WITH THE EYES ON NON-CODING RNAS

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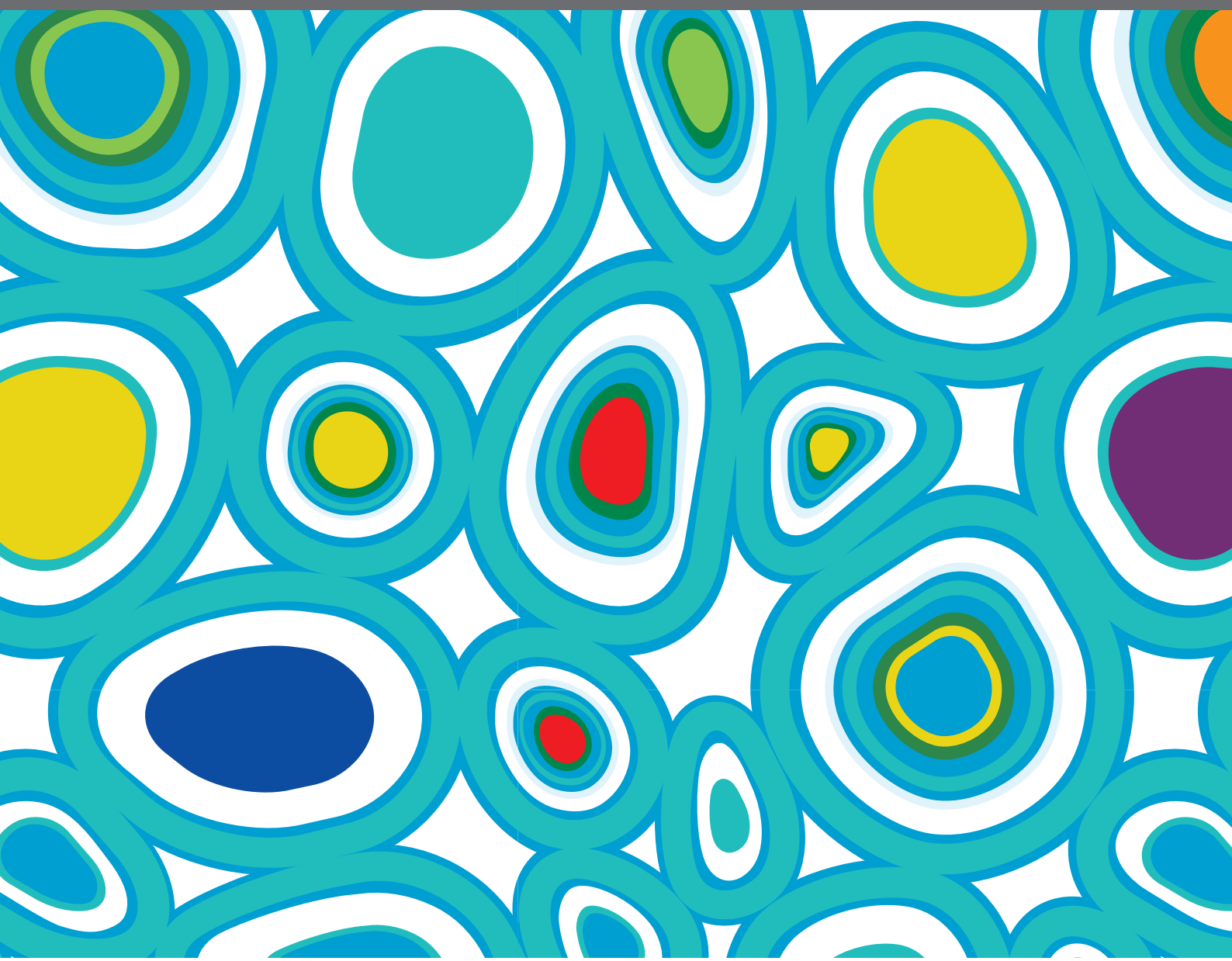

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WITH THE EYES ON NON-CODING RNAS

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EDITORIAL
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Editorial: With the Eyes on Non-coding RNAs

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Keywords: lncRNA, miRNAs, retina, visual system, eye

Editorial on the Research Topic

With the Eyes on Non-coding RNAs

Non-coding RNAs (ncRNAs) are a class of structural and regulatory RNAs that have received increasing attention because of their implication in the disease development and a variety of physiological and pathological processes. These includes tissue-specific and "housekeeping" ncRNAs, including long non-coding RNAs, microRNAs, circular-RNA, natural antisense transcripts (NATs), and several other poorly characterized ncRNAs that regulate gene expression in a variety of ways at epigenetic, chromatin remodeling, transcriptional, and translational levels. Regulatory ncRNAs exhibit dynamic temporal and spatial expression profiles in specific cellular contexts and contribute to tissue patterning and to the control of different cellular programs, including proliferation, differentiation, apoptosis, migration, and invasion. The 12 selected papers in this special Research Topic entitled "With the Eyes on Non-Coding RNAs" highlights the significant scientific breakthroughs on the physiological and pathological role of ncRNAs, offering additional insights into the challenges in understanding the functions of ncRNAs in the eye and improve the diagnostic ability, making them as targets of future novel therapies to treat ocular diseases.

The most compelling evidence for the contribution of individual miRNAs to photoreceptor cell homeostasis and functions has been well-documented by Pawlick et al. They summarize recent advances in how single miRNA can regulate hundreds of mRNAs, highlights the emerging contribution of miR-124 and the miR-183/96/182 cluster in photoreceptors in health and disease. They also discuss the experimental validation and manipulation approaches to study complex miRNA/mRNA regulatory networks in several animal models.

Focusing on the role of regulatory ncRNAs (e.g., miRNAs, lncRNAs, circular RNAs, and antisense RNAs), Carrella et al. examine emerging novel regulation and functions of these classes of ncRNAs in conferring robustness to photoreceptor development and function. First, this review examines the evidence for the role of miRNAs to photoreceptor pathophysiology and discusses the role of specific lncRNAs and circRNAs in targeting miRNAs for the proper control of photoreceptor homeostasis and function. Moreover, Carrella et al. further summarizes the current knowledge on miRNAs involvement in mitochondrial eye diseases and delve on the therapeutic implications including identification of miRNAs as biomarkers and for treatment of retinal pathologies.

Importantly, in this topic Konar et al. gave an outstanding overview about the role of specific miRNAs in the control of Müller Glia reprogramming during retina regeneration as well as putative future therapeutic targets for treatment of visual disorders and damage. In addition, they discuss about main model systems and technologies that are used to evaluate miRNAs in the retina, along with their advantages and limitations.

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miRNAs' function in peripheral tissues and cells also likely play a crucial role in keeping retina health. As reviewed by Wei et al., alteration of the expression profile and function of miRNAs induces a dysfunction of immune system leading to autoimmune disorders, which in part affect the eye. In this review, Wey et al. highlight the roles of miRNAs as main actors implicated in most representative ocular autoimmune disorders, including autoimmune uveitis, Grave's ophthalmopathy, and Sjögren's syndrome dry eye. Importantly, they discuss the potential of circulating miRNAs as biomarkers for autoimmune-mediated eye disorders, along with a future exploration of miRNA-based therapeutic approaches.

Intartaglia et al. assessed the contribution of the emerging roles of miRNAs in the function and health of the Retinal Pigment Epithelial (RPE) cells and on the future exploration of miRNA-based therapeutic approaches to counteract blinding diseases. More specifically, they highlight the crosstalk between miRNAs function and circadian regulation of phagocytosis and POS cell clearance as active part in the visual cycle and function.

In addition, six original research papers also provided evidence on the role of miRNAs involved in regulating the visual system. Fernando et al. explored the molecular pathways in miR-223 KO mice demonstrating the functional role of this miRNA in controlling inflammation in retinal and circulating immune cells during retinal degeneration. They highlight the relevance of miR-223 in physiological normal conditions and in a photo-oxidative damage model of retinal degeneration, discussing future perspectives on the relevance in defining the therapeutic utility to modulate miR-223 expression and function in retinal degenerative diseases.

Interestingly, Chen et al. reported the retinal consequences of loss of function of the most abundant circular RNA, namely Cdr1as. They carried out an extensive study demonstrating that the abundance Cdr1as is required for retinal development and maintenance and discussing future perspectives on the role of this circRNA in the visual system. In this topic focusing on the role of ncRNAs involved in regulating the differentiation and maintenance of fovea in primate's retina, Fishman et al. characterized the transcriptional landscape of the developing rhesus monkey retina and found differentially expressed miRNAs during fovea development. Notably, this study not only provides novel datasets for a more comprehensive understanding of fovea formation and function, but it led to hypothesize that dysfunction of specific miRNA could contribute to fovea disease, such as age-related degeneration and noise- or toxic fovea damage. A pioneer study aimed at defining the function of miRNAs in the in reactive Müller Glia cells was carried out by Kang et al., through the conditional inactivation of Dicer in Muller cells profiled both miRNAs and mRNAs in reactive Müller Glia cells after light damage. This study points to miRNA-based therapeutic approaches to attenuate gliosis. The study by Xu et al. provided compelling evidence for the involvement of miR-183/96/182 cluster in human cause IRD. They carried out a mutational screening that took into account the three members of the miR-183 cluster and identified six sequence variants in a large cohort of patients, three in pre-miR-182 and three in pre-miR-96 and highlighted the potential roles of these miRNAs in the susceptibility to IRDs. Finally, along a clinical investigation in identifying pathophysiological roles of ncRNAs in ocular tissues and visual function, Anand et al. constructed the miRNAs-mRNA gene regulatory networks downstream to Tdrd7, a Tudor domain protein involved in post transcription regulation of gene expression and mutated in congenital cataracts. The comprehensive dataset of miRNAs and mRNA identified in the study point to novel pathways involved in maintaining lens function and homeostasis throughout life.

