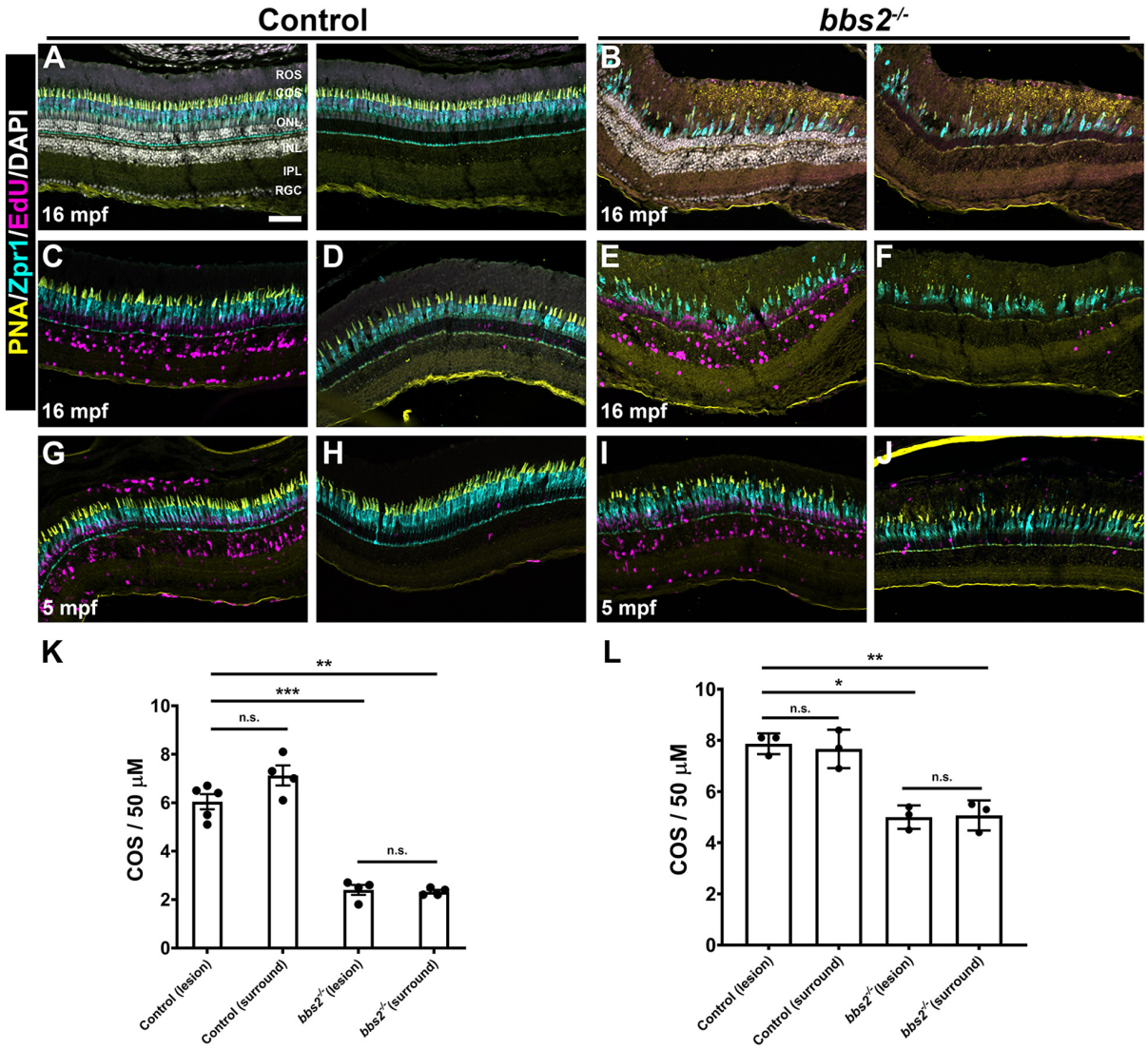

FIGURE 8 | Photoreceptors regenerate to pre-damage density in control and *bbs2*$^{-/-}$ mutants. Transverse cryosections of undamaged retinas from control **(A)** and *bbs2*$^{-/-}$ mutant **(B)** adults (16 mpf). Cryosections were stained with PNA (yellow), Zpr1 (cyan), EdU (magenta) and DAPI (grays). Right panels omit DAPI channel to permit better visualization of EdU labeling. **(C–J)** Images of transverse cryosections of light damaged 16 mpf control animals **(C,D)** and *bbs2*$^{-/-}$ mutants **(E,F)** or 5 mpf control animals **(G,H)** and *bbs2*$^{-/-}$ mutants after recovery. Left panels show the regions of the dorsal retina where damage and proliferation occurred (e.g., lesion). Right panels show more distal regions of the dorsal retina that were undamaged (e.g., surround). **(K)** Quantification of cone outer segment (COS) density in lesion and surround areas for 16 mpf control (n = 5) and *bbs2*$^{-/-}$ mutants (n = 4). **(L)** Quantification of cone outer segment (COS) density in lesion and surround areas for 5 mpf control (n = 3) and *bbs2*$^{-/-}$ mutants (n = 3). Each data point represents quantification from an individual animal. *$p < 0.01$; **$p < 0.002$. ***$p < 0.0001$. Scale bar: 50 µm. RGC, retinal ganglion cells; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; COS, cone outer segment layer; ROS, rod outer segment layer.

at P30 in mice with a cone-specific knockout of *Bbs8* (Dilan et al., 2018). The zebrafish may, therefore, offer a unique opportunity to investigate the role of Bbs proteins in cone photoreceptor function.

In both mouse and zebrafish, acute retinal injury triggers resident microglia to rapidly migrate and accumulate near the site of injury or disease and release pro-inflammatory cytokines. These microglia release numerous growth factors and inflammatory cytokines that stimulate Müller glia to regenerate lost neurons (Goldman, 2014; Gorsuch and Hyde, 2014; Mitchell et al., 2018, 2019). The cone degeneration observed in zebrafish *bbs2*$^{-/-}$ mutants triggers a significant inflammatory response but this is not sufficient to initiate a regenerative response from the Müller glia.

Following light induced retinal damage, we found photoreceptor loss in the dorsal retina of *bbs2*$^{-/-}$ mutants, followed by significant Müller glia proliferation in the INL. Taken together, these results suggested that Müller glia do not require Bbs2 function for reprogramming and proliferation and that *bbs2*$^{-/-}$ mutants retained the capacity for regeneration

following light-induced photoreceptor damage. Although retinal regeneration is not possible in humans, retinal regeneration in zebrafish is to be viewed as a means to develop strategies to treat progressive retinal diseases in humans (Wan and Goldman, 2016). For regeneration to be a viable therapeutic option in advanced cases of retinal disease, restoring photoreceptor density should be a goal. After light damage, the density of regenerated cones within the lesion of *bbs2*$^{-/-}$ mutants was similar to that in the neighboring undamaged, but diseased retina. Furthermore, the density of regenerated *bbs2*$^{-/-}$ photoreceptors was significantly lower than the density of regenerated wild-type photoreceptors, regardless of the age of the *bbs2*$^{-/-}$ mutants. These results suggest that the environment in the degenerated *bbs2*$^{-/-}$ retina may not be permissive to a full regenerative response.

These studies contribute to the body of work documenting the essential role of BBS proteins to both rod and cone photoreceptor function across vertebrates. Without normal BBSome function, cone function and photoreceptor outer segment morphogenesis is compromised. The degeneration phenotypes observed in *bbs2*$^{-/-}$ zebrafish is similar to that found in zebrafish *cep290*$^{-/-}$ and the *eys*$^{-/-}$ mutants (Yu et al., 2016; Lessieur et al., 2019). In all three cases, photoreceptors undergo progressive degeneration without significant Müller glia proliferation or regeneration of cones. Our work also illustrates that additional work is needed to more fully understand the relationship between retinal degeneration, inflammation, and the signals required to stimulate Müller glia regeneration.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Cleveland Clinic Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

PS, JF, and BP conceived and designed experiments. PS, JF, LC, RS, and BP performed experiments and analyzed the data. BP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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New Technologies to Study Functional Genomics of Age-Related Macular Degeneration

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Age-related macular degeneration (AMD) is the most common cause of irreversible vision loss in people over 50 years old in developed countries. Currently, we still lack a comprehensive understanding of the genetic factors contributing to AMD, which is critical to identify effective therapeutic targets to improve treatment outcomes for AMD patients. Here we discuss the latest technologies that can facilitate the identification and functional study of putative genes in AMD pathology. We review improved genomic methods to identify novel AMD genes, advances in single cell transcriptomics to profile gene expression in specific retinal cell types, and summarize recent development of *in vitro* models for studying AMD using induced pluripotent stem cells, organoids and biomaterials, as well as new molecular technologies using CRISPR/Cas that could facilitate functional studies of AMD-associated genes.

Keywords: age-related macular degeneration, retina, CRISPR/Cas, induced pluripotent stem cell models, single cell transcriptomics, organoids

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of central vision loss among people over 50 years old in developed countries (Klein et al., 1992; Mitchell et al., 1995). Due to the increase of the aging population, the prevalence of AMD is estimated to affect 196 million globally in 2020, reaching 288 million by 2040 (Wong et al., 2014). Early and intermediate AMD are characterized by minimal visual symptoms, pigmentary changes in the macula and formation of drusen beneath the retina (Hernández-Zimbrón et al., 2018). Advanced AMD is classified into two types: atrophic and neovascular. In atrophic AMD, gradual vision loss occurs due to retinal pigment epithelium (RPE) degeneration and photoreceptor death. Neovascular AMD often causes sudden vision loss due to the formation of abnormal, new blood vessels, usually developing in the choroid and invading through Bruch's membrane into the neuroretina. When this occurs there is usually fluid leakage, hemorrhage, and if left untreated, irreversible scarring will occur which affects the RPE and photoreceptors at the macula, ultimately leading to severe loss of central vision (Velez-Montoya et al., 2014). Research on AMD pathogenesis has been largely focused on the contribution of RPE and Bruch's membrane, but lately there is also renewed interest in the role of the choroid. There is increasing evidence suggesting that pathological changes in the choroid may play an early role in the pathogenesis of AMD (Farazdaghi and Ebrahimi, 2019). For example, loss of choriocapillaris endothelial cells is one of the earliest detectable events in this disease, which can drive progression to more advanced stages due to subsequent loss of metabolic support to the outer retina

(Chirco et al., 2017). Altogether, photoreceptor cells, RPE, Bruch's membrane and choroid depend upon each other to maintain normal vision. Future research should explore all these domains to gain a better insight into factors at play throughout the course of the disease.

Although many AMD associated genes have been identified from genome-wide association study (GWAS), currently there is a lack of understanding of the biological significance of many of these genes and their functions in AMD pathophysiology. This remains a major roadblock to finding specific treatments for AMD, especially in terms of a preventative therapy or cure. In neovascular AMD, past treatment strategies with laser and photodynamic therapy, as well as current therapies using anti-VEGF treatments are not treating the underlying cause, just the consequences of the disease (Colquitt et al., 2008; Brown et al., 2009; Guymer et al., 2014; Khanna et al., 2019). Thus, even when appropriate treatments are delivered, with time atrophy and fibrosis will often affect the outcomes and lead to longer term vision loss (Jaffe et al., 2019). Furthermore, there is no approved treatment available for atrophic AMD (Hernández-Zimbrón et al., 2018). This is partly because we are only beginning to uncover the complex interaction between genetics, environmental factors and the aging process that contribute to AMD. A deeper insight into how these different components play a role in pathogenesis will help us develop more effective treatments and preventative strategies for AMD. Critically, two key questions need to be addressed in order to translate the knowledge on AMD genetics and identify new therapeutic targets: 1) What are the expression profiles of AMD genes in the outer retina and choroid? and 2) What are the functions of these AMD-associated genes? Understanding in more detail the contribution of genes that underlie AMD pathophysiology could help us discover novel biomarkers to detect early AMD in those at risk of progression to vision-threatening late AMD, as well as identifying new therapeutic targets for AMD treatment. Here we summarize recent research on the genetic contributions to AMD, with a focus on improved genomic methods to identify novel AMD genes, as well as novel technologies including single-cell RNA sequencing (scRNA-seq), CRISPR, and *in vitro* stem cell models that will help us better understand how AMD-associated genes contribute to disease mechanisms (**Figure 1**).

GENOME-WIDE ANALYSIS TO STUDY GENETIC CONTRIBUTIONS TO AMD

Advances in genome-wide analyses have helped tremendously in our understanding of the genetics of AMD. Several genome-wide analysis techniques have been developed to identify genetic associations with diseases, such as GWAS, which detects single nuclear polymorphisms (SNPs) associated with diseases (Chang et al., 2018). Using GWAS, studies have now identified 69 SNPs related to AMD (Fritsche et al., 2013, 2016; Han et al., 2020), including loci that confer major susceptibility such as *CFH* and *ARMS2/HTRA1* (Hageman et al., 2005; Klein et al., 2005; Rivera et al., 2005).

CFH is an important inhibitor of the alternative complement pathway (Rivera et al., 2005). Within the *CFH* gene, the Y402H variant appears to have the strongest association to AMD susceptibility (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005; Souied et al., 2005; Zareparsi et al., 2005; Magnusson et al., 2006). This polymorphism has been demonstrated to decrease CFH binding to C reactive protein, heparin, Streptococcus M protein, malondialdehyde epitopes, oxidized phospholipids, as well as heparan sulfate and dermatan sulfate glycosaminoglycans within Bruch's membrane (Skerka et al., 2007; Yu et al., 2007; Ormsby et al., 2008; Clark et al., 2010; Weismann et al., 2011; Shaw et al., 2012; Molins et al., 2016). This reduced binding can cause inappropriate complement regulation, which results in chronic inflammation, abnormal physiological homeostasis and cell damage. The link between *CFH* and AMD suggests an inflammatory role in the pathogenesis of AMD, with increased complement cascade activity promoting AMD development in genetically predisposed individuals (Hageman et al., 2005; Despriet et al., 2006).

In addition to *CFH*, the *ARMS2/HTRA1* loci are identified as a major contributor to the risk of developing AMD. The mechanisms as to how *ARMS2* plays a role in the progression of AMD are not well understood. A previous study suggested that *ARMS2* interacts with extracellular matrix proteins, including fibulins and EMILIN-2, which help assemble and stabilize extracellular matrix structures of the Bruch's membrane (Kortvely et al., 2010). *ARMS2* has also been shown to participate in the phagocytosis function of RPE, which may be a mechanism that contributes to the development of AMD (Xu et al., 2012). Another study has reported that *ARMS2* functions as a surface complement regulator – recombinant *ARMS2* binds to human apoptotic and necrotic cells and initiates complement activation to clear cellular debris (Micklisch et al., 2017). On the other hand, *HTRA1* encodes for a secreted protease that is involved in cell signaling, organization of extracellular matrix, and skeletal development (Hadfield et al., 2008; Mauney et al., 2010; Vierkotten et al., 2011; Graham et al., 2013; Supanji et al., 2013). Additionally, *HTRA1* was reported to play a role in the development of a variety of cancers (Baldi et al., 2002; Chien et al., 2004; Mullany et al., 2011), Alzheimer's disease and osteoarthritis (Grau et al., 2005, 2006; Launay et al., 2008).

While the role of *CFH* in the complement pathway has been well studied, there is still lack of consensus as to which gene or genetic variant in the *ARMS2/HTRA1* loci is functionally relevant to AMD pathology (Kanda et al., 2007; Kortvely et al., 2010; Xu et al., 2012; Micklisch et al., 2017; Tosi et al., 2017). Since *ARMS2/HTRA1* region exhibits high linkage disequilibrium, it remains controversial as to which of the two genes is causally linked to AMD pathogenesis by statistical means (Fritsche et al., 2008; Kanda et al., 2010; Yang et al., 2010; Friedrich et al., 2011). Several papers supported that *HTRA1* variants confer AMD risk (Tosi et al., 2017; Lin et al., 2018; Orozco et al., 2020), while other studies suggested *ARMS2* variants confer AMD risk (Kanda et al., 2007; Grassmann et al., 2017). Despite the strong linkage equilibrium, *ARMS2/HTRA1* region still displays some level of recombination, producing rare recombinant haplotypes (Grassmann et al., 2017). These recombinant haplotypes can

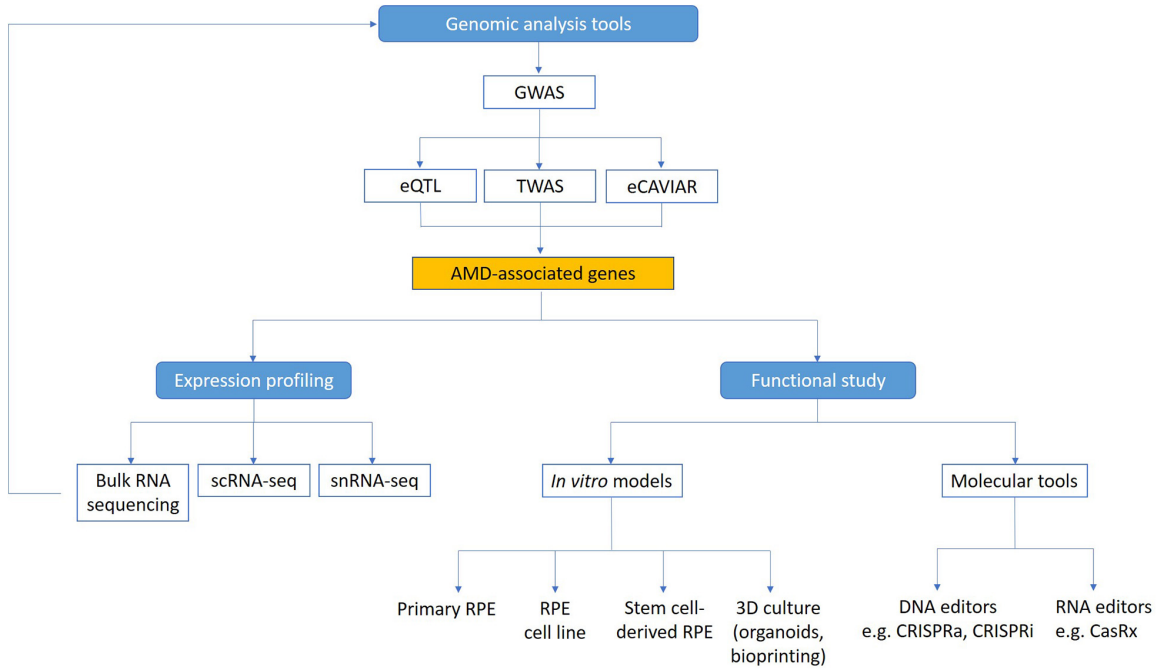

FIGURE 1 | Technologies to study functional genomics of AMD. Genome-wide analysis methods (GWAS, eQTL, TWAS, and eCAVIAR) can be used to identify candidate genes linked to AMD. Expression of these genes in the retina can be mapped using transcriptomic technologies. Functional study of AMD-associated genes can be facilitated using *in vitro* AMD models and molecular tools. scRNA-seq, single cell RNA sequencing; snRNA-seq, single nuclei RNA sequencing.

be used to dissect a disease-associated genomic region in a similar fashion to gene mapping in monogenic diseases. One study has analyzed rare recombinant haplotypes in 16,144 AMD cases and 17,832 controls from the International AMD Genomics Consortium (Fritsche et al., 2016). Using logistic regression analysis, the findings suggested that variants in or close to *ARMS2* but not *HTRA1* are responsible for disease susceptibility (Grassmann et al., 2017).

Other than identifying major AMD susceptibility genes, GWAS has also been used to study the commonalities and differences between two advanced AMD subtypes, atrophic and neovascular (Fritsche et al., 2016). While these two subtypes were found to share the majority of genetic risk, one variant was identified to be specific to one subtype – a variant near *MMP9* was exclusively associated with neovascular but not with atrophic AMD. This finding suggested that individuals that have high risk of developing neovascular AMD also have high risk of atrophic AMD. Future therapeutic strategies should therefore aim to target variants that confer risk for both neovascular and atrophic AMD.

IMPROVED METHODS TO IDENTIFY AMD-ASSOCIATED GENES BY INTEGRATION OF GENE EXPRESSION DATA

Genome-wide association study is a valuable tool to identify disease associated loci, however, there are several limitations to this method. Firstly, although GWAS findings reveal SNPs that are associated with the disease, these SNPs do not necessarily establish causal variants and genes. Linkage disequilibrium can conceal causal variants responsible for the association, such as in the case of *ARMS2/HTRA1*, making it difficult to pinpoint variants that have an effect on the trait from GWAS data alone (Wainberg et al., 2019). Secondly, as SNPs are often in non-coding regions and may be up to 2 Mbps away from the affected genes, it is challenging to pinpoint the genes they impact on the disease (Brodie et al., 2016; Gusev et al., 2016; Zhu et al., 2016; Cannon and Mohlke, 2018). Moreover, this method yields a large number of hits, which could make functional characterization challenging (Tam et al., 2019). Importantly, additional studies are required to confirm if these causal genes are driving disease association.

To address this, GWAS studies can be complemented with expression quantitative trait loci (eQTL) studies to prioritize causal variants and genes at GWAS loci. Using gene expression dataset, eQTL identifies genetic variants that influence gene expression levels. These eQTLs can act in *cis* (locally) or in *trans* (at a distance) to a gene and affect gene expression at the level of transcription or translation (Majewski and Pastinen, 2011). To date, studies on AMD genetics have mostly focused on investigating *cis*-eQTLs (Ratnapriya et al., 2019; Han et al., 2020; Orozco et al., 2020) so it would be interesting to perform *trans*-eQTL studies in the future.

Recently, several methods were described to improve the integration of GWAS and eQTL studies. For instance, transcriptome-wide association study (TWAS) could be used

to identify genes that mediate effects of genetic variants on phenotype, thereby prioritizing candidate causal genes and tissues underlying GWAS loci. This method involves the use of an expression panel such as eQTL to train a predictive model of expression from genotype. This model is then used to predict gene expression for individuals in the GWAS cohort and identify association with the trait (Wainberg et al., 2019). AMD-associated genes discovered by TWAS would provide better understanding into the influence of gene regulation on phenotypic consequences in this disease. TWAS has been used to investigate the relevance of AMD pathology in tissues other than the retina. Even though AMD pathology is observed in the posterior pole of the eye, several studies have suggested a systematic expression profile of AMD associated genes throughout the body (Morohoshi et al., 2009; Camelo, 2014; Cougnard-Grégoire et al., 2014; Paun et al., 2015; Kiel et al., 2017). A TWAS study discovered 106 genes significantly associated with AMD variants in at least one of 27 tissues investigated, which suggests the expression of AMD-associated genes is not only limited to retinal tissue but also observed in other tissues throughout the whole body (Strunz et al., 2020). Based on these results, future studies on therapeutic strategies for AMD should consider the systemic expression profile of AMD-associated genes and processes underlying AMD pathology.

Similarly, eCAVIAR is another method that uses GWAS and eQTL studies to determine colocalization of causal variants (Hormozdiari et al., 2016). eCAVIAR measures the probability that the same variant is causal in both a GWAS and eQTL study. The underlying concept is that if the same variant underlying GWAS locus also influences gene expression, then the relevant gene and tissue could contribute to disease mechanism.

Leveraging methods such as eQTL, TWAS, and eCAVIAR can help reveal new biological mechanisms of AMD GWAS risk loci. Using eQTL in human retinal samples, an elegant study by Ratnapriya et al. (2019) identified potential target genes in six novel AMD GWAS loci, and three additional genes using TWAS. In addition to eQTL and TWAS, this study also used eCAVIAR to prioritize the most plausible target genes. Lead GWAS SNPs were connected to specific target genes at known AMD-associated loci, including *B3GLCT, BLOC1S1, SH2B3, PLA2G12A, PILRB*, *TMEM199*, and *POLDIP2*. A subsequent study used co-localization of GWAS and eQTL and found 15 candidate causal genes for AMD, which included genes highly expressed in RPE, such as *TRPM1* and *TSPAN10*, and previously identified genes such as *BLOC1S1* and *TMEM199* (Orozco et al., 2020). These two studies demonstrated that the combination of different techniques allow better identification of genes associated with AMD.