To conclude, this Research Topic summarized the current information on the relevance of ncRNAs in visual system. The number and selection of papers considered within this Research Topic demonstrate the complex, multilevel activity of ncRNAs in the pathogenesis of both monogenic and complex genetic disorders and summarizes the current effort to improve diagnosis and develop treatments to eye pathologies.

AUTHOR CONTRIBUTIONS

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ORIGINAL RESEARCH
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MicroRNA-223 Regulates Retinal Function and Inflammation in the Healthy and Degenerating Retina

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Introduction: MicroRNAs (miRNAs) are small, non-coding RNA molecules that have powerful regulatory properties, with the ability to regulate multiple messenger RNAs (mRNAs) and biological pathways. MicroRNA-223-3p (miR-223) is known to be a critical regulator of the innate immune response, and its dysregulation is thought to play a role in inflammatory disease progression. Despite miR-223 upregulation in numerous neurodegenerative conditions, largely in cells of the myeloid lineage, the role of miR-223 in the retina is relatively unexplored. Here, we investigated miR-223 in the healthy retina and in response to retinal degeneration.

Methods: miR-223-null mice were investigated in control and photo-oxidative damage-induced degeneration conditions. Encapsulated miR-223 mimics were intravitreally and intravenously injected into C57BL/6J wild-type mice. Retinal functional responses were measured using electroretinography (ERG), while extracted retinas were investigated by retinal histology (TUNEL and immunohistochemistry) and molecular analysis (qPCR and FACS).

Results: Retinal function in miR-223$^{-/-}$ mice was adversely affected, indicating that miR-223 may be critical in regulating the retinal response. In degeneration, miR-223 was elevated in the retina, circulating serum, and retinal extracellular vesicles. Conversely, retinal microglia and macrophages displayed a downregulation of miR-223. Further, isolated CD11b^{+} inflammatory cells from the retinas and circulation of miR-223-null mice showed an upregulation of pro-inflammatory genes that are critically linked to retinal inflammation and progressive photoreceptor loss. Finally, both local and systemic delivery of miR-223 mimics improved retinal function in mice undergoing retinal degeneration.

Conclusion: miR-223 is required for maintaining normal retinal function, as well as regulating inflammation in microglia and macrophages. Further investigations are

required to determine the targets of miR-223 and their key biological pathways and interactions that are relevant to retinal diseases. Future studies should investigate whether sustained delivery of miR-223 into the retina is sufficient to target these pathways and protect the retina from progressive degeneration.

Keywords: microRNA-223, retinal degeneration, macrophage, neuroinflammation, retinal function, photoreceptor cell death, microglia, microRNA

INTRODUCTION

Immune system dysregulation is a critical process involved in the onset and progression of retinal degenerative diseases (Ambati et al., 2013), including age-related macular degeneration (AMD), retinitis pigmentosa (RP), and diabetic retinopathy (DR). Subtle but chronic changes within the retinal microenvironment, such as the steady build-up of reactive oxygen species (ROS), can lead to a larger inflammatory response building in the retina (Xu et al., 2009). Microglia, the resident immune cells of the central nervous system (CNS), migrate to the site of injury or degeneration, where they may also recruit blood-borne macrophages (Penfold et al., 2001; Karlstetter et al., 2015), leading to cytokine secretion, complement system activation, and phagocytosis of degenerating and living photoreceptors (Zhao et al., 2015; Akhtar-Schafer et al., 2018; Silverman and Wong, 2018; Wooff et al., 2019). Understanding how abnormal immune responses can be controlled is therefore paramount in order to develop effective treatments for reducing inflammation in retinal diseases.

MicroRNAs (miRNAs) are highly conserved, small non-coding RNA molecules that are approximately 22 nucleotides in length that can regulate specific gene targets. By binding to the 3′ untranslated region (3′ UTR) of a messenger RNA (mRNA), the mRNA is targeted for translational repression or degradation (reviewed in Bartel, 2004). A single miRNA has the ability to regulate the expression of hundreds of mRNAs due to seed sequence similarity in the 3′ UTRs of many mRNAs (Bartel, 2004). Often, many targets of a single miRNA exist within associated or the same biological pathways; hence, miRNAs can be powerful regulators (Bartel, 2004). The immune system is extensively modulated by miRNA signaling, with many miRNAs well characterized to participate in inflammatory regulation (reviewed in O'Connell et al., 2012). Specifically within the retina, miRNAs are known to play a major role in the development and disease of the photoreceptors, bipolar cells, and Müller cells (Zuzic et al., 2019), including the regulation of retinal inflammation (Anasagasti et al., 2018; Chu-Tan et al., 2018).

In a systematic review of studies on miRNAs and neurodegenerative diseases (including Alzheimer's disease, multiple sclerosis, Parkinson's disease, amyotrophic lateral sclerosis, and AMD), miR-223-3p was identified as one of the top miRNAs upregulated in diseased conditions, alongside miR-146a-5p and miR-155-5p (Juźwik et al., 2019). Although both mature strands of miR-223 (miR-223-5p and miR-223-3p) are functional and each regulates a different set of mRNAs, miR-223-3p appears to be heavily associated with neurodegeneration (Juźwik et al., 2019). Therefore, from here on, miR-223-3p will be referred to as miR-223 and is the focus of this study. miR-223 is produced primarily within cells of the hematopoietic system, with the highest levels of expression observed in myeloid cells, particularly neutrophils and macrophages (reviewed in Haneklaus et al., 2013; Yuan et al., 2018). miR-223 is thought to be expressed by a "myeloid-like" gene under tight regulation during granulopoiesis (Fukao et al., 2007). In its mature form, miR-223 is known to regulate immune cell functions through several proposed mechanisms, including myeloid activation and regulation (Fazi et al., 2005; Gilicze et al., 2014; Galloway et al., 2019), NLRP3 inflammasome suppression (Bauernfeind et al., 2012; Yang et al., 2015; Neudecker et al., 2017b), IGF1R signaling (Qadir et al., 2015), cathepsin activity (Gantier, 2013), suppression of chemokines (Dorhoi et al., 2013), downregulation of the NF-κB pathway (Zhou et al., 2018), and alteration of JAK/STAT signaling (Chen et al., 2012). In the CNS, miR-223 may be a circulating biomarker in stroke (Wang et al., 2014) and multiple sclerosis (Fenoglio et al., 2013), and is also elevated in the plasma (Ertekin et al., 2014) and in peripheral blood cells (Litwinska et al., 2019) of neovascular AMD patients. In addition, exosomal miR-223 has been demonstrated to play an important role in cell signaling in stroke and dementia (Chen et al., 2017; Wei et al., 2018).

In the retina, elevated levels of miR-223 have also been found in models of experimental autoimmune uveoretinitis (EAU) (Watanabe et al., 2016), Müller cell disruption (Chung et al., 2016), amyloid-beta-induced retinal degeneration (Huang et al., 2017), optic nerve crush injury (Fuller-Carter et al., 2015), and in photo-oxidative damage (Saxena et al., 2015). However, the role that miR-223 plays in the healthy retina and in disease progression is unknown. Here, we conducted an exploratory investigation into the role of miR-223 in the retina under normal conditions and in a photo-oxidative damage model of retinal degeneration (Natoli et al., 2016a), using miR-223-null mice. We demonstrate that miR-223 has an important role in maintaining normal retinal function. We also show that miR-223 may modulate inflammation in retinal and circulating immune cells during disease.

MATERIALS AND METHODS

Animal Handling

All experiments were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and with approval from the Australian National University (ANU) Animal Experimentation Ethics

Committee (Ethics ID: A2017/41). Adult wild-type (WT) C57BL/6J mice (ANU Phenomics Facility) and miR-223-null mutant mice (miR-223$^{-/-}$, B6.Cg-*Ptprc*^{a} *Mir223*^{tm1Fcam}/J, #013198, Jax Laboratories) were utilized for all experiments. Animals were born and reared under normal lighting conditions (12:12-h light to dark cycle of ∼5 lux light) and were aged at 50–70 post-natal days at the time of use. All animals were screened for *Crb1*^{rd8}, an inherited retinal degeneration present in some commercial lines, using primers described previously (Mattapallil et al., 2012) and were deemed free of this mutation (ANU Phenomics Facility and JCSMR Biomolecular Resource Facility). Sample sizes (N) are included in each figure legend.