POTENTIAL BIOLOGICAL ROLE OF NOVEL AMD-ASSOCIATED GENES

Notably many of the recently identified AMD-associated genes are not well studied in the retina. Understanding their biological roles within the retina would provide critical insights in AMD pathogenesis. Interestingly, several novel AMD-associated genes have a potential role in protein degradation, including autophagy and ubiquitin-mediated degradation. For instance, *POLDIP2* has been identified as a significant gene for AMD susceptibility in several publications (Ratnapriya et al., 2019; Han et al., 2020; Strunz et al., 2020). It has been implicated in other diseases, such as Alzheimer's disease, in which it was found to be a novel regulator of Tau aggregation (Kim et al., 2015). Overexpression of *POLDIP2* caused impairments in autophagy, which led to increased Tau aggregate formations. Conversely, downregulation of *POLDIP2* reduced reactive oxygen species (ROS)-induced Tau aggregation. Since there is evidence that suggests defective autophagy and oxidative stress contribute to AMD pathophysiology (Jarrett and Boulton, 2012; Mitter et al., 2012; Golestaneh et al., 2017), it would be interesting to investigate the association of *POLDIP2* to oxidative damage and autophagy and determine how it contributes to AMD pathogenesis.

Similarly, *BLOC1S1* was identified as a potential causal gene from the RDH5-CD63 AMD risk locus in two eQTL studies (Ratnapriya et al., 2019; Orozco et al., 2020). BLOC1S1 is part of the octameric protein complex BLOC-1, which is associated with the biogenesis of organelles related to the endosome–lysosome system (Falcón-Pérez et al., 2002; Langemeyer and Ungermann, 2015). The BLOC-1 complex is associated with several biological processes, such as the sorting of synaptic vesicle proteins and postsynaptic receptors, cytoskeleton modulation, membrane fusion and macroautophagy, which malfunction could result in the development of a wide array of disorders (Hartwig et al., 2018). Notably, *BLOC1S1* was also identified as a potential causal gene using human RPE samples, where phagocytic (endocytic) and autophagy pathways play a major role in retinal homeostasis (Orozco et al., 2020). It is possible that disruption of these regulatory processes could result in accumulated stress for retinal cells within the macula, ultimately causing macular degeneration. Nevertheless, functional studies in retinal cells are required to validate the association of *BLOC1S1* in AMD pathogenesis.

TMEM199 encodes an accessory component of the V-ATPase proton pump which is required for endo-lysosomal acidification. Human *TMEM199* mutations have been identified to disrupt Golgi homeostasis and cause glycosylation defects (Jansen et al., 2016). However, the role of *TMEM199* in the V-ATPase function remains unknown. In a recent study, Miles et al. reported that disruption of *TMEM199* resulted in intracellular iron depletion, thereafter impairing the activity of Iron(II) prolyl hydroxylase enzymes, which hydroxylate the HIF1α subunit and facilitate its proteasomal degradation. Prevention of HIF1α degradation leads to HIF activation, revealing an important role of *TMEM199* linking between the V-ATPase, iron metabolism and HIFs (Miles et al., 2017). As V-ATPase has been shown to engage in autophagic processes (Mijaljica et al., 2011), *TMEM199* may participate in response to chronic oxidative stress, hypoxia, and disturbed autophagy, which are crucial to the pathology of neovascular processes in AMD.

NPLOC4 is another novel AMD gene that has been implicated in ubiquitin-mediated protein degradation. NPLOC4 forms a ternary complex with UFD1 and VCP, which binds ubiquitinated proteins and exports misfolded proteins from

endoplasmic reticulum to the cytoplasm. As the NPLOC4-UFD1-VCP complex is involved in the proteasomal ubiquitin-dependent pathway, mutations of the complex have been reported to associate with multisystem proteinopathy that causes inclusion body myopathy, Paget's disease of bone, and frontotemporal dementia (Blythe et al., 2019). In the retina, the ubiquitin-proteasome system and autophagy are essential for the degradation and recycling of cellular waste such as all-*trans* retinal in RPE cells (Blasiak et al., 2019). As such, disruption of *NPLOC4* may cause impaired protein degradation and contribute to the pathologic development of AMD. In summary, we have discussed several novel AMD-associated genes that could contribute to AMD pathogenesis through deregulation of protein degradation pathways. Biological roles of these genes in AMD as well as other novel genes can be explored using the technologies discussed in this review.

NEW TRANSCRIPTOMIC TECHNOLOGIES TO MAP EXPRESSION OF AMD-ASSOCIATED GENES IN THE RETINA

Recent advances in transcriptomic technologies have greatly facilitated our understanding of gene expression profiles of the human retina, providing a key step to better identify AMD-associated genes from GWAS and facilitate functional studies of these AMD-association genes. Previous studies have used bulk RNAseq to profile the human retina (Farkas et al., 2013; Whitmore et al., 2014; Pinelli et al., 2016; Hoshino et al., 2017). However, results of bulk RNA-seq represent an average signal of gene expression profile in all retinal cell types, as such gene expression in less abundant cell types would be obscured. Recent development of scRNA-seq technology overcomes this issue by resolving cell heterogeneity and profiling gene expression at a single cell level, providing a unique opportunity to reveal gene expression in specific retinal cell types. Using scRNA-seq, single cells from a complex tissue could be separated using microfluidic systems and tagged with a unique barcode to generate cDNA libraries for individual cells (Kulkarni et al., 2019). scRNA-seq technology can overcome the heterogeneity issue in complex tissue and provide a powerful method to analyze the transcriptome landscape of the retina.

Our team and others have recently utilized scRNA-seq to establish the transcriptome of major retinal cell types in the human retina (Lukowski et al., 2019; Menon et al., 2019; Voigt et al., 2019). Critically, these dataset could be used to study expression profiles of AMD-associated genes in the retina and characterize the effect of AMD on gene expression changes across different cell types and tissues. For instance, scRNA-seq has been used to analyze gene expression of 34 AMD risk loci identified by GWAS (Menon et al., 2019). Menon et al. (2019) found that within the retina, the majority of genes surrounding the 34 risk loci were expressed in Müller glia and astrocytes, including leading AMD genes (nearest gene to lead GWAS variant) such as *CFI*, *VEGFA*, *TIMP3*, and *COL4A3*. They also identified the cell types within the retina that are most predictive of AMD risk are cone photoreceptors, glial, and vascular cells. These results suggest that in addition to photoreceptors and RPE, which are the major cell types affected by AMD, glia and vascular cells are also potentially important in AMD pathogenesis.

On the other hand, single-nucleus RNA-seq (snRNA-seq) provides an alternative method for gene expression profiling in complex tissues from frozen samples at single cell levels (Grindberg et al., 2013). Compared to scRNAseq, snRNA-seq analyze gene expression within the nuclei instead of intact cells. It should be noted that there could be potential differences between the RNA type and expression levels between nucleus and cytosol. As observed in a previous study comparing nuclear and whole cell transcriptome in mouse neurons at single cell levels, a subset of genes associated with mitochondrial respiration was almost exclusively detected in the whole cell transcriptome (Lake et al., 2017). In addition, since nascent transcripts are naturally abundant in nuclei, there is a difference in maturity levels of transcripts detected between the nucleus and cytosol. Shorter genes were better detected in whole cells while longer genes showed better detection in the nuclei, and as a result, an additional normalization step was required to reduce technical bias. In regards to AMD, recent snRNA-seq studies have established the transcriptome of the macula and peripheral regions of the human retina (Liang et al., 2019; Orozco et al., 2020). In particular, Orozco et al. identified substantial gene expression differences between the macula and peripheral retina, including several AMD-associated genes such as *CFI*, *HTRA1*, *B3GLCT*, *TSPAN10*, and *PILRA* (Orozco et al., 2020). In addition, the authors also showed that the majority of AMD-associated genes are present in non-neuronal cells: 38% of the genes are expressed in RPE cells, 29% in Müller glia, and 27% in astrocytes. These studies highlighted the potential of using snRNA-seq to identify the retinal cells that contribute to AMD pathogenesis.

In summary, single cell transcriptomics provided useful tools in understanding the genetic signals within the retina and identifying the potential cell types within the retina that contribute to AMD pathogenesis. Future studies using single cell transcriptomics to analyze the retinal gene expression profiles in AMD patients could allow us to map the molecular deregulation in specific retinal cells that lead to AMD.

NEW *IN VITRO* MODELS TO FACILITATE FUNCTIONAL STUDY OF AMD-ASSOCIATED GENES

Recent development of *in vitro* cell systems provides better models to study AMD. This includes the use of primary RPE and choroidal endothelial cells, immortalized cell lines, and cells derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) (Malek et al., 2020). These *in vitro* models are useful for evaluating various cellular responses associated with AMD development, such as morphology, metabolism, cell proliferation, oxidative stress, and cell viability.

Primary RPE cells can be obtained from human donor eyes to study AMD, including human fetal RPE cells and adult RPE

cells (Malek et al., 2020). However, there are several limitations associated with using primary cells. Firstly, access to donor human eyes can be limited and establishing primary culture is time-consuming and labor-intensive. Secondly, there could be potential variability between different donors. Finally, primary cells have limited proliferative capacity and will eventually senescence in culture. To address this, several human RPE cell lines have been developed to facilitate *in vitro* studies, which include spontaneously transformed cell lines H80HrPE-6, ARPE-19, D407 RPE, RPE-340, as well as immortalized cell lines hTERT RPE-1, h1RPE-7, and h1RPE-116 (Kuznetsova et al., 2014). In particular, ARPE-19 is the most commonly used human RPE cell line to date, which has morphological and functional characteristics of native RPE, including the abilities to phagocytose the photoreceptor outer segments (POS) (Finnemann et al., 1997) and secret endogenous growth factors (Ablonczy et al., 2011). However, ARPE-19 do not exhibit several properties of the native RPE, such as the absence of pigmentation and reduced expression levels of some RPE markers (e.g., *RPE65* and *CRALBP*) and low transepithelial resistance (Ablonczy et al., 2011). Treatment with nicotinamide could improve some RPE characteristics of ARPE-19, such as expression of RPE65 and better polarization (Hazim et al., 2019). Despite these limitations, ARPE-19 cell line represents a valuable *in vitro* model to study functions of AMD genes in RPE since it is commercially available and easy to culture.

Besides primary cells and cell lines, pluripotent stem cells provide an attractive *in vitro* model to study AMD. Somatic cells from patients can be reprogrammed into iPSC (McCaughey et al., 2016), which can be subsequently differentiated into the clinically relevant cell types for AMD, such as RPE and photoreceptors. iPSC provided a feasible strategy to derive patient-specific models to study ocular diseases, as we described previously (Khan et al., 2016; Hung et al., 2017; Wong et al., 2017). In particular, stem cell-derived RPE cells demonstrate similar characteristics to those of native RPE, such as polygonal morphology, pigmentation, phagocytosis of POS and ability to metabolize vitamin A (Malek et al., 2020). iPSC-derived RPE are also highly similar to human fetal RPE in terms of gene expression and enriched transcription factor motif profiles (Lidgerwood et al., 2016; Galloway et al., 2017; Smith et al., 2019). Also, an elegant study by Hallam et al. (2017) reported an iPSC model from AMD patients homozygous for low- and high-risk CFH (Y402H) polymorphism. Interestingly, iPSC-derived RPE cells derived from patients with high-risk genotypes resembled many key AMD features such as elevated inflammation and cellular stress, impaired autophagy, deposition of drusen-like deposits, and lipid droplets accumulation. Collectively, these studies support the feasibility of using patient-specific iPSC-derived RPE as an *in vitro* model to better study macular degeneration.

There are limitations to the use of monolayer RPE cultures to study AMD, as they cannot recapitulate the complex retina structure with supporting tissues around the RPE, such as photoreceptors, Bruch's membrane and choroid. To address this, several three-dimensional (3D) culture systems are developed, providing better models to investigate cellular interaction within the microenvironment of the retina, such as retinal organoids derived from pluripotent stem cells. Early reports demonstrated that ESCs or iPSCs can self-organize and form retinal organoids, which are 3D structures that recapitulate *in vivo* development of the retina (Eiraku et al., 2011; Nakano et al., 2012). Subsequently, several groups have modified the retinal organoid differentiation process by promoting cell specification using exogenous transcription factors or modulators/growth factors of signaling pathways (Zhou et al., 2015; Hasegawa et al., 2016; Völkner et al., 2016). For example, inhibition of Notch signaling could promote retinal differentiation and generate retinal organoids that are enriched with photoreceptors (Völkner et al., 2016). Photoreceptors with mature features can also be derived using retinal organoids, including the connecting cilia, inner and outer segments (Gonzalez-Cordero et al., 2017). However, a key limitation of the organoid differentiation method is that it is time-consuming, with up to 6–7 months needed for the derivation of light sensitive photoreceptors (Zhong et al., 2014). Further research to understand the differentiation signaling could improve the kinetic of the retinal organoid method. Altogether, patient-specific organoid system provides a unique 3D model which can be used to study the influences of genetic signaling on cell–cell interaction between retinal cells.