Separation of Retinal Microglia and Macrophages by FACS

We employed a fate-mapping strategy for the separation of retinal microglia and recruited monocyte-derived macrophages (CX3CR1^{+}) by using fluorescence-activated cell sorting (FACS), according to a previously described model by O'Koren et al. (2016). In brief, tamoxifen (Sigma Aldrich, St. Louis, MO, United States) was dissolved in corn oil at 37°C at a concentration of 20 mg/ml. P60 CX3CR1$^{YFP-CreER/wt}$:R26^{RFP} mice (ANU Phenomics Facility) were intraperitoneally (IP) injected with tamoxifen (75 mg/kg) twice, 24 h apart, to induce Cre recombinase and RFP expression in all CX3CR1^{+} cells. Following a "wash out" period of 60 days, mice were exposed to 5 days of photo-oxidative damage. In this model, the "wash out" period allows for RFP expression to be lost in circulating monocytes due to turnover, but RFP expression to be retained by long-lived resident microglia (O'Koren et al., 2016). Following photo-oxidative damage, CX3CR1$^{YFP-CreER/wt}$:R26^{RFP} retinas were collected via corneal incision and digested using mechanical dissociation and papain digestion (LS003126; Worthington Biochemicals, Lakewood, NJ, United States), as described previously (Mulfaul et al., 2020; Wooff et al., 2020b). YFP^{+} microglia/macrophages were FACS-isolated based on relative expression of RFP (BD FACS Aria III; JCSMR Imaging and Cytometry Facility). Microglia are YFP^{+}RFP^{+}, whereas recruited macrophages are YFP^{+} only.

Blood Collection and Preparation for FACS

Peripheral blood was collected to measure miR-223 in circulating serum and to isolate CD11b^{+} cells for examining their inflammatory status. A sub-mandibular blood collection method (Golde et al., 2005) was used to collect blood from the submandibular vein using a lancet (Goldenrod animal lancet 4 mm point length; MEDIpoint Inc, Mineola, NY, United States). To collect serum for miRNA extraction, 200 μl peripheral blood was collected into 1.5 ml Eppendorf tubes and left at room temperature for 30 min to clot. Samples were centrifuged at 1500 g for 10 min at 4°C, following which the separated serum was used for miRNA extraction.

For FACS isolation of CD11b^{+} cells, peripheral blood was collected into Microtainer MAP microtubes containing K2EDTA (#363706; BD, Franklin Lakes, NJ, United States); 100 μl of each blood sample was mixed with 900 μl of distilled water for exactly 20 s (to initiate lysis of erythrocytes) and then lysis was stopped with 100 μl of 10× phosphate-buffered saline (PBS). Samples were spun at 500 *g* for 5 min at 4°C, and the pellets were resuspended in 900 μl of distilled water for exactly 20 s, before adding 100 μl of 10× PBS and centrifuging at 500 *g* for 5 min at 4°C. Pellets were resuspended and stained using a PE anti-mouse/human CD11b antibody (1:500 in 1× PBS, clone M1/70, #101207; BioLegend, San Diego, CA, United States). After staining for 40 min, samples were sorted by FACS (BD FACS Aria III; JCSMR Imaging and Cytometry Facility) into 1× PBS, which was replaced by TRIzol (Thermo Fisher Scientific) for RNA extraction.

Delivery of miR-223 Mimics

To achieve local and systemic transfection of synthetic miR-223 mimics, a miR-223-3p mimic (#MC12301, hsa-miR-223-3p; Thermo Fisher Scientific, Waltham, MA, United States) and a negative control mimic (#4464058; Thermo Fisher Scientific) were each encapsulated in Invivofectamine 3.0 (#IVF3001; Thermo Fisher Scientific) and sterile endotoxin-free 1× PBS (pH 7.4, Thermo Fisher Scientific) according to our previously published methods (Chu-Tan et al., 2020).

Wild-type animals were anesthetized using a mixture of Ketamine (100 mg/kg body weight; Troy Laboratories, Glendenning, NSW, Australia) and Ilium Xylazil-20 (12 mg/kg body weight; Troy Laboratories), delivered through IP injection. For retinal transfection, encapsulated mimics were intravitreally (IVT) injected at 1 μg/μl (1 μg per eye), according to our previously described methods (Chu-Tan et al., 2020). Injections were performed 3 h prior to photo-oxidative damage, with our previous study indicating that retinal transfection is effective for 3–4 days post-injection and can be detected in all layers of the retina (Chu-Tan et al., 2020).

For systemic intravenous (IV) delivery of mimics, tail vein injections were performed on restrained mice with the aid of a heat lamp. Each mouse received 0.5 mg/kg of encapsulated mimic, as recommended by the manufacturer. Injections were performed at 2 days into a 5-day photo-oxidative damage paradigm, as mimics may have a shorter half-life in circulation.

Photo-Oxidative Damage

To induce retinal degeneration using photo-oxidative damage (PD), animals were subject to continuous white LED light exposure at 100 K lux for a period of either 1, 3, 5, or 7 days, according to our previously described pigmented mouse model (Natoli et al., 2016a). Animals were administered pupil dilator eye drops twice daily (1% w/v Minims atropine sulfate; Bausch and Lomb, Garden City, NY, United States). Dim-reared control animals with no photo-oxidative damage (12:12-h light to dark cycle of ∼5 lux light) were used for comparison.

Electroretinography

Electroretinography (ERG) was used to measure retinal function in mice in response to full-field flash stimuli under scotopic conditions (with dark adaptation for approximately 16 h). A single-flash paradigm was used to elicit mixed (rod and cone)

responses, over an intensity range of -2.0 to 1.6 log cd.s/m^2 using the Celeris full-field ERG system (Diagnosys LLC, Lowell, MA, United States). Measurements of the amplitudes of the a-wave (photoreceptor activity) and b-wave (ON-bipolar and Müller cell activity) were performed as an assessment of retinal function using Espion V6 software (Diagnosys LLC).

Histological Analysis of Retinal Cryosections

Following euthanasia of the animal using carbon dioxide, whole eyes were enucleated and cryosectioned at 12 μm in the parasagittal plane for histological analysis (CM1850; Leica, Wetzlar, Germany). To detect photoreceptor cell death in the outer nuclear layer (ONL), the terminal deoxynucleotidyl transferase (Tdt) dUTP nick end labeling (TUNEL) assay (Roche Diagnostics, Basel, Switzerland) was used on retinal cryosections, according to previously described protocols (Maslim et al., 1997; Natoli et al., 2010). Sections were counterstained using a DNA label (Bisbenzimide or Hoechst's stain, DAPI, 1:10,000; Sigma Aldrich) for visualization of the cellular layers and the retinal pigment epithelium (RPE).

For immunohistochemistry in retinal cryosections, antigen retrieval (ImmunoSolutions, Brisbane, QLD, Australia) was performed prior to incubation with primary antibodies. To visualize the migration and recruitment of microglia and macrophages to the outer retina, a primary antibody against ionized calcium-binding adaptor molecule 1 (rabbit α-IBA1, 1:500, #019-19741; Wako, Osaka, Japan) was used according to previously described protocols (Rutar et al., 2011b, 2015). A primary antibody against glial fibrillary acidic protein (rabbit α-GFAP, 1:500, #Z0334; Dako, Agilent, Santa Clara, CA, United States) was used to determine reactive gliosis, with staining localized to Müller cells and astrocytes. Sections were counterstained with a DNA label (DAPI) for visualization. Negative controls were incubated with no primary antibody.

$TUNEL^+$ photoreceptors and $IBA1^+$ cell counts in the outer retina (ONL-RPE) were performed along the full length of retinal cryosections (supero-inferior) in duplicate. The number of rows of photoreceptor cell nuclei was quantified in the superior retina (approximately 1 mm superior to the optic nerve) to determine ONL thinning, as described previously (Fernando et al., 2016). For each section, three measurements were taken, and sections were counted in duplicate.

For GFAP staining, immunofluorescence was quantified in each cryosection using two methods. Firstly, the ratio of the GFAP staining area to the whole retinal area was taken. Each image was binarized in ImageJ (NIH, Bethesda, MD, United States). The threshold function was then used to isolate the stained area. The area of GFAP staining, as well as the whole retinal area between the inner limiting membrane (ILM) and the outer limiting membrane (OLM), as observed through DAPI staining of the cellular layers, was graphed as a ratio. This represents GFAP staining in Müller cells and astrocytes in the whole retina. Secondly, as GFAP staining is localized to both Müller cells and astrocytes located in the inner retina, GFAP levels can be very intense between the ILM and the ganglion cell layer (GCL). Fluorescence intensity in the other layers of the retina was determined as an indication of GFAP staining in the Müller cell processes. This was performed by determining the intensity of staining in all layers between the inner plexiform layer (IPL) and the OLM in ImageJ. This represents GFAP staining in the Müller cell processes through all layers between the IPL and the OLM in the retina.

Primary Cell Cultures

Primary retinal $CD11b^+$ microglia were isolated from dim-reared mouse retinas and sorted by FACS (BD FACS Aria III; JCSMR Imaging and Cytometry Facility) into a 48-well plate at ∼1500 cells per well, as previously described (Mulfaul et al., 2020; Wooff et al., 2020b). Cells were stained with a PE anti-mouse/human CD11b antibody (clone M1/70, #101207; BioLegend) for 40 min prior to FACS. Isolated primary microglia were cultured for 4 weeks in Dulbecco's Modified Eagle Medium (DMEM)-F12 (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), 1% antibiotic-antimycotic (Thermo Fisher Scientific), 3% L-glutamine (Thermo Fisher Scientific), 0.25 ng/ml GM-CSF (Stem Cell Technologies, Vancouver, BC, Canada), and 2.5 ng/ml M-CSF (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described (Mulfaul et al., 2020; Wooff et al., 2020b).