The use of synthetic and natural polymeric scaffolds provided an alternative method to model the Bruch's membrane in a 3D culture system (Trese et al., 2012). Previous studies showed that the Bruch's membrane can be mimicked using porous scaffold constructed with polycaprolactone (McHugh et al., 2014; Xiang et al., 2014). This biomaterial creates a highly porous membrane which has been demonstrated to promote growth of RPE cells, as well as cell–cell interaction and migration. In particular, "artificial Bruch's membrane" can be incorporated with human RPE cells and primate choroidal cells to construct a 3D co-culture model to better model the complexity of the retina (Shokoohmand et al., 2017). Similarly, cell sheet engineering can be applied to construct artificial substitutes of the choroid, by harvesting sheets of cultured cells along with their extracellular matrix, and integrating them to create tissue-like structures (Mokhtarinia et al., 2018). For instance, Djigo et al. (2019) have tissue-engineered a choroidal stromal model by stacking sheets of choroidal stroma fibroblasts and their extracellular matrix together. In addition, this choroidal stromal model can be integrated with RPE cells, choroidal melanocytes, and human umbilical vein endothelial cells to better recapitulate the environment of the native choroid. This model could help provide insight into RPE-choroid interactions and pathophysiological mechanisms affecting the choroid. Several hypotheses have been proposed regarding the relationship between RPE and choroid in AMD. One hypothesis for atrophic AMD is that RPE loss preceded the death of choriocapillaris (McLeod et al., 2009). RPE has been shown to produce VEGF, which is a vasodilator, endothelial cell survival factor, and angiogenic factor (Adamis et al., 1993). When VEGF is no longer in areas of RPE atrophy, choriocapillaris constricts or degenerates and eventually atrophies. However, choriocapillaris loss has also been observed

in the absence of RPE atrophy in early AMD (Seddon A. W. R. et al., 2016), suggesting other factors are also involved. In contrast, the loss of choroidal vasculature may be the initial insult that causes RPE atrophy in neovascular AMD. Choriocapillaris degeneration results in hypoxia in RPE due to a reduced blood supply. It has been suggested that hypoxic RPE produces VEGF, which stimulates the formation of new vessels from choriocapillaris and results in choroidal neovascularization (Bhutto and Lutty, 2012; Seddon J. M. et al., 2016). Future studies using *in vitro* RPE-choroidal models could provide novel insights and help us determine the mechanism of tissue damage and disease progression in AMD.

Another method to engineer a layered retinal model is bioprinting, which has been increasingly used to create 3D tissue constructs. 3D bioprinter systems allow precise positioning and deposition of bio-components layer-by-layer, providing an attractive strategy to engineer complex tissues (Tasoglu and Demirci, 2013). A recent study by Masaeli et al. (2020) demonstrated the potential of using bioprinting to recreate retinal components. Using cells isolated from post-mortem donor eye, RPE cells were first cultured on a thin layer of gelatin methacrylate to mimic the Bruch's membrane/RPE complex. Photoreceptors were then bioprinted onto RPE cell sheets, creating a layered structure. Notably, the authors showed that both RPE and photoreceptors retained expression of structural markers, while the RPE cells retain functional activities in VEGF secretion and phagocytosis (Masaeli et al., 2020). Future research that incorporates 3D bioprinting using retinal cells derived from stem cells would be important to overcome the problem with donor shortage to obtain cellular source for tissue engineering. Altogether, advances in pluripotent stem cells, biomaterials and 3D bioprinting provide new methods to develop better *in vitro* cell models that could accelerate functional study of AMD-associated genes and drug development.

NEW MOLECULAR TOOLS TO FACILITATE FUNCTIONAL STUDY OF AMD-ASSOCIATED GENES

Conventional strategies to study gene functions involve either gain-of-function studies by transgene overexpression, as well as loss-of-function studies by RNAi knockdown or homologous recombination-mediated knockout. The emergence of clustered regularly interspaced short palindromic repeats (CRISPR) technology provides exciting opportunities to facilitate functional studies of AMD-associated genes. Earlier use of CRISPR technology focuses on gene knockout for loss-of-function studies. In a widely used CRISPR system for gene editing, a guide RNA (gRNA) is used to direct the Cas9 nuclease derived from *Streptococcus pyogenes* (SpCas9) to cut a specific target DNA sequence and generate a double-strand DNA cleavage, which results in permanent and heritable gene knockout (Cho et al., 2013; Cong et al., 2013; Liu and Li, 2019). On the other hand, CRISPR/Cas can also be used for gene editing to correct SNPs at loci that confer high risks of AMD, such as the *CFH* locus using CRISPR-mediated base editing (Tran et al., 2019). Subsequent research have derived Cas9 nucleases from other organisms, such as SaCas9 derived from *Staphylococcus aureus* which is smaller in size and would facilitate packaging into viral vectors for gene delivery (Ran et al., 2015).

Beside gene editing, CRISPR can also be repurposed for gene activation or repression. In particular, a nuclease inactive or dead Cas9 (dCas9) can be fused with transcriptional activator(s) or repressor(s) to regulate gene expression, termed CRISPR activation (CRISPRa) or interference (CRISPRi), respectively (Boettcher and McManus, 2015). Numerous CRISPRa systems have been developed in recent years. The first generation of CRISPRa system consisted of dCas9 fused to a VP64 activator (Gilbert et al., 2013), which is capable of activating expression of silent genes or upregulating expression of active genes (Gilbert et al., 2013; Mali et al., 2013; Perez-Pinera et al., 2013). However, the activation levels observed with dCas9-VP64 in mammalian cells were relatively small using a single sgRNA. Subsequently, several improved CRISPRa systems have been developed, including VPR, SunTag, and SAM (Tanenbaum et al., 2014; Chavez et al., 2015, 2016; Konermann et al., 2015). The VPR system employs three potent activators VP64-p65-Rta fused to a dCas9. Similarly, the SAM system uses multiple transcriptional activators, dCas9-VP64 paired with MS2-p65-HSF1, to induce a synergistic effect. On the other hand, the SunTag system involves fusing dCas9 to a tandem array of peptides that can recruit multiple copies of the VP64 effector. Also, our group has reported a cloning-free pipeline that harnesses CRISPRa for gene activation, which can be used as a rapid workflow for gain-of-function studies (Fang et al., 2019). On the other hand, CRISPRi utilizes dCas9 that is fused to a repressor complex such as Krupper-associated box (KRAB), which allows repression of the target gene (Gilbert et al., 2013). An enhanced version of CRISPR repressor is dCas9-KRAB-MeCP2, which showed improved repression compared to dCas9-KRAB for the majority of genes tested (Yeo et al., 2018). Notably, CRISPRi systems are capable of multiplex gene repression, with high knockdown levels and minimal off-target effects (Housden and Perrimon, 2016). These technologies could be used to decipher functional roles of novel AMD-associated genes such as those identified in recent GWAS studies (Fritsche et al., 2016; Ratnapriya et al., 2019). Collectively, these CRISPRa and CRISPRi systems allow robust activation of multiple genes simultaneously with high efficiency and specificity, providing valuable tools for functional studies of AMD-associated genes.

Notably, CRISPRa and CRISPRi pooled libraries have been developed for genome-wide screens to study gene functions (Gilbert et al., 2014; Konermann et al., 2015). This provides an exciting strategy to identify novel genes that contribute to pathological processes leading to AMD, such as degeneration of RPE cells. In CRISPR pooled screens, the cells are transduced with a gRNA library to activate or inhibit a pool of target genes, followed by cell selection based on the phenotype of interest (e.g., RPE degeneration). Subsequently, the cell samples can be

processed for next generation sequencing to identify the sgRNAs and the target genes that cause the phenotype of interest (Joung et al., 2017; Sanson et al., 2018). Genome-scale CRISPR functional screens offer a powerful modality to interrogate gene function that may take years to identify using conventional strategies. Future genome-wide screens using RPE models could enable identification of genes that are important in RPE degeneration and provide insights into AMD pathology.

Beyond gene function studies, CRISPRa and CRISPRi could also be applied to study regulatory regions of AMD genes. Since the majority of genetic variation associated with complex human disease is found within non-coding regions of the genome (Thurman et al., 2012), understanding how regulatory regions within the non-coding DNA regulate gene expression could help us understand pathogenesis of AMD. In this regard, CRISPRa or CRISPRi could be used to target non-coding regions of GWAS loci to identify novel regulatory regions of AMD-associated genes (Klann et al., 2018).

In addition to DNA-targeting CRISPR systems, RNA-targeting Cas9 enzymes are also available, such as CasRx which showed robust knockdown of gene expression (Konermann et al., 2018). Interestingly, CasRx can also be used to target pre-mRNA to manipulate alternative splicing (Konermann et al., 2018). Deregulation of alternative splicing has been implicated in the aging process (Li et al., 2017) and observed in several age-associated diseases such as amyotrophic lateral sclerosis and Alzheimer's disease (Lin et al., 1998; Spillantini et al., 1998; Glatz et al., 2006). In regards to AMD, Allikmets et al. (1997) have shown that a point mutation (G5196A) in the Stargardt disease gene ABCA4, eliminates a 5′ donor splice site and increases the risk of AMD. However, a subsequent GWAS study with larger cohorts could not confirm this association between ABCA4 and AMD (Fritsche et al., 2016). Overall, the association of AMD pathophysiology with alternative splicing regulation remains unclear and CasRx technology could facilitate research in this understudied area. In summary, recent development of CRISPR/Cas technology has greatly expanded the toolbox to carry out functional study of AMD-associated genes, providing new tools that can modulate gene expression by targeting at the DNA level, RNA level as well as the splicing variants.

CONCLUSION

Despite extensive research in AMD and considerable success in identifying genetic associations with this disease, we still do not fully understand the pathological mechanisms leading to AMD. Recent advances in the field of genomics, cell biology, molecular biology and tissue engineering offer an unprecedented opportunity for functional genomics studies and help to efficiently decipher the genetic contribution to AMD pathogenesis. Whilst identification of novel AMD-associated genes is greatly facilitated by new development in genome-wide analysis methods, more study is required to understand the functional role of these genes in AMD. Single cell transcriptomics offers a unique opportunity to understand how these genes affect specific cell types in the retina. Advances in stem cells and biomaterials provide better *in vitro* systems to study the function of AMD-related genes in the clinically relevant cell types, while new CRISPR technologies provide an efficient method to perform gain-of-function and loss-of-function studies for AMD-related genes. Future studies that utilize the technologies discussed here would provide novel insights into AMD genetics and accelerate identification of new therapeutic targets.

AUTHOR CONTRIBUTIONS

TN, CL, RG, and RCBW provided conceptual framework of this work. TN, DU, RHCL, CL, RG, and RCBW contributed to manuscript writing. All authors approved the manuscript.

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REVIEW
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Age-Related Macular Degeneration Revisited: From Pathology and Cellular Stress to Potential Therapies

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Age-related macular degeneration (AMD) is a neurodegenerative disease of the aging retina, in which patients experience severe vision loss. Therapies available to patients are limited and are only effective in a sub-population of patients. Future comprehensive clinical care depends on identifying new therapeutic targets and adopting a multi-therapeutic approach. With this goal in mind, this review examines the fundamental concepts underlying the development and progression of AMD and re-evaluates the pathogenic pathways associated with the disease, focusing on the impact of injury at the cellular level, with the understanding that critical assessment of the literature may help pave the way to identifying disease-relevant targets. During this process, we elaborate on responses of AMD vulnerable cells, including photoreceptors, retinal pigment epithelial cells, microglia, and choroidal endothelial cells, based on *in vitro* and *in vivo* studies, to select stressful agents, and discuss current therapeutic developments in the field, targeting different aspects of AMD pathobiology.