Primary bone marrow-derived macrophages (BMDMs) were extracted from the hind leg bone marrow of wild-type dim-reared mice and were cultured for 6 days in DMEM (high glucose with sodium pyruvate; Thermo Fisher Scientific) supplemented with 10% FBS (Sigma Aldrich), 1% L-glutamine (Thermo Fisher Scientific), 30% L929-conditioned media, 1% penicillin and streptomycin (Thermo Fisher Scientific), and 1% non-essential amino acids (Thermo Fisher Scientific), as described previously (Man et al., 2016; Fox et al., 2020). Cells were seeded at 0.5×10^6 cells/well in a 24-well plate in antibiotic-free media 24 h prior to experimentation.

Immortalized Cell Cultures

Immortalized cell lines for photoreceptor-like cells [mouse 661W; Dr. Muayyad R. Al-Ubaidi, University of Oklahoma Health Sciences Centre, Oklahoma City, OK, United States (al-Ubaidi et al., 1992)], RPE-like cells [human ARPE-19, CRL-2302; ATCC, Manassas, VA, United States (Dunn et al., 1996)], microglia-like cells [mouse C8B4, CRL-2540; ATCC (Alliot et al., 1996)], and Müller-like cells [human MIO-M1; Moorfield's Institute of Ophthalmology, London, United Kingdom (Limb et al., 2002)] were also used in this study. Immortalized cell lines were authenticated by CellBank (Sydney, Australia) and were cultured with DMEM (high glucose with sodium pyruvate; Thermo Fisher Scientific) supplemented with 10% FBS (Sigma Aldrich), 1% antibiotic-antimycotic (Thermo Fisher Scientific), and 3% L-glutamine (Thermo Fisher Scientific). Cells were incubated at 37°C in a humidified atmosphere (5% CO_2).

Cells were seeded in 24-well plates at varying densities (661W at 2.5×10^4 cells/well, ARPE19 at 10×10^4 cells/well, C8B4 at 15×10^4 cells/well and MIO-M1 at 2×10^4 cells/well) and were incubated in reduced serum (1% FBS) DMEM media

with additives, as described above, for 24 h prior to the start of experiments.

In vitro Stimulation for Oxidative Stress and Inflammation

661W photoreceptor-like cells were stimulated with 15,000 lux white light exposure for 5 h, according to previously described protocols (Natoli et al., 2016b). Cells with no light exposure were used as unstimulated controls. C8B4 cells, primary retinal CD11b$^+$ microglia, and primary BMDMs were stimulated with 20 ng/ml lipopolysaccharide (LPS from *E. coli* 0111:B4, #N4391; Sigma Aldrich) for 4 h, followed by 5 mM adenosine triphosphate (ATP, #A6419; Sigma Aldrich) for 0.5 h. MIO-M1 cells were stimulated with 10 ng/ml TNF-α (#210-TA; R&D Systems, Minneapolis, MN, United States) for 24 h, followed by 5 mM ATP for 0.5 h. ARPE19 cells were stimulated with 50 ng/ml recombinant human interleukin-1α (IL-1α) protein (#ab9615; Abcam, Cambridge, United Kingdom) for 24 h, followed by 5 mM ATP for 0.5 h. Unstimulated cells were used as controls for comparison within each cell type. For sample collection, cells were washed in 1× PBS, then lysed and homogenized in TRIzol reagent (Thermo Fisher Scientific), prior to RNA extraction.

Isolation of Retinal Extracellular Vesicles

For molecular analysis, retinas were excised using corneal incision. For RNA extraction from retinal extracellular vesicles, each sample contained two pooled retinas (from one mouse). Isolation of small-medium extracellular vesicles (s-mEVs) from papain-digested retinas was performed according to previously published methodology (Wooff et al., 2020a) and in accordance with the MISEV2018 guidelines (Théry et al., 2018). Characterization of the properties of isolated retinal s-mEVs (which include exosomes) has been published, detailing transmission electron microscopy, size distribution analysis, and western blot analysis of these s-mEVs (Wooff et al., 2020a).

qPCR for miR-223 and Inflammatory Genes

Isolated retinas, serum, retinal s-mEVs, and cell lysates were processed for RNA extraction using a combination of TRIzol (Thermo Fisher Scientific) and a RNAqueous Micro Total RNA Isolation Kit (Thermo Fisher Scientific), or a *mir*Vana miRNA Isolation Kit (Thermo Fisher Scientific), according to the manufacturer's protocol and our previously described protocols (Natoli et al., 2008). RNA samples were tested for purity and concentration using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, United States; JCSMR Biomolecular Resource Facility). Samples were stored at -80°C.

cDNA was prepared from 250 to 500 ng RNA (dependent on sample type) using a Tetro cDNA Synthesis Kit (Bioline, London, United Kingdom), according to the manufacturer's protocol. For miRNA conversion to cDNA, a TaqMan miRNA Reverse Transcription Kit (Thermo Fisher Scientific) was used with specific RT primers from TaqMan miRNA assays (refer to **Table 1**).

TABLE 1 | TaqMan assays used for qPCR (Thermo Fisher Scientific).

Gene symbol	Gene name	Catalog number
C1qa	Complement component 1, q subcomponent, alpha polypeptide	Mm00432142_m1
C3	Complement component 3	Mm00437858_m1
Ccl3	Chemokine (C-C motif) ligand 3	Mm00441259_g1
Ctse	Cathepsin E	Mm00456010_m1
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1
Il-1β	Interleukin-1β	Mm00434228_m1
Nlrp3	NLR family pyrin domain containing 3	Mm00840904_m1
Stat3	Signal transducer and activator of transcription 3	Mm01219775_m1
miR-223	miR-223-3p	#002295
U6	U6 snRNA	#001973

Quantitative real-time polymerase chain reaction (qPCR) was performed to measure gene and miRNA expression using a combination of cDNA, TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) and TaqMan assays (refer to **Table 1**) according to the manufacturer's protocol. qPCR reactions were performed in duplicate in a 384-well plate format and run using a QuantStudio 12K Flex instrument and software (Thermo Fisher Scientific; JCSMR Biomolecular Resource Facility). Analysis was performed using the comparative cycle threshold (CT) method ($\Delta\Delta$CT), which was normalized to the *Gapdh* reference gene. miR-223 expression was normalized to U6 expression.

Genes for pro-inflammatory panels were selected on the following basis: (1) *Ccl3*, *Nlrp3,* and *Stat3* are validated targets of miR-223-3p (Bauernfeind et al., 2012; Chen et al., 2012; Dorhoi et al., 2013) and are known to be involved in retinal degenerative diseases (Doyle et al., 2012; Kohno et al., 2014; Chen et al., 2016, 2019; Fernando et al., 2016; Wooff et al., 2020b); (2) we have shown that *C1qa*, *C3,* and *Il-1*β are all critically linked to retinal disease progression in photo-oxidative damage (Natoli et al., 2017a,b; Jiao et al., 2018); and (3) *Ctse* is a predicted target of miR-223-3p and is involved in key inflammatory cell functions (Gantier, 2013).

Imaging and Statistical Analysis

Fluorescence labeling of retinal cryosections was visualized and images were acquired using either an LSM800 confocal microscope and software (ZEISS, Oberkochen, Germany) or a Nikon A1$^+$ confocal microscope and software (Nikon, Tokyo, Japan). Images were processed using ImageJ. All graphing and statistical analyses were performed using Prism 6 (GraphPad Software, La Jolla, CA, United States). Significance testing was performed using unpaired Student's *t*-tests ($P < 0.05$). Significance testing of ERG datasets was analyzed using two-way analysis of variance (ANOVA) with *post hoc* Sidak's multiple comparisons test to determine statistical significance ($P < 0.05$). Graphs were generated showing mean ± SEM values.