Keywords: age-related macular degeneration, oxidative stress, retinal pigment epithelial (RPE), choroidal endothelial cells, therapy

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible central vision loss in the Western hemisphere (Wong et al., 2014). It has been postulated that with the growing aging population, the prevalence and burden of AMD will continue to rise. In the early stages of the disease, visual deficits include impaired dark adaption, but otherwise may be minimal. However, as the disease evolves, vision becomes progressively more compromised, the retinal tissue degenerates, and suffers permanent damage. Traditionally, AMD has been classified broadly into two clinical sub-types; dry or non-exudative and wet/neovascular or exudative (Ferris et al., 2013; Spaide et al., 2018). In developed countries, approximately 10% of the population over the age of 65 years and 25% over the age of 75 years are purported to have been diagnosed with AMD. It is further estimated that in the US, about 11 million people (∼85% of all AMD) have dry AMD, while 1.5 million (∼15% of all AMD) are affected by the advanced stages of the disease (Joachim et al., 2015; Chou et al., 2016), with an estimated 70,000 new cases of wet AMD identified each year (Rudnicka et al., 2015). Though select treatment options targeting vascular leakage and stability are available for patients presenting with the wet or neovascular form of the disease, it has been shown to be effective in only a subpopulation of patients (Nagai et al., 2016). Importantly, no treatment options are available for the early and intermediate stages of AMD. The lack of treatments is in part due to the complexity of the disease, as not only multiple genetic and environmental risk factors but also different cell types within the

inner and outer retina, have been shown to be involved in the pathophysiology of AMD (Malek and Lad, 2014; Malek et al., 2018; Choudhary and Malek, 2020). Therefore, it is vital to further understand the molecular mechanisms underlying disease development and progression, in concert with the temporal development of pathological changes that occur in the retina. This is necessary in order to identify potential therapeutic targets. Herein, we will review the pathology and visual deficits associated with the different clinical subtypes of AMD and outline the pathogenic pathways linked to the development of AMD, with a focus on the growing body of evidence indicating that stress and injury to AMD vulnerable cells including photoreceptors, retinal pigment epithelial cells (RPE), retinal immune cells and choroidal endothelial cells, is a crucial component of the disease.

AMD CLASSIFICATION AND GRADING

The hallmark lesions of the *early stages of dry AMD*, often referred to as non-neovascular or non-exudative AMD (**Figure 1**), are sub-RPE deposits called drusen, derived from the German word for node or geode. Drusen formation have been noted in the peripheral regions with age, however, in early dry AMD they become larger and are found within the macula. Other indicators of dry AMD are RPE abnormalities, hyperpigmentation, and atrophy, as well as choriocapillary loss (Mullins et al., 2011; Malek and Lad, 2014) distinctive morphology from that seen in the normal posterior pole (**Figures 1P–T**). Clinically, drusen are small, yellowish appearing lesions located between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane (a penta-laminar extracellular matrix, upon which the RPE cells reside) (**Figures 1A–E**). Histological evaluation of AMD donor tissue along with *in vivo* imaging of the posterior pole of AMD patients using optical coherence tomography (OCT) has revealed the presence of a variety of deposits characteristic of aging and AMD, beyond drusen, including basal laminar deposits, present between the RPE and its basal lamina, containing lipid-rich material and wide-spaced collagen; basal linear deposits containing phospholipids and located between the RPE basal lamina and Bruch membrane, within the same plane as drusen; and sub-retinal drusenoid deposits containing some established drusen markers (e.g., unesterified cholesterol, apolipoprotein E and complement factor H), but not all (e.g., esterified cholesterol) (Rudolf et al., 2008; Chen et al., 2020). Drusen size has been instrumental in classifying the severity of disease with small drusen, sized up to 63 μm in diameter; intermediate, sized between 64 and 125 μm in diameter; and large drusen exceeding 125 μm in diameter. These deposits have further been categorized based on their shape and boundaries, referred to as hard when they present with well-demarcated borders, soft with poorly demarcated borders and confluent when drusen are continuous without clear borders. In general, an eye with large, soft, and confluent drusen is at a higher risk of progressing to either of the advanced forms of AMD, geographic atrophy or choroidal neovascularization, relative to an eye with only hard drusen.

The *non-neovascular advanced stage of dry atrophic AMD* also known as geographic atrophy involves degeneration of the RPE, retina and the choriocapillaris with well-demarcated borders, resembling the map of a 'continent' (**Figures 1F–J**). The atrophic regions tend to be multi-focal, may or may not involve the foveal center (Ferris et al., 2013; Spaide et al., 2018) and often present bilaterally (Mann et al., 2011). The *wet or neovascular advanced form of AMD* is characterized by the presence of vascular growth from the choroid penetrating Bruch's membrane, referred to as choroidal neovascularization, within the macula (**Figures 1K–O**). Though wet AMD is less frequent than dry AMD, the need for successful therapies is paramount as it is responsible for 90% of acute blindness. The clinical manifestations of neovascular AMD are varied and include subretinal and intraretinal fluid, retinal, subretinal, or sub-RPE hemorrhage, lipid exudates, plaque-like yellow-green choroidal neovascular membranes, RPE detachment, and RPE tear. In the end-stages, the neovascular membrane may evolve into a 'disciform scar' (hypertrophic, fibrovascular, or atrophic macular scar) causing permanent central vision loss (Ferris et al., 2013; Spaide et al., 2018).

It is important to note that geographic atrophy and choroidal neovascularization are not mutually exclusive as the atrophic retina may result in the development of a neovascular lesion (mostly at the edge of the atrophic region, especially if the contralateral eye is wet), and wet AMD may proceed to macular atrophy.

EPIDEMIOLOGY AND RISK FACTORS OF AMD

The complexity of AMD lies not only in the variety of pathologies associated with the disease, but it is also reflected in the number of risk factors identified to date. Formative population-based investigations and genome-wide association studies, have revealed significant knowledge about AMD prevalence and genetic risk, respectively. The landmark study from 1992, "The Beaver Dam Study," provided one of the first estimates of the prevalence of features of maculopathy including soft drusen, pigmentary abnormalities, choroidal neovascularization, and geographic atrophy, over a broad spectrum of ages (Klein et al., 1992). In general, the prevalence of advanced forms of the disease (wet AMD and geographic atrophy) was discovered to increase with each decade of life, being the highest after 75 years of age (Mitchell et al., 1995; Vingerling et al., 1995; Congdon et al., 2004; Joachim et al., 2015). The higher frequency of more severe macular pathology in the elderly, especially in the aging western population, brought to light the severity of this disease as an ongoing public health problem. Epidemiologic studies have also identified key risk factors for AMD, with advanced age acknowledged as the main one and cigarette smoking coming in second. Additional risk factors include but are not limited to positive family history, sex (female), hyperopia, light iris color, hypertension, hypercholesterolemia, cardiovascular diseases, obesity, and elevated inflammatory

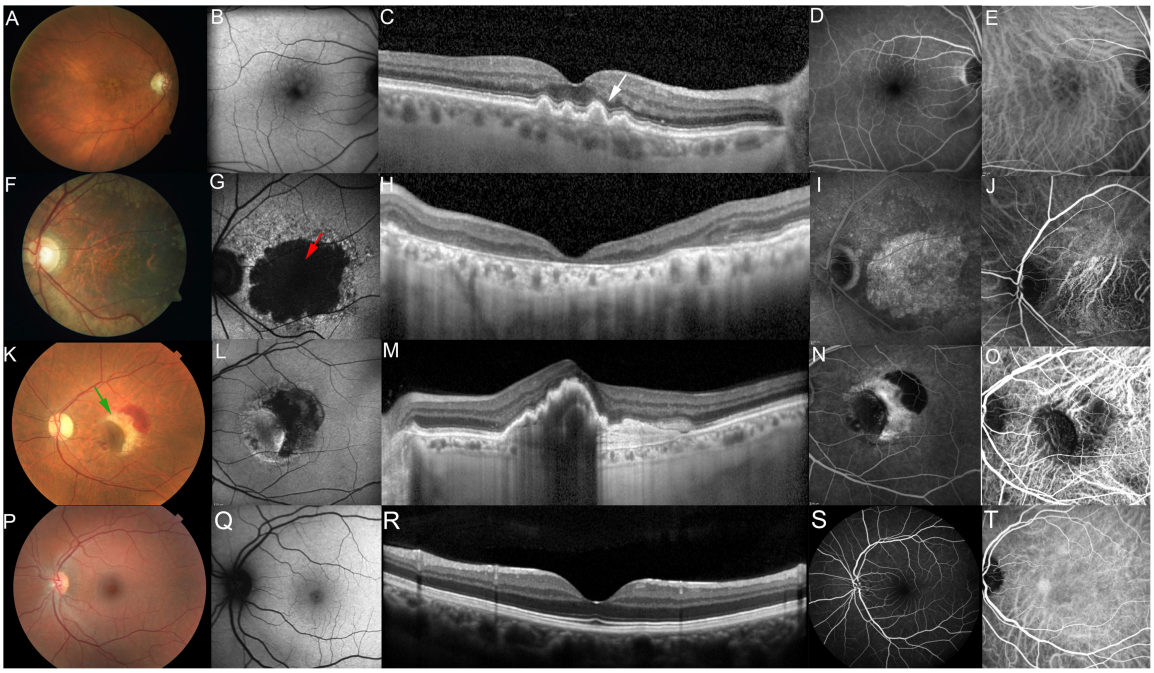

FIGURE 1 | Photomicrographs showing different stages of AMD. Photomicrographs showing different stages of AMD compared to normal macula. Non-exudative AMD, intermediate (**A–E**, fundus photo, fundus autofluorescence, optical coherence tomography, fundus angiography and indocyanine green, respectively) showing drusen (white arrow), non-exudative AMD, advanced with subfoveal involvement **(F–J)** showing large, central GA (red arrow), Exudative AMD **(K–O)** showing Choroidal Neovascular Membrane (CNVM) and retinal hemorrhage (green arrow), Normal macula **(P–T)**.

markers (Seddon et al., 1996, 2003; Age-Related Eye Disease Study Research Group, 2000; Hyman et al., 2000; Smith et al., 2001; Klein et al., 2003; Tomany et al., 2004; Malek and Lad, 2014; Armstrong and Mousavi, 2015). Importantly, the prevalence of the advanced forms of AMD appears to vary in different ethnic and racial groups, with the highest risk reported in the Caucasian population (5.4%) and lowest in African-Americans (2.4%); and the risk for Hispanics and Asians falling in between (4.2 and 4.6%, respectively) (Frank et al., 2000; Klein et al., 2004; Choudhury et al., 2016; Cheung et al., 2017).

Large genome-wide association studies of AMD, to date, have identified 52 genetic variants at 34 genetic loci associated with AMD. These genes harbor mutations that affect various biological pathways, including complement regulation, lipid metabolism, extracellular collagen matrix, angiogenesis, and all-*trans*-retinaldehyde metabolism, to name a few. Two major susceptibility genes for AMD that have been the focus of intense investigation, are the well-characterized CFH (1q31) that codes complement factor H, and poorly understood ARMS2 (10q26) (Hageman et al., 2005; Jakobsdottir et al., 2005; Klein et al., 2005; Scholl et al., 2005; Souied et al., 2005). The CFH mutation confers a 4.6- and 7.6-fold increased risk for AMD, while the ARMS2 mutation confers a 2.7- and 8.2-fold in heterozygotes and homozygotes, respectively. Most recently family-based exome sequencing studies have identified rare coding variants for novel candidate genes at eight previously reported loci, with 13 additional candidates detected outside of known regions, further highlighting the multi-factorial nature of AMD (Fritsche et al., 2013; Cheng et al., 2015; Fritsche et al., 2016; Gorin et al., 2016; Han et al., 2020). Genetic testing is currently available for AMD, but it is controversial and not officially recommended, given the limited treatment options available to patients. However, with the rapid advancements in this research field, this is likely to change soon (Edwards, 2006; Chew et al., 2015; Stone, 2015; Cascella et al., 2018; Warwick and Lotery, 2018).

AMD-DRIVING PATHOGENIC PATHWAYS

Despite extensive research, we still do not fully understand critical drivers involved in the initiation of AMD and progression from the early to advanced stages. This, in turn, has made predicting progression and effective treatments difficult. However, breakthroughs in identifying probable pathogenic pathways and molecular mechanisms associated with disease, born out of a consolidation of AMD pathologies, identified through observations of *in vivo* and *ex vivo* tissues, epidemiological studies, and in particular high-risk genes linked with AMD development, have been instrumental in the pursuit of animal models and potential therapies. These pathways, which are also often related to aging, include but are not limited to: complement activation, lipid trafficking and metabolism, vitamin A cycle/metabolism, proteostasis, bioenergetics, autophagy/mitophagy, extracellular matrix turnover, choroidal vascular dropout, and last but not least oxidant-induced and non-oxidant associated cellular injury and stress (Pool et al.,

2020). The possible roles of each of these pathways in AMD warrant a special review in and of themselves. However, the rest of this review will focus on the impact of various stress modalities on cells vulnerable in AMD, whose induction has been attributed to modifiable dietary and environmental factors as well as factors that remain unknown.

IMPACT OF OXIDANT AND NON-OXIDANT STRESS AND INJURY ON AMD

Oxidative stress is often defined as a disturbance in the equilibrium between the amounts of reactive oxygen species and antioxidant production/detoxification capacity of cells. This equilibrium is critical for cell and tissue survival such that the consequence of any imbalance would be tissue injury. Injury to cells, however, can also occur in response to environmental factors and aging in general, compromising the tissues ability to respond effectively and counter stress (Luo et al., 2020). Importantly, the cells response to injury can also vary in accordance with the level of stress (low versus high) and the length of exposure (acute versus chronic), such that young healthy cells may counter acute stress more formidably that aged cells, specifically effecting cellular processes including autophagy, phagocytosis, proteosomal degradation, toxic clearance and metabolism, among others. Thus, it is not surprising that stress also has a major impact on aging neurodegenerative diseases such as AMD. Beyond the age factor, the retina is particularly vulnerable to photo-oxidative stress as it is chronically exposed to light (B Domènech and Marfany, 2020). Visual transduction pathways can result in reactive oxygen species production in response to oxidation of the building blocks of the photoreceptor outer segments, polyunsaturated fatty acids. Other mechanisms that put the retina in the line of fire for vulnerability to stress include modifiable behavioral risks, including smoking and indulging in diets rich in high fat and cholesterol (Klettner et al., 2013). Additional evidence for stress comes from proteomic studies of Bruch's membrane tissue from AMD donors, revealing the presence of oxidative products (Beattie et al., 2010; Yuan et al., 2010), and the AREDS studies, which have shown an association between reduced prevalence of AMD and high dietary intake of antioxidants (Chew et al., 2013, 2014). With all this in mind, it is not surprising that there is a large body of evidence pointing to oxidant and non-oxidant stress as a bona fide pathobiologial process in AMD, including *in vitro* and *in vivo* studies examining AMD-vulnerable cells and tissues, which we will further review below (**Figure 2**).

Retinal Ganglion Cells

The innermost retinal layer is primarily composed of ganglion cells. Though this layer is not a primary vulnerable site in AMD, thinning has been reported in dry AMD patients (Yenice et al., 2015) and as a consequence of retinal remodeling following photoreceptor degeneration (Garcia-Ayuso et al., 2018). Furthermore, a subset of ganglion cells contain melanopsin and are light-sensitive, significant given this layer is exposed to chronic light (Garcia-Ayuso et al., 2015). In response to overproduction of reactive oxygen species including superoxide anions, hydrogen peroxide, and hydroxyl radicals, ganglion cells die (Cao et al., 2015). However, they have also been shown to be remarkably resistant to cell death induction by these stressors in part due to their endogenous peroxides (Kortuem et al., 2000). As expected, this has led to a quest for neuroprotectives. *In vitro* and *in vivo* studies have revealed protective roles for master antioxidant defense regulators including the nuclear transcription factor kB (NF-kB) and nuclear factor – erythroid 2 – related factor 2 (NRF2) as well as polysaccharides, growth factors, including transforming growth factor beta, nerve growth factor, and brain-derived neurotrophic factor; endogenous antioxidant factors including glutathione, superoxide dismutase, and catalase to name a few (Pietrucha-Dutczak et al., 2018).

Microglia

The retinal microglia are resident immune cells thought to be critical to the initiation of retinal inflammation (Rashid et al., 2019). A major consequence of oxidative and non-oxidant stress is inflammation. Though traditionally, abnormal microglial activity has been associated with retinal diseases including diabetic retinopathy, hereditary retinopathies, and glaucoma, recent evidence points to a role in AMD as well (Fletcher, 2020). Enlarged amoeboid microglia have been found adjacent to RPE cells overlying drusen in AMD retinal sections and may be a potential source for NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome activation of RPE cells and increased pro-inflammatory cytokines such as IL-1 beta (Madeira et al., 2015). *In vitro* culture studies have shown that conditioned media from reactive microglial cells can trigger caspase-mediated photoreceptor cell death (Madeira et al., 2018). *In vivo* studies have also provided evidence for a role of microglia in retinal phenotypes associated with AMD. For example, in mice exposed to bright white, photoreceptor cell death, and retinal degeneration occurs along with migration of microglial cells to the outer retina (Wang et al., 2014). Another mouse model that presents with dry AMD phenotypes involves immunizing mice with carboxyethylpyrrole-adducted proteins. In these mice there is evidence of infiltrating phagocytes around degenerating photoreceptors and RPE cells (Hollyfield et al., 2008; Hollyfield, 2010). The question as to whether or not infiltrating macrophages are beneficial or detrimental is a complex one and ties into why the cells accumulate to begin with, which may be due to an increase in migration of monocytes into the retina or failure of immune cell clearance. In the carboxyethylpyrrole immunized mouse model, though sub-retinal macrophages are present, they are not seen in areas with severe RPE degeneration, suggesting that they may have a beneficial effect, perhaps in removing debris. Importantly in the wet laser-induced experimental mouse model of AMD, recruitment of ameboid microglia and mononuclear phagocytes are seen within the neovascular lesion, the number of which varies with the severity and nature of the lesion (fibrotic versus leaking), reflecting the dynamic nature of these cells (Crespo-Garcia et al., 2015; Zhou et al., 2017). As a consequence of microglial recruitment to the retina, there is enhanced phagocytosis and production of pro-inflammatory factors. This,

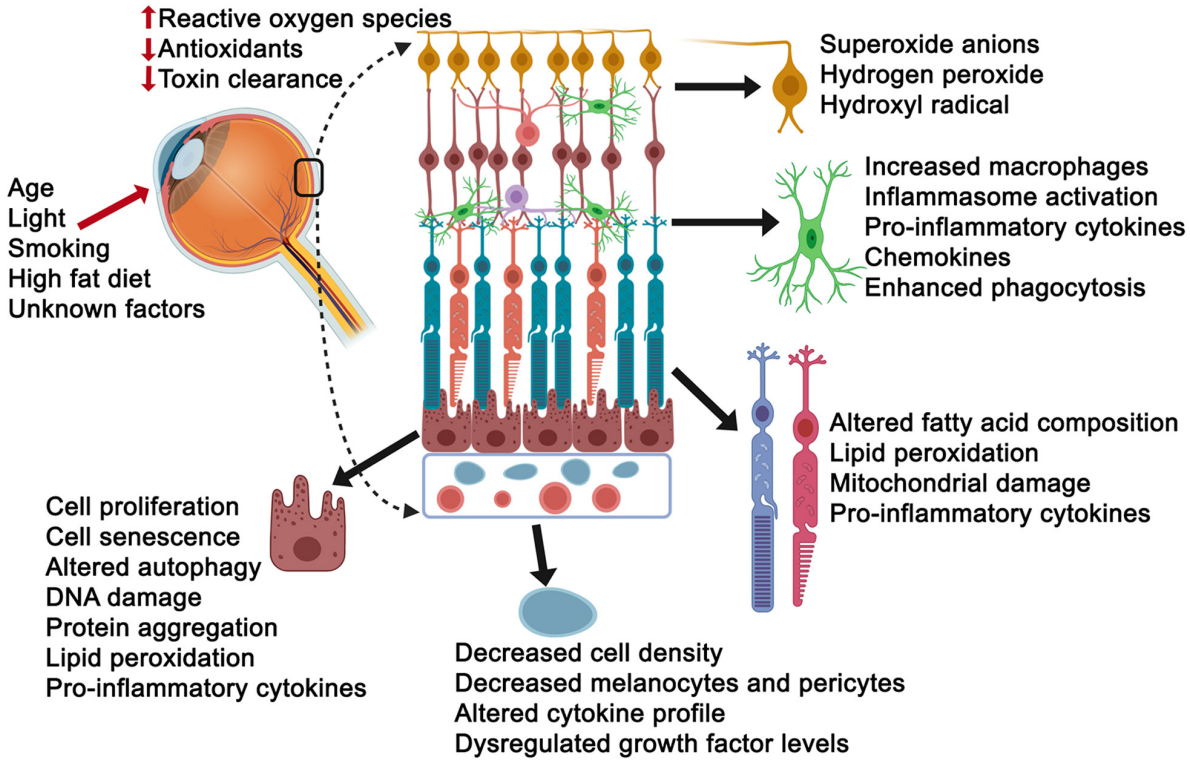

FIGURE 2 | Consequences of stress exposure on retinal cells. Age, light exposure, smoking, high fat diet and unknown factors contribute to elevated reactive oxygen species, decreased intracellular anti-oxidant levels and toxin clearance mechanisms of retinal cells including the ganglion cells (yellow), microglial cells (green), photoreceptors (aqua/orange or light purple/red), retinal pigment epithelial cells (burgundy) and choriocapillaris/endothelial cells (light blue). Select consequences have been listed next to each cell.

in turn, can impact the integrity of the neural retina resulting in thinning of the outer nuclear layer (Karlstetter et al., 2015; Zhao et al., 2015).

The effect of oxidative stress on retinal microglia in AMD is still a relatively new area of research, the mechanism of which is unknown. Though much may be extrapolated regarding microglial cells from other retinal diseases such as glaucoma, in which the effect of adenosine blockade, a neuromodulator which works through its receptor $A_{2A}R$ present on microglial cells, has been investigated (Santiago et al., 2014). Blockade of the receptor has been associated with decreased reactive oxygen species levels and morphological changes in microglial cells associated with pressure changes including retinal degeneration. Studies using minocycline in light-induced retinal damage models and their impact on microglial cells have varied showing both a decrease in immunolabeling of CD11b of microglial cells, which may protect against loss of photoreceptors through inhibition of retinal microglia activation, and a delay in photoreceptor cell death, which was independent of a reduction in retinal microglial cells, following depletion of microglia cells using liposomal chlodronate (Yang et al., 2009; Peng et al., 2014; Ferrer-Martin et al., 2015). *In vitro* studies also support this hypothesis, in which conditioned media from activated microglia cells induce apoptosis in the 661W transformed photoreceptor cell line (Roque et al., 1999). Hypoxia can cause retinal microglial cells to produce IL-1 beta and TNF alpha, and this has been associated with retinal microvascular degeneration by inducing semaphorin 3A in neurons (Rivera et al., 2013). Finally, in an *ex vivo* retinal culture model for oxidative injury induced by hydrogen peroxide, a dose-dependent increase in microglia and elevation of CD11b expression was observed followed oxidative stress induction, with a time-dependent increase in IL-1 beta, iNOS, HSP70 at day 3 and TNF alpha and IL-1 beta at day 8 (Hurst et al., 2017). Therapeutically, hypothermia used to counter hypoxia as a potential therapy for retinal degenerations has been shown to protect microglia numbers as well as CD11b expression (Maliha et al., 2019). Finally, in a mouse model of retinitis pigmentosa, MutY homolog-mediated (MUYTH-mediated) base excision repair (BER) in oxidative microglial activation has been proposed to be a novel target to dampen disease progression, able to suppress microglial activation and photoreceptor cell death (Nakatake et al., 2016).

Photoreceptors

The major light-sensing neurons in the retina are the rod and cone photoreceptors, vulnerable in AMD in part due to their high metabolic demand. As mentioned earlier given the degree of photo-oxidative stress photoreceptors are exposed to, including light pollution by artificial light originating from commonly used technologies, a sundry of studies have been

devoted to understanding the pathways impacted by oxidative stress. Using hypoxia as an inducer of stress, mice carrying the retinal degeneration 8 mutation, presented with accelerated photoreceptor degeneration and rosette formation, thinning of the central retina, and increased NADPH oxidase 4 in the outer nuclear layer (Lajko et al., 2017). Cool white light exposure (200 lux light-emitting diode) in mice has been shown to lead to photoreceptor cell death and alterations in fatty acid composition, specifically a decrease in docosahexanoic acid levels concomitant with an increase in stearic acid (Benedetto and Contin, 2019). In 661W cells, murine photoreceptor-like cells, knockdown of Nrf2 resulted in an increase in reactive oxygen species levels suggesting Nrf2 is again a key endogenous protective factor (Chen W. J. et al., 2017). Therapeutically, edaravone, a free radical scavenger, has been tested in a mouse model exposed to *N*-methyl-*N*-nitrosourea and found to inhibit outer nuclear layer thinning, cell death and oxidative stress markers (Tsuruma et al., 2012). Other treatments that have been tested include celastrol, a naturally occurring quinone methide triterpene, which demonstrated photoreceptor cell death suppression in BALB/c mice exposed to bright white light (Bian et al., 2016) and overexpression of cytochrome b5 in the *Drosophila melanogaster*, which resulted in suppression of blue light-induced retinal degeneration and lipid peroxidation (Chen X. et al., 2017). Importantly, it should be noted that age-related macular degeneration does not affect one cell type and when considering therapy the complex tissue should be studied. Indeed overexpression of catalase, an antioxidant, in RPE cells has been shown to protect its neighboring cells, the photoreceptors, from light damage, resulting in reduced 4 hydroxynonenal and nitrotyrosine levels, two markers of oxidative stress (Rex et al., 2004).