RESULTS

miR-223 Signaling in the Normal Retina

We sought to determine whether miR-223 plays a regulatory role in the retina under normal conditions. We used dim-reared adult miR-223-null (miR-223$^{-/-}$) mice that were confirmed to have decreased miR-223 expression, compared to wild-type (WT) age-matched dim-reared control mice (**Figure 1A**, $P < 0.05$). When investigating the retinal response, miR-223$^{-/-}$ animals demonstrated a significantly reduced ERG a-wave (**Figure 1B**, $P < 0.05$), indicating less photoreceptor function, and a significantly decreased ERG b-wave (**Figure 1C**, $P < 0.05$), indicative of reduced ON-bipolar and Müller cell activity. This indicates that an absence of miR-223 may contribute to disruptions in normal retinal function. We investigated reactive gliosis by staining for the marker GFAP, which showed no significant change in the intensity or area of GFAP staining between miR-223$^{-/-}$ and WT groups (**Figures 1D–F**, $P > 0.05$). Although miR-223$^{-/-}$ retinas showed significantly reduced TUNEL^{+} photoreceptors and IBA1^{+} cells in the outer retina compared to WT controls (**Figure 1G**, $P < 0.05$), overall miR-223$^{-/-}$ retinas showed more cumulative photoreceptor layer thinning (**Figure 1H**, $P < 0.05$). qPCR for a suite of pro-inflammatory cytokines, complement components, and innate immune system contributors [several of which are targets of miR-223 regulation (Bauernfeind et al., 2012; Chen et al., 2012; Dorhoi et al., 2013; Gantier, 2013)] was performed on whole retinal tissue. It was demonstrated that there was a small but significant decrease in complement component 1qa (*C1qa*) and significantly increased expression of cathepsin E (*Ctse*) in miR-223$^{-/-}$ retinas (**Figure 1I**, $P < 0.05$).

miR-223 Production in Retinal Damage

miR-223 is thought to be primarily produced by cells of the myeloid lineage in many tissues, particularly in neutrophils and macrophages (Haneklaus et al., 2013). However, its expression has not yet been characterized in retinal degenerative conditions. We investigated the expression of miR-223 using a photo-oxidative damage model of retinal degeneration, in which central, focal photoreceptor degeneration and loss of retinal function are elicited (Natoli et al., 2016a). Using this model, we have demonstrated that the presence of outer retinal microglia and macrophages within the photoreceptor layer and subretinal space is critically linked with progressive degeneration and lesion expansion (Natoli et al., 2017a). Here, we showed that following 3–7 days of photo-oxidative damage, levels of miR-223 are substantially increased in WT retinas compared to dim-reared WT control retinas (**Figure 2A**, $P < 0.05$). There was a ∼100% increase at 5 days light exposure, which is concurrent with a peak in cell death and outer retinal IBA1^{+} microglia and macrophages in this model (Natoli et al., 2016a).

We then sought to determine the modulation in expression levels of miR-223 within retinal cell types both *in vivo* and *in vitro*. As miR-223 is thought to be primarily expressed by cells of the myeloid lineage (Haneklaus et al., 2013), we used a fate-mapping strategy that distinguishes resident microglia from recruited macrophages (O'Koren et al., 2016), as these are the main classes of immune cells present in the degenerating retina. Microglia and macrophages from photo-oxidative damaged retinas were compared to dim-reared control microglia/macrophages. This reference control population contains primarily microglia, as the retina contains few macrophages without damage (Natoli et al., 2017a). On a per-cell basis, FACS-isolated microglia (YFP^{+}RFP^{+}) and macrophages (YFP^{+}) both have reduced miR-223 levels at 7 days photo-oxidative damage, compared to dim-reared control cells (**Figure 2B**, $P < 0.05$). Further, resident microglia isolated from 7-day photo-oxidative damaged retinas showed a larger decrease in miR-223 than recruited macrophages at the same time point (**Figure 2B**).

In vitro, stimulated cultures of microglia and macrophages (C8B4 microglia, retinal CD11b^{+} cells and BMDMs) all showed reduced miR-223 compared to unstimulated controls (**Figure 2C**), with BMDMs showing a significant change ($P < 0.05$). Müller cell cultures (MIO-M1) also showed a significant decrease in miR-223 under stimulated conditions (**Figure 2C**, $P < 0.05$), whereas ARPE-19 cells showed no change in miR-223 expression. Interestingly, a significant increase in miR-223 was detected in 661W photoreceptor-like cells (**Figure 2C**, $P < 0.05$) after stimulation with light. However, when investigating the CT values of miR-223 expression between cell types in both unstimulated and stimulated conditions, only microglia and macrophages showed robust expression of miR-223 (lower CT values for miR-223, **Figure 2D**). MIO-M1, ARPE-19, and 661W cells demonstrated only low miR-223 expression (high CT values of 34–36, **Figure 2D**). Therefore, in this *in vitro* comparison, microglia and macrophages were heavily abundant in miR-223 compared to Müller, RPE, and photoreceptor cell types.

Taken together, these data indicate that retinal miR-223 increases in photo-oxidative damage. Although microglia and macrophages are likely to be the primary producers of miR-223 in the retina, their expression of miR-223 decreases on a per-cell basis in retinal degeneration. Photoreceptors may also contribute towards an increase in miR-223 levels seen in retinal degeneration.

The Role of miR-223 in Retinal Degeneration

As miR-223 levels were shown to peak at 5 days during photo-oxidative damage (**Figure 2A**), we next looked at the role of miR-223 in retinal degeneration at this time point using miR-223$^{-/-}$ mice and age-matched WT control mice. Again, we confirmed a decrease in miR-223 expression in miR-223$^{-/-}$ animals compared to WT controls (**Figure 3A**, $P < 0.05$). However, after 5 days of photo-oxidative damage, there was no difference in the ERG a-wave between groups (**Figure 3B**, $P > 0.05$), unlike dim-reared controls. Earlier, it was shown that dim-reared miR-223$^{-/-}$ animals already had a lower a-wave than WT controls (**Figure 1B**). The data shown in **Figure 3B** indicate that after damage, WT animals had a faster decline in the a-wave response compared to miR-223$^{-/-}$ animals. However,

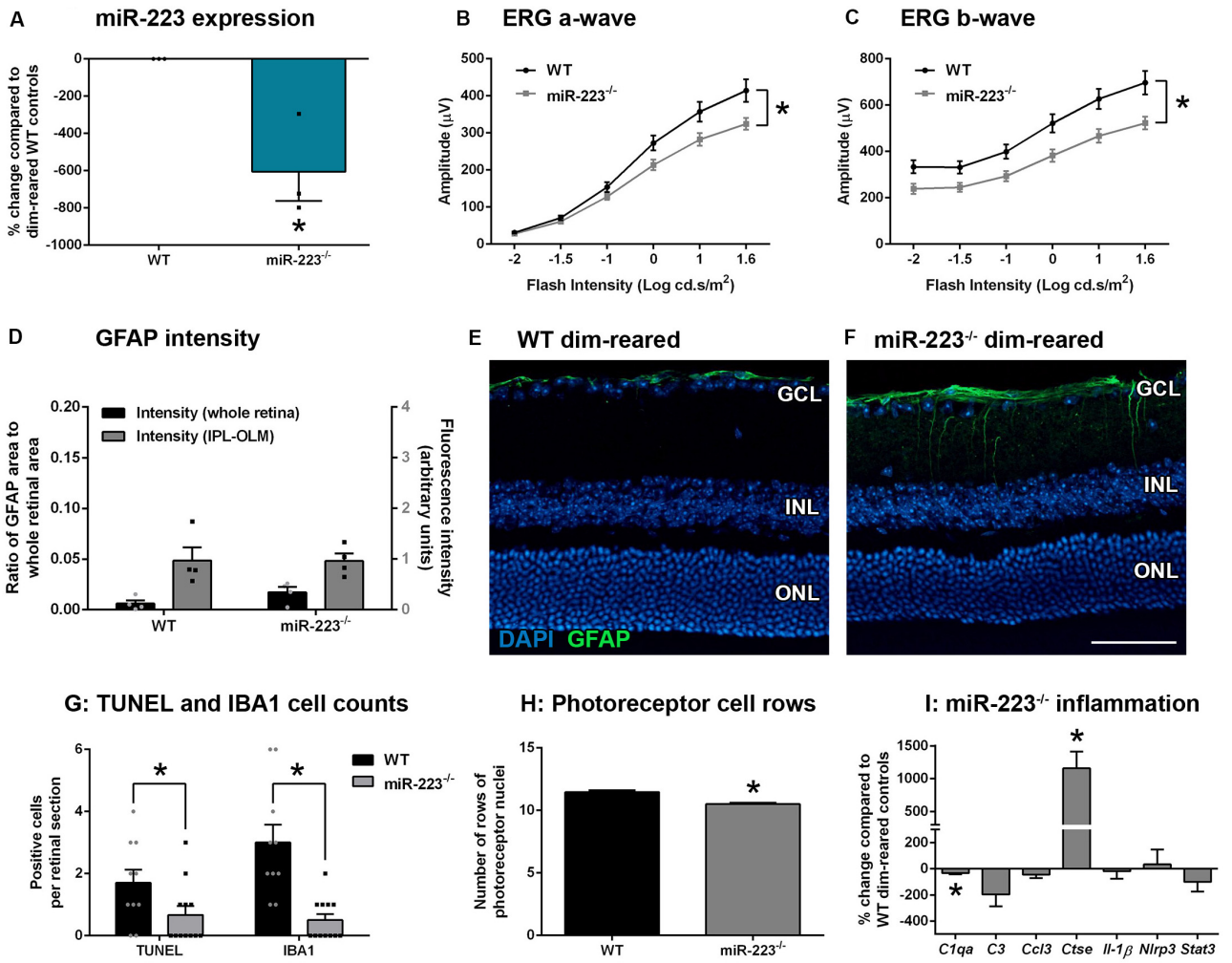

FIGURE 1 | Retinas of wild-type (WT) and miR-223$^{-/-}$ mice under normal, dim-reared conditions. **(A)** miR-223$^{-/-}$ mice were confirmed to have a significant reduction in retinal miR-223 expression compared to WT mice ($P < 0.05$). **(B,C)** Electroretinography (ERG) of the a-wave **(B)** and b-wave **(C)** demonstrated that miR-223$^{-/-}$ mice had reduced retinal function compared to WT controls ($P < 0.05$). **(D–F)** No significant differences in GFAP levels (green) were observed between groups ($P > 0.05$). Units for whole retinal GFAP on left *Y*-axis (black) and units for IPL to OLM GFAP on right *Y*-axis (gray). **(G)** TUNEL^{+} and IBA1^{+} cells were reduced in the outer retina of miR-223$^{-/-}$ mice compared to WT controls ($P < 0.05$). **(H)** However, miR-223$^{-/-}$ retinas had more photoreceptor layer thinning than WT retinas ($P < 0.05$). **(I)** *C1qa* and *Ctse* retinal expressions were significantly changed in miR-223$^{-/-}$ retinas compared to WT controls ($P < 0.05$). Expression of *C3*, *Ccl3*, *Il-1*β, *Nlrp3,* and *Stat3* were unchanged ($P > 0.05$). IPL, inner plexiform layer; OLM, outer limiting membrane; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar is 50 μm. Sample size is $N = 3$–6. *Denotes significance ($P < 0.05$).

there was still a significant decrease in the ERG b-wave in miR-223$^{-/-}$ animals compared to WT controls after photo-oxidative damage (**Figure 3C**, $P < 0.05$), consistent with the change in the b-wave in dim conditions (**Figure 1C**).

An assessment of reactive gliosis using the GFAP marker demonstrated that there was a significant increase in GFAP intensity in the Müller cell processes (all layers between the IPL and OLM) in miR-223$^{-/-}$ retinas compared to WT controls (**Figures 3D–F**, $P < 0.05$). However, the area of GFAP staining was unchanged between WT and miR-223$^{-/-}$ in whole retinas (**Figure 3D**, $P > 0.05$). Although there was no difference in TUNEL^{+} photoreceptors undergoing cell death (**Figure 3G**, $P > 0.05$), cumulatively there were more rows of photoreceptor cells present in the miR-223$^{-/-}$ retinas compared to WT controls, indicating lower levels of photoreceptor loss in the knockout animals (**Figure 3H**, $P < 0.05$). Upon investigating the inflammatory status of the retina, it was found that there were significantly less IBA1^{+} microglia and macrophages in the outer retinas of miR-223$^{-/-}$ mice compared to WT controls (**Figures 3J–L**, $P < 0.05$). In WT animals, there were more IBA1^{+} cells present closer to the photoreceptor layer (**Figure 3K**), whereas in miR-223$^{-/-}$ animals, these were situated closer to the RPE and were fewer in number (**Figure 3L**). qPCR for a range of pro-inflammatory cytokines, complement components, and

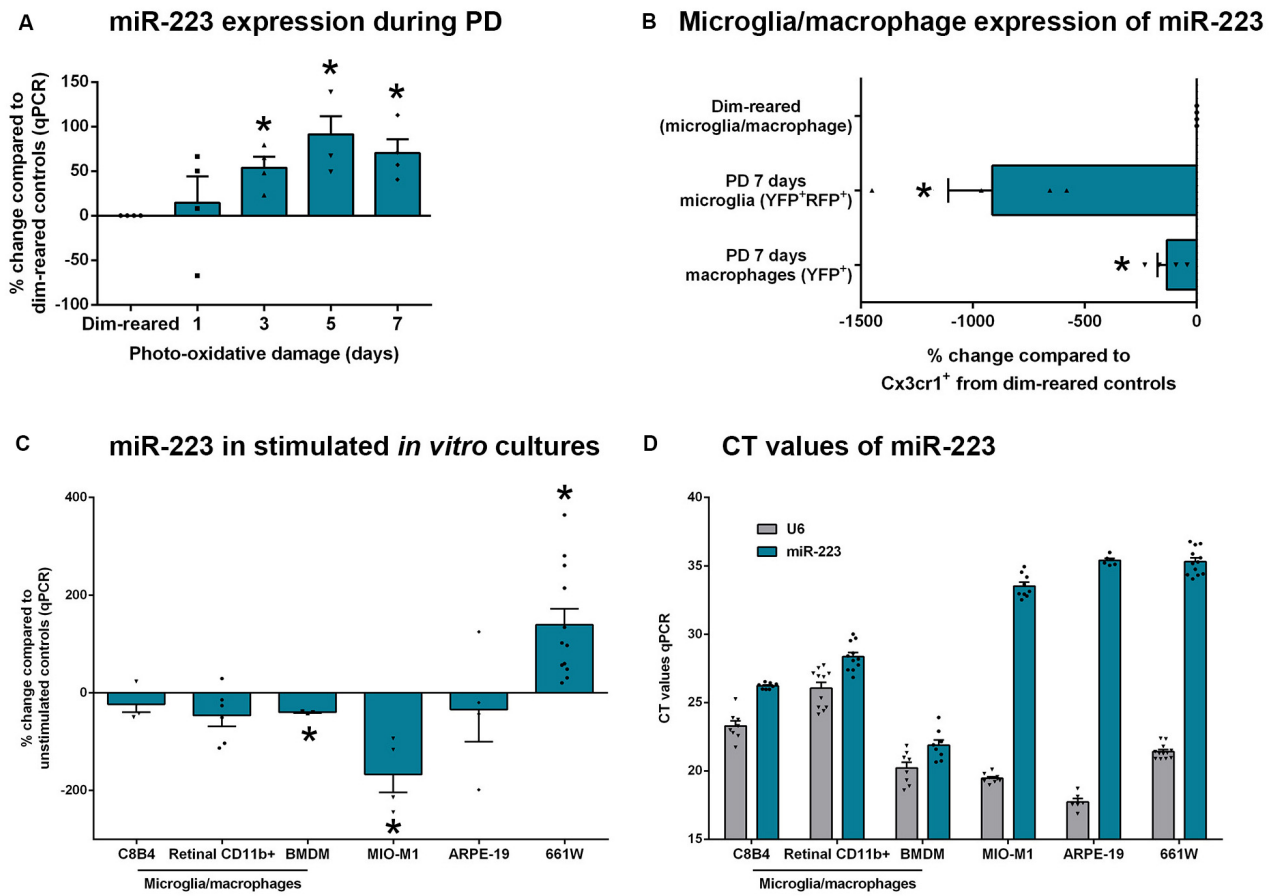

FIGURE 2 | Retinal expression of miR-223 during stimulation. **(A)** During photo-oxidative damage (PD) in wild-type (WT) mice, miR-223 in the retina is significantly upregulated between 3 and 7 days, peaking at 5 days ($P < 0.05$), compared to dim-reared control levels. **(B)** Compared to dim-reared microglia/macrophages (Cx3cr1$^+$ cells), isolated microglia (YFP$^+$RFP$^+$) and macrophages (YFP$^+$) from 7-day PD mice had significantly reduced miR-223 levels ($P < 0.05$). **(C)** BMDMs and MIO-M1 cells had significantly reduced miR-223 after stimulation in culture, whereas 661W cells showed an increase in miR-223 ($P < 0.05$). C8B4 cells, retinal CD11b$^+$ cells, and ARPE-19 cells showed no change in the expression of miR-223 between conditions ($P > 0.05$). **(D)** When comparing the CT values of miR-223 and U6 (reference) across cell types in both unstimulated and stimulated conditions, it is shown that microglia/macrophages have a higher expression of miR-223 (lower CT) relative to other cell types (higher CT of 34–36 indicates low expression of miR-223). Sample size is $N = 3$–12. *Denotes significance ($P < 0.05$).

innate immune system contributors [several of which are targets of miR-223 regulation (Bauernfeind et al., 2012; Chen et al., 2012; Dorhoi et al., 2013; Gantier, 2013)] only revealed a significant increase in *Ctse* in miR-223$^{-/-}$ retinas (**Figure 3I**, $P < 0.05$).

miR-223 in the Retina and Circulation in Retinal Degeneration

Circulating and s-mEV levels of miR-223 have been shown to be dysregulated in several neurological disorders (Fenoglio et al., 2013; Wang et al., 2014; Chen et al., 2017; Wei et al., 2018). We assessed whether miR-223 was changed in response to photo-oxidative damage in both serum and retinal s-mEVs. We found that alongside elevated levels of retinal miR-223 at 5 days of photo-oxidative damage, there was also a significant increase in serum miR-223, compared to dim-reared controls (**Figure 4A**, $P < 0.05$). Retinal s-mEVs isolated at 5 days of photo-oxidative damage also showed an elevation in miR-223, compared to s-mEVs from dim-reared controls (**Figure 4A**, $P < 0.05$).

Circulating CD11b$^+$ and CD11b$^-$ leukocytes were then isolated from peripheral blood by FACS following 5 days of photo-oxidative damage (**Figure 4B**). While approximately equal amounts of CD11b$^+$ and CD11b$^-$ cells were identified in the blood of WT animals (mean of 46.4 and 46.8%, respectively, of cells within the leukocyte gate), miR-223$^{-/-}$ animals had a large increase in the ratio of CD11b$^+$ cells to CD11b$^-$ cells (mean of 86.8 and 7.5%, respectively). This may indicate that miR-223$^{-/-}$ animals undergoing photo-oxidative damage have a higher proportion of circulating CD11b$^+$ cells compared to WT controls, potentially due to either increased CD11b$^+$ cell generation, or loss of CD11b$^-$ cells as a consequence of miR-223 deficiency.