Retinal Pigment Epithelium (RPE)

The retinal pigment epithelium (RPE) cells are hexagonal, polarized epithelial cells in close contact with photoreceptor outer segments at their apical side and Bruch's membrane along their basal side. These highly specialized cells have many vital functions essential to retinal health, including daily phagocytosis and degradation of photoreceptor outer segments, light absorption, vitamin A metabolism, and heat exchange (Strauss, 2016). Additionally, RPE cells maintain the outer blood-retinal barrier and provide selective entry and removal of oxygen, nutrients, and metabolites (Strauss, 2005). With these multiple and diverse functions, RPE cells help maintain the photoreceptors and choriocapillaris' health and function, thus playing a significant part in AMD's pathogenesis.

Prolific investigations in AMD have proposed that oxidative stress is a common consequence of multiple risk factors involved in its pathogenesis. Macular high oxygen demand makes this part of the retina particularly susceptible to disturbed oxygen homeostasis. Various aerobic metabolism pathways produce reactive oxygen species; however, the primary source of their production is the mitochondria (Mao et al., 2014). For example, the identification of polymorphisms in mitochondrial MTND2*LHON4917G, NADH dehydrogenase subunits, and mitochondrial superoxide dismutase 2, suggests a role for oxidative stress in AMD's pathogenesis (Kraja et al., 2019). The LOC387715 polymorphism additionally supports this statement (Tong et al., 2010; Yang et al., 2010). The sources of oxidative stress in RPE cells range from high oxygen tension attributed to its close proximity to the outer retinal blood supply, the choriocapillaris, to the accumulation of autofluorescent lipid-protein aggregates that occur with aging, called lipofuscin (Sparrow and Yamamoto, 2012). Upon exposure to oxidative stress, intracellularly, not only are RPE proteins, lipids and DNA damaged, but also the mitochondria. Similar to photoreceptors, the post-mitotic nature of RPE cells preclude the rapid clearance of damaged mitochondria through cell division (Cai et al., 2000; Plafker et al., 2012). Like photoreceptors and ganglion cells, the role of Nrf2 for protection against phototoxic stress in RPE cells has been examined with *in vitro* studies demonstrating that sulforaphane, an Nrf2 activator can protect RPE cells from blue light-induced damage (Gao and Talalay, 2004). Other antioxidants tested in RPE cells range from glutathione, which in its reduced form has been shown to be protective against tert-butylhydroperoxide induced injury of RPE cells, potentially directly reacting with photooxidized components of lipofuscin (Sternberg et al., 1993; Yoon et al., 2011); to vitamins and their analogs including alpha-tocopherol (vitamin E), ascorbic acid (vitamin C) and beta-carotene, a precursor of vitamin A (Kagan et al., 2012).

Choriocapillaris

The choriocapillaris is the complex fenestrated capillary layer of the choroid providing oxygen and nutrients to the RPE/neural retina. It is located immediately adjacent to Bruch's membrane. Recently, the importance of the integrity of the choriocapillaris in all three clinical sub-types of AMD has been brought to the light with seminal studies demonstrating its vulnerability in non-neovascular or dry AMD (Chirco et al., 2017). Studies of human donor tissue from dry AMD patients revealed a loss in the density of the choriocapillaries (choriocapillary dropout), represented as an increase in non-perfused capillary segments also known as 'ghost vessels' (Mullins et al., 2011), while OCTA studies indicate thinning of the choroid, concomitant with increased average choriocapillaris signal void size, compared to eyes without neovascular AMD (Choi et al., 2015). Interestingly, in flatmount analyses of the choroidal tissue from geographic atrophy patients, the choriocapillaris appears intact in some regions adjacent to RPE loss, suggesting vulnerability in these patients is initially at the level of the RPE and perhaps secondarily effecting the choriocapillaris (McLeod et al., 2009). Extensive choriocapillary loss is seen in neovascular AMD, even in regions where the RPE appears to be intact (Moreira-Neto et al., 2018).

The impact of stress on the choriocapillaris is a burgeoning area of research with few studies so far, some in which photo-oxidative stress has served as the measurable endpoint. Most have involved the use of *in vitro* cultures exposed to blue-light or oxidative stress inducers such as hydrogen peroxide. Others include light-induced lipid peroxides localized to the choroid in the choroidal endothelial cells and melanocytes of albino (BALB/cJ) mice. An additional *in vivo* study tested the effect of

overexposure to green light induced oxidative stress in choroidal endothelial cells in albino mice, observing oxidative damage to DNA impacting melanotyes and pericytes. Interestingly light-induced photo-oxidative stress resulted in activation of the NF-κB signaling pathway, which has been shown to be in response to oxidative stress (Wu et al., 2005). These studies primarily use albino mice as photo-oxidative stress induction in pigmented mice has been difficult. Therapeutically, sirt 1 (silent information regulator 1), which is activated when changes in cellular redox state occur, has been proposed as a potential target. *In vitro* studies using a monkey choroidal endothelial cell line (RF6A) exposed to sirtuin inhibitors points to a significant increase in reactive oxygen species production (Balaiya et al., 2017). Translocator protein (TSPO), a cholesterol-binding protein involved in mitochondrial cholesterol transport has been found to be expressed in the mitochondria of choroidal endothelial cells (Biswas et al., 2018). When exposed to TSPO ligands, production of reactive oxygen species by choroidal endothelial cells are reduced and there is an increase in antioxidant capacity, and reduction of pro-inflammatory cytokines induced by oxidized low-density lipoproteins, suggesting TSPO may be a potential therapeutic means to reduce oxidative stress in the choroidal endothelial cells. Finally, tert-butylhydroperoxide (tBH) mediated oxidative stress reduces survival of choroidal endothelial cells *in vitro*, and RPE cells exposed to tBH-mediated oxidative stress secrete increasing amounts of bFGF but not vascular endothelial growth factor (VEGF) in culture and support proliferation of choroidal endothelial cells, suggesting a mechanism leading to neovascularization as seen in wet AMD (Eichler et al., 2008). In conditions in which there is elevated VEGF, choroidal endothelial cells produce increased levels of reactive oxygen species, which can be prevented by NADPH oxidase inhibitors, as confirmed in the laser-induced choroidal neovascularization model (Monaghan-Benson et al., 2010). Finally, a mouse model lacking the anti-oxidant enzyme CuZn superoxide disumutase have been reported to develop neovascular lesions (Imamura et al., 2006).

Pigment epithelial-derived factor (PEDF) expression has also been shown to impact the oxidative state of choroidal endothelial cells. PEDF is an endogenous inhibitor of angiogenesis. Choroidal endothelial cells isolated from PEDF knockout mice demonstrated heightened sensitivity to hydrogen peroxide challenge with an increase in apoptotic cells, oxidative stress, and pro-inflammatory cytokine profile, along with increased cellular proliferation, decreased adhesion and migration (Park et al., 2011). Polypoidal choroidal vasculopathy, a late stage of neovascular AMD, is characterized by abnormal branching in the vascular networks and the presence of polypoidal or aneurysmal dilations, with the choroidal vessels displaying hyalinization. These dilations have been suggested to be the result of alternations in elastin, homocysteine-associated oxidative stress, and endothelial dysfunction. Interestingly, pretreatment of RF/6A cells subjected to paraquat to induce oxidative stress, with fenofibrate, a peroxisome proliferator activated receptor (PPAR) alpha agonist, resulted in decreased cellular apoptosis, diminished changes in mitochondrial membrane potential, increased expression of peroxiredoxin, thioredoxin, Bcl-2 and Bcl-xl and reduced BAX, pointing to fenofibrates anti-oxidant properties, as a potential adjunct therapy (Hsu et al., 2020). The receptor TNF alpha R2 is expressed in choroidal vascular cells, RPE, and Mueller cells and it has been shown that TNF alpha contributes to choroidal neovascularization by upregulating VEGF through reactive oxygen species activation of the beta-catenin signaling pathway (Wang et al., 2016). The expression of thrombospondin-1, which is another endogenous inhibitor of angiogenesis and inflammation, has been shown to regulate choroidal endothelial cells. Interestingly thrombospondin 1 knock out in choroidal endothelial cells results in increased levels of thrombospondin 2, phosphorylated endothelial and inducible nitric oxide synthase, which are associated with significantly high levels of nitric oxide and oxidative stress (Fei et al., 2014). In addition to supplementation with carotenoids such as zeaxanthin and lutein, potential therapies targeting reactive oxygen species production in the choroid tested *in vitro* and *in vivo*, have been the use of resveratrol, which showed to inhibit proliferation of hypoxic choroidal endothelial cells in association with an increase in caspase 3, and may serve as a therapeutic option to be considered for targeting stress in choroidal neovascularization (Balaiya et al., 2013; Nagai et al., 2014).

SUCCESSES AND FAILURES OF AMD THERAPIES AND THE PIPELINE

Over two decades ago, a diagnosis of wet AMD was a dreadful one as no treatment options were available to patients. However, a breakthrough came when the Food and Drug Administration (FDA) approved the first anti-angiogenic drug, Macugen (Pegaptanib sodium injection, Eye Tech Pharmaceuticals, currently OSI Pharmaceuticals, Long Island, NY, United States), to be used in the treatment of wet AMD. Since then, the field has blossomed with an AMD disease prognosis changing to one in which therapeutic options leave more than 90% of patients maintaining their vision [losing <15 ETDRS (Early Treatment Diabetic Retinopathy Study) letters] after 1 year of treatment (Heier et al., 2012). In recent years, though Macugen showed promise in slowing down vision loss in patients with wet AMD it has quickly been replaced by more effective medications. Currently, the three most widely used drugs provide an anti-angiogenesis effect by blocking VEGF. Two are FDA approved, and one is being used off-label. The FDA approved ranibizumab (Lucentis, Genentech) in 2006, a recombinant humanized antibody fragment (Fab) that binds and inhibits all active forms of VEGF-A and their functional degradation products (Brown et al., 2006; Rosenfeld et al., 2006). Aflibercept (Regeneron) was approved by the FDA shortly after in 2011. It is a soluble protein that acts as a VEGF receptor decoy by combining ligand-binding elements of the extracellular domains of VEGFR1 and two fused to the constant region (Fc) of the immunoglobulin G (IgG). Because of its greater half-life, the drug can be used in a bimonthly regimen, significantly reducing the number of necessary intravitreal injections (Schmidt-Erfurth et al., 2014).

Finally, an off-label drug for AMD treatment, Bevacizumab (Genentech), is a full-length humanized monoclonal antibody against VEGF, with a longer systemic half-life than other anti-VEGF agents (e.g., about 21 days for bevacizumab, vs. 2.2 h for ranibmizumab) (Ferrara et al., 2004; Wang et al., 2004; Yang and Wang, 2004; Heier et al., 2012; Bakall et al., 2013; Busbee et al., 2013; Rofagha et al., 2013; Ferrone et al., 2014; Grewal et al., 2014; Schmidt-Erfurth et al., 2014; Bhisitkul et al., 2015; Avery et al., 2017). It is important to note that increased oxidative stress plays an important role in AMD, triggering the expression of VEGF-A, in this case, believed to serve as a survival factor. It follows that anti-VEGF therapy may negatively impact cell survival under oxidant injury conditions, the extent to which can only be determined through a systematic study examining the impact of anti-VEGF on reactive oxygen species levels following oxidative stress.