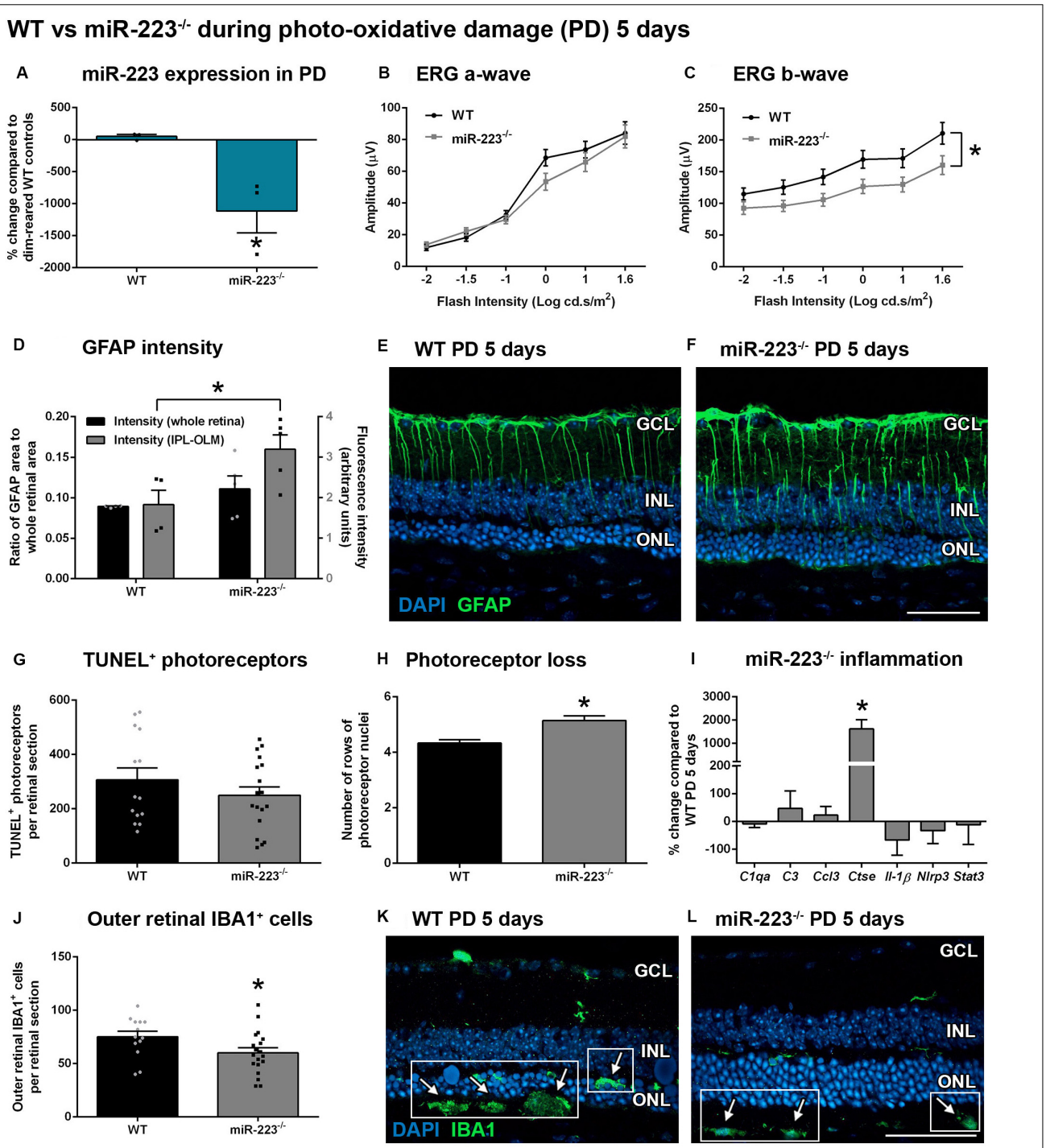

FIGURE 3 | Retinas of wild-type (WT) and miR-223$^{-/-}$ mice during 5 days of photo-oxidative damage (PD). **(A)** miR-223$^{-/-}$ mice were again confirmed to have a significant reduction in retinal miR-223 expression compared to WT mice ($P < 0.05$). **(B,C)** Electroretinography (ERG) demonstrated that there was no change in the a-wave [**(B)**, $P > 0.05$], but a significant change in the b-wave [**(C)**, $P < 0.05$] in miR-223$^{-/-}$ mice compared to WT controls. **(D–F)** GFAP intensity (green) was higher in all layers between the IPL and the OLM of miR-223$^{-/-}$ retinas compared to WT retinas ($P < 0.05$). Units for whole retinal GFAP on left *Y*-axis (black) and units for IPL to OLM GFAP on right *Y*-axis (gray). **(G)** No change in TUNEL was observed between groups ($P > 0.05$). **(H)** miR-223$^{-/-}$ retinas had more photoreceptor cell rows than WT retinas ($P < 0.05$), indicating less cumulative photoreceptor death. **(I)** *Ctse* retinal expression was significantly increased in miR-223$^{-/-}$ retinas compared to WT controls ($P < 0.05$); however, no changes in any other inflammatory genes assayed were observed ($P > 0.05$). **(J–L)** miR-223$^{-/-}$ retinas had a significant reduction in IBA1^{+} (green) microglia and macrophages in the outer retina (arrows, white boxes), compared to WT controls ($P < 0.05$). These were mainly present in the subretinal space. In WT animals, IBA1^{+} cells were increased in number and situated closer to the photoreceptor layer (arrows, white boxes). IPL, inner plexiform layer; OLM, outer limiting membrane; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar is 50 μm. Sample size is $N = 3–9$. *Denotes significance ($P < 0.05$).

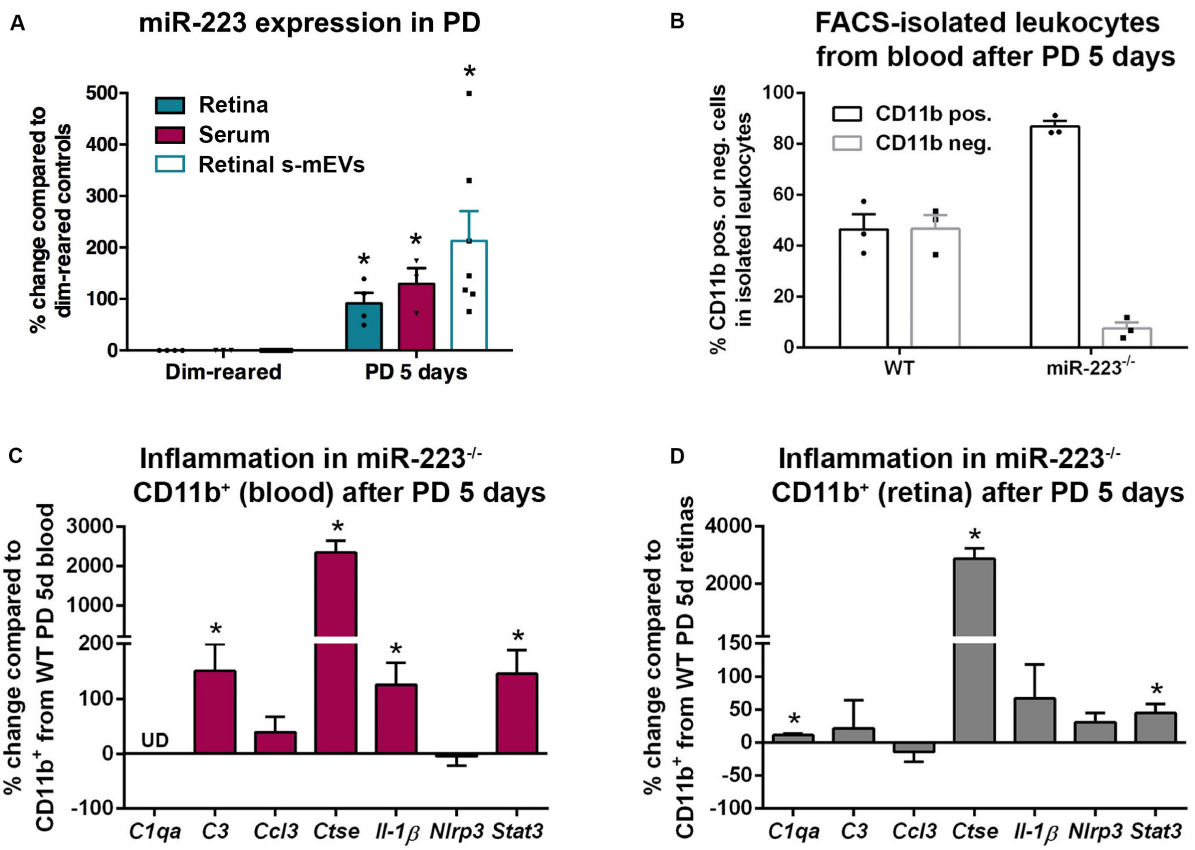

FIGURE 4 | miR-223 in the retina and circulation during retinal degeneration. **(A)** miR-223 was shown to be significantly increased in the retina, serum, and retinal small-medium extracellular vesicles (s-mEVs) at 5 days of photo-oxidative damage (PD), compared to dim-reared control retinas, serum, and s-mEVs, respectively ($P < 0.05$). **(B)** During FACS-isolation of leukocytes after 5 days of PD, the number of $CD11b^+$ cells to $CD11b^-$ cells was skewed in the miR-$223^{-/-}$ mouse blood compared to wild-type (WT) mouse blood, where the proportion between positive and negative populations was more even. **(C)** In $CD11b^+$ cells isolated from blood after PD, *C3*, *Ctse*, *Il-1β,* and *Stat3* were all significantly upregulated compared to $CD11b^+$ cells from WT ($P < 0.05$). The expression of *C1qa* was undefined (UD). **(D)** In the retina, $CD11b^+$ cells had a significantly higher expression of *C1qa*, *Ctse*, and *Stat3* after PD compared to WT retinal $CD11b^+$ cells ($P < 0.05$). Sample size is $N = 3$–7. *Denotes significance ($P < 0.05$).