Despite the availability of treatments for wet AMD patients, about a third of patients have visual decline by 15 letters or more (Moutray and Chakravarthy, 2011; Rofagha et al., 2013; Bhisitkul et al., 2015). Importantly, repeated intravitreal injections lead to significant socioeconomic burden (Brown et al., 2005). Although available therapies are grossly successful, wet AMD still remains the center of interest of leading pharmaceutical companies. Numerous new injectable medications are coming down the pipeline, only some of which we have space to review here (see also **Table 1**). Already approved by the FDA (October of 2019) is brolucizumab, developed by Novartis and Alcon Labs. This humanized single-chain antibody fragment that inhibits all isoforms of VEGF-A has already proven to achieve the clinical endpoint on the 12-week dosing interval following the induction (Dugel et al., 2020). The real-world experience to follow the clinical trial results is still mandated to make this medication more competitive. In 2019, a new anti-VEGF agent, conbercept by Lumitin (China), was approved locally to treat wet AMD and reported to be safe and efficient (Călugăru and Călugăru, 2019; Liu et al., 2019). From Roche/Genentech currently under investigation is a drug that simultaneously inhibits VEGF-A and angiopoietin-2, faricimab (Hussain et al., 2019). Aerpio is developing ARP-1536, a humanized monoclonal antibody that targets the extracellular domain of vascular endothelial protein tyrosine phosphatase (Al-Khersan et al., 2019). Opthea is developing OPT-302, a soluble form of human VEGF receptor-3 that blocks VEGF-C and VEGF-D to be used combined with an anti-VEGF-A agent (Al-Khersan et al., 2019). Kodiak Sciences is working on a novel, anti-VEGF antibody biopolymer conjugate to treat wet AMD (KSI-301). The first results on treatment naïve eyes with neovascular AMD are expected in 2020 (Al-Khersan et al., 2019). Regenxbio is developing a gene therapy, RGX-314, as a one-time subretinal injection. It consists of the NAV AAV8 vector encoding a VEGF inhibiting antibody fragment (Al-Khersan et al., 2019). Allergan is in Phase 3, successfully exploring a novel agent with designed ankyrin repeat proteins (DARPin) technology to be used as an intravitreal injection to inhibit all isoforms of anti-VEGF-A. Thus far, in *in vitro* experiments, the VEGF-A binding affinity of abicipar pegol was found to be similar to that of aflibercept and greater than that of ranibizumab and bevacizumab (Callanan et al., 2018; Rodrigues et al., 2018; Moisseiev and Loewenstein, 2020; Sharma et al., 2020). Regenxbio and Adverum Biotechnologies are developing gene therapies, RGX-314 and ADVM-022, respectively, as one-time subretinal injections. Regenxbio's approach utilizes the NAV AAV8 vector encoding a VEGF inhibiting antibody fragment (Al-Khersan et al., 2019), while ADVM-022 is an AAV.7m8-aflibercept gene therapy product. PanOptica is going with a less invasive, topical application option (once-a-day drop) of pazopanib (PAN-90806), a molecule that blocks VEGF receptor 2 via tyrosine kinase inhibition (Hussain and Ciulla, 2017; Patra et al., 2018; Al-Khersan et al., 2019).

Unlike for wet AMD, to date, there are no approved treatments for dry AMD. The groundbreaking Age-Related Eye Disease Study (AREDS) initially conducted from 1992 to 2001 concluded that daily supplementation with high antioxidants levels and zinc might reduce the risk of progression in about 25% of patients. Recently conducted supplemental studies have revealed that some patients may experience up to 85% risk reduction, while others may encounter a threefold increased risk of progression while on supplementation, depending on their genetic make-up (Seddon et al., 2016; Vavvas et al., 2018). Significant effort has been made in dry AMD treatment research, and currently, there are ongoing, promising clinical trials (see also **Table 2**). There is an ongoing effort in China to transplant human embryonic stem cells derived from RPE into the subretinal space of patients with advanced dry AMD (NCT03046407). Additionally, the Bionic Vision system PRIMA (retinal prosthesis) is being developed by Pixium Vision (NCT03392324). Hemera Biosciences is investigating AAVCAGsCD59, an ocular gene therapy product that causes normal retinal cells to increase their expression of a soluble form of CD59. Conveniently, the compound can be injected in the physician's office. This soluble recombinant version of CD59 is designed to inhibit the formation of the membrane attack complex, the terminal step of complement-mediated cell lysis, to protect retinal cells (NCT03144999). Allegro Ophthalmics is planning a phase III trial to evaluate the safety and exploratory efficacy of risuteganib (Luminate) on dry AMD (NCT03626636). Risuteganib regulates mitochondrial dysfunction and downregulates oxidative stress response in order to restore retinal homeostasis. Opthotech is evaluating avacincaptad pegol (Zimura, a novel complement C5 inhibitor) when intravitreally administered in subjects with geographic atrophy (NCT02686658). Regenerative Patch Technologies has initiated a clinical trial to assess the feasibility of delivery and safety of human embryonic stem cell-derived retinal pigment epithelial cells on a parylene membrane (CPCB-RPE1) in patients with advanced, dry AMD (NCT02590692).

TABLE 1 | Approved or advanced in trials Wet AMD treatments.

Generic	Brand name	Manufacturer	Target	FDA approved/year	Phase in trials
Pegabtanib	Macugen	OSI Pharmaceuticals	165 isoforms VEGF-A/Pegylated RNA aptamer	Yes/2004	Concluded
Ranimizumab	Lucentis	Genentech	All isoforms VEGF-A/Monoclonal anti-VEGF (Fab) fragment	Yes/2006	Concluded
Bevaclzumab	Avastin	Genentech	All isoforms VEGF-A/Monoclonal Ab	No	Concluded/used off-label
Aflibercept	Eylea	Regeneron	All isoforms of VEGF-A, VEGF-B, and PlGF15/Fusion protein: VEGFR-1,2 fused with lgG1 Fc	Yes/2011	Concluded
Brolucizumab	Beovu	Novartis/Alcon	VEGF-A.B, PlGF/Single-chain anti-VEGF Ab/fragments (scFv)	Yes/2019	Concluded
Conbercapl		Lumitin	All isoforms of VEGF-A, VEGF-B, VEGF-C, and PlGF32/Fusion protein: VEGFR-1,2 fused with lgG1 Fc	No/approved in China	Phase 3/NCT03577899
Faricimab		Genentech/Roch	All isoforms VEGF-A and Ang-2/Bispecific monoclonal Ab	No	Phase3/NCT03823287
ARP-1536		Aerpio	All isoforms of VEGF-A (inactivate), Tie2 (activate)/reactivating monoclonal antibody	No	Predinical development
OPT-302		Opthea	VEGF-C and VEGF-D/Trap' molecule (VEGF-C and VEGF-D)	No	Phase2/NCT03345082
KSI-301		Kodiak Science	All isoforms of VEGF-A/anti-VEGF antibody biopolymer conjugate	No	Phase 1/NCT04049266
Abicipar pegol		Allergan	Ankyrin repeat proteins (DARPin)Zall isoforms of anti-VEGF A	No	Phase3/NCT02462928
RGX-314		Regenxbio	All isoforms of VEGF-A/NAV AAV8 vector containing a gene encoding for a monoclonal antibody fragment	No	Phase2/NCT03066258
ADVM-022		Adverum Biotechnologies, Inc.	All isoforms of VEGF-A/AAVJmS-aflibercept	No	Phase 1/NCT03748784
PAN-90S06		Panoptica	VEGFR2/small-molecule tyrosine kinase inhibitor	No	Phase2/NCT03479372

Some additional potential treatments for dry AMD, advanced in clinical trials, are listed below. Alkeus Pharmaceuticals, Inc. propose visual cycle modifications as a treatment option (ALK001-P3001, NCT03845582), via the use of a modified form of vitamin A that replaces natural vitamin A in the body, thus slowing the production of damaging vitamin A dimers, postulated to slow the accumulation of toxic end products and therefore slow the progression of AMD (2019d). Soliris (Alexon), Genentech, and Apellis Pharmaceuticals are successfully investigating the role of complement inhibition in slowing down the progression of dry AMD. Their products, Eculizumab, Lampalizumab, and Pegcetacopan (respectfully), are currently undergoing Phase 2 and 3 clinical trials (NCT00935883, NCT03972709, NCT02247531, and NCT0350054) (Yehoshua et al., 2014; Yaspan et al., 2017; Holz et al., 2018). Additionally, anti-inflammatory agents have also been proposed to slow down dry AMD advancement. Genentech/Roche proposes the use of FHTR2163 (Genentech/Roche), a new antibody delivered by intravitreal injection that inhibits HTRA1, a serine protease gene HTRA1 as a major risk factor for wet AMD [Phase 2/NCT03972709 (Dewan et al., 2006)]. A Phase 2 clinical trial conducted by Allergen (NCT02087085) is investigating the neuroprotective role of intravitreal brimonidine for geographic atrophy, administered by a delayed-delivery system implant (2020a). Finally, Jenssen Pharmaceuticals is assessing non-stem cell-based therapy with Palucorcel (CNTO-2476), which uses human umbilical cord tissue-derived cells (hUTC), while Astellas Pharma is assessing a stem cell-based approach using human embryonic stem cells (hESC) as cell-based approach therapies to treat advanced dry stages of AMD

TABLE 2 | Advanced in trials Dry AMD treatments.

Generic	Brand name	Manufacturer	Target	FDA approved/year	Phase in trials
PRIM A FS-US		Pixium Vision	Bionic Vision	No	N/A/NCT03046407
Risuteganib	Luminate	Allegro Ophthalmic	Mitochondrial dysfunction (oxidative stress)/integrin inhibitor	No	Phase 2/NCT03626636
ALK001-P3001		Alkeus Pharmaceuticals, Inc.	Modified vitamin A decreases rate of toxic dimer formation	No	Phase 3/NCT038455B2
AAVCAGsCDSS		Hamera Biosciences	MAC inhibition via CD59/gene therapy	No	Phase1/NCT03144999
Eculizumab		Soliris, Alexon	A humanized monoclonal antibody derived from the murine anti-human C5 antibody	No	Phase2/NCT00935883
Lampalizumab		Genentech	Antigen-binding fragment (Fab) of a humanized monoclonal antibody that acts as a selective inhibitor of complement factor D	No	Phase 2/MCT03972709
Pegcetacoplan (APL-2)		Apellis Pharmaceuticals	Synthetic molecule that selectively inhibits C3, effectively downregulating all three complement pathways	No	Phase 2/NCT03500549
Avacincaptad pegol	Zirnura	Ophthotech/Iveric	Complement factor- C5 inhibitor	No	Phase 3/NCT02686658
CPCB-RPE1		Regenerative Patch Tech.	Human Embryonic Stem RPEs/RPE transplantation	No	Phase 2/NCT02590692
Brimo DDS		Allergan	Brimonidine implant/neuroprotection	No	Phase 2/NCT0208708S
CNTO-2476		Janssen Pharmaceuticals	Biological/non-stem cell-based therapy with palucorcel (CNTO-2476), which uses human umbilical cord tissue-derived cells (hUTC)	No	Phase 2/NCT01226628
hESC MA09-hRPE		Astellas Pharma Inc.	biological/sub-retinal Transplantation of hESC Derived RPE (MA09-hRPE)	No	Phase 2/NCT01344993
		Chinese Academy of Sciences	Human Embryonic Stem RPEs/RPE transplantation	No	Phase 2/NCT03046407
FHTR2163		Genentech/Roche	Antibody delivered by intravitreal injection that inhibits a serine protease gene (HTRAI)	No	Phase 2/NCT03972709

[Phase 2/NCT01226628 and NCT03046407 (Lund et al., 2007; Schwartz et al., 2015)].

CONCLUSION

As presented above, significant research is being done to investigate new therapeutics for both dry and wet AMD. The most successful therapies so far address aspects of wet AMD, leaving a large gap to be filled with therapies for dry AMD. Unfortunately, a large number of potential medications have been tested for dry AMD and have failed. Currently more candidates are undergoing clinical trials, some targeting the impact of stress on mitochondria as well as inflammation, emphasizing the importance of these pathways in the pathogenesis of AMD. Nevertheless, the very nature of the complex etiology of AMD dictates that future therapeutic protocols, will require treatments directed to more than one aspect of the pathobiology of AMD, thus advocating for additional effort to be invested in a multi-targeted approach to AMD treatment.

AUTHOR CONTRIBUTIONS

MH and GM contributed to the conceptualization, writing, and editing of this review. Both authors contributed to the article and approved the submitted version.

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