Next, we quantified levels of pro-inflammatory cytokines, complement components, and innate immune system contributors by qPCR in $CD11b^+$ cells isolated from blood (**Figure 4C**). In circulating $CD11b^+$ cells from miR-$223^{-/-}$ mice, there was a significant increase in complement component 3 (*C3*), *Ctse*, interleukin-1β (*Il-1*β), and signal transducer and activator of transcription 3 (*Stat3*) compared to WT controls (**Figure 4C**, $P < 0.05$). Pro-inflammatory genes *C3* and *Il-1*β are associated with progressive retinal degeneration in this model (Natoli et al., 2017a,b). Chemokine C-C motif ligand 3 (*Ccl3*) and NLR family pyrin domain containing 3 (*Nlrp3*) did not change (**Figure 4C**, $P > 0.05$), and *C1qa* was undetected in circulating $CD11b^+$ cells.

From photo-oxidative damaged retinas, $CD11b^+$ cells (primarily retinal microglia and macrophages) were also isolated for qPCR. *C1qa*, *Ctse,* and *Stat3* were significantly upregulated in $CD11b^+$ cells isolated from miR-$223^{-/-}$ retinas compared to WT retinas (**Figure 4D**, $P < 0.05$). No significant changes in *C3*, *Ccl3*, *Il-1*β, or *Nlrp3* were observed (**Figure 4D**, $P > 0.05$).

These data demonstrate that levels of miR-223 increase in the retina, circulation, and retinal s-mEVs in response to photo-oxidative damage. When no systemic miR-223 is present, the circulating inflammatory cell profile may be significantly altered towards a more pro-inflammatory activation state.

Delivery of miR-223 Mimics to the Degenerating Retina

Transfection of encapsulated miR-223 mimics into the retina via intravitreal (IVT) delivery, as well as systemic via tail vein (IV) delivery, was used to increase the bioavailability of miR-223 in the circulating or retinal system. As retinal microglia and recruited macrophages both contribute toward progressive photoreceptor loss in degeneration (Natoli et al., 2017a), we investigated the injection of miR-223 both locally and systemically to supplement

these cells, as they lose miR-223 expression during degeneration (**Figure 2**). WT animals were IVT-injected with 1 μg/μl into each eye immediately prior to photo-oxidative damage. Following 5 days photo-oxidative damage, the ERG a-wave and b-wave were both significantly increased after injection with the miR-223 mimic, compared to scrambled mimic negative controls (**Figures 5A,B**, $P < 0.05$), indicating a positive effect on the retinal response. The retinal level of miR-223 was significantly increased at 5 days of photo-oxidative damage at approximately 10,000% higher than negative controls (**Figure 5C**, $P < 0.05$). No difference in TUNEL$^+$ photoreceptors or outer retinal IBA1$^+$ microglia and macrophages was observed (**Figures 5D,E,GŰJ**; $P > 0.05$). However, there was a small but significant increase in the number of photoreceptor cell rows following injection with miR-223 mimics (**Figure 5F**, $P < 0.05$), consistent with an improved retinal response.

Wild-type animals were also injected systemically (IV) with miR-223 mimics (0.5 mg/kg) at 2 days into a 5-day photo-oxidative damage protocol. This time point was chosen for the IV injections as Invivofectamine 3.0 is thought to have a shorter half-life in circulation compared to the eye, where retinal transfection has been shown to last 3–4 days (Chu-Tan et al., 2020). At

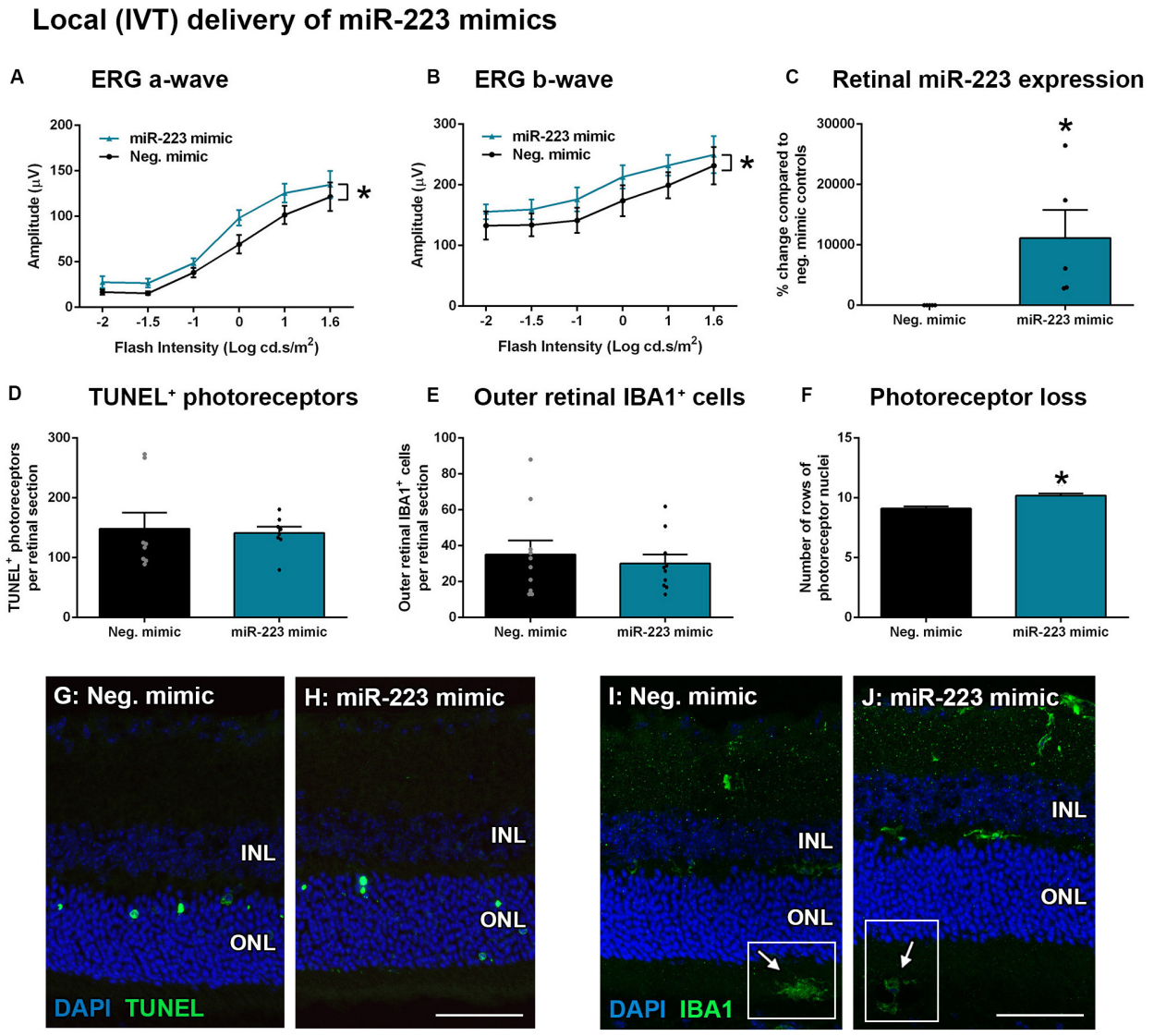

FIGURE 5 | Local intravitreal (IVT) delivery of miR-223 mimics to the retina during 5 days of photo-oxidative damage. **(A,B)** WT animals injected with miR-223 mimics had a significantly higher a-wave **(A)** and b-wave **(B)** than negative controls ($P < 0.05$), as assessed by electroretinography (ERG). **(C)** Retinal expression of miR-223 was significantly higher at 5 days after mimics were intravitreally injected ($P < 0.05$). **(D,E)** There was no significant difference in TUNEL$^+$ **(D)** or IBA1$^+$ cells **(E)** in the outer retina between groups ($P > 0.05$). **(F)** Retinas injected with miR-223 mimics had a significantly higher number of photoreceptor rows compared to controls ($P < 0.05$). **(G,H)** Representative images depicting TUNEL$^+$ photoreceptors (green). **(I,J)** Representative images depicting IBA1 immunohistochemistry (green), with arrows pointing towards IBA1$^+$ cells in the subretinal space (white boxes). INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar is 50 μm. Sample size is $N = 4–5$. *Denotes significance ($P < 0.05$).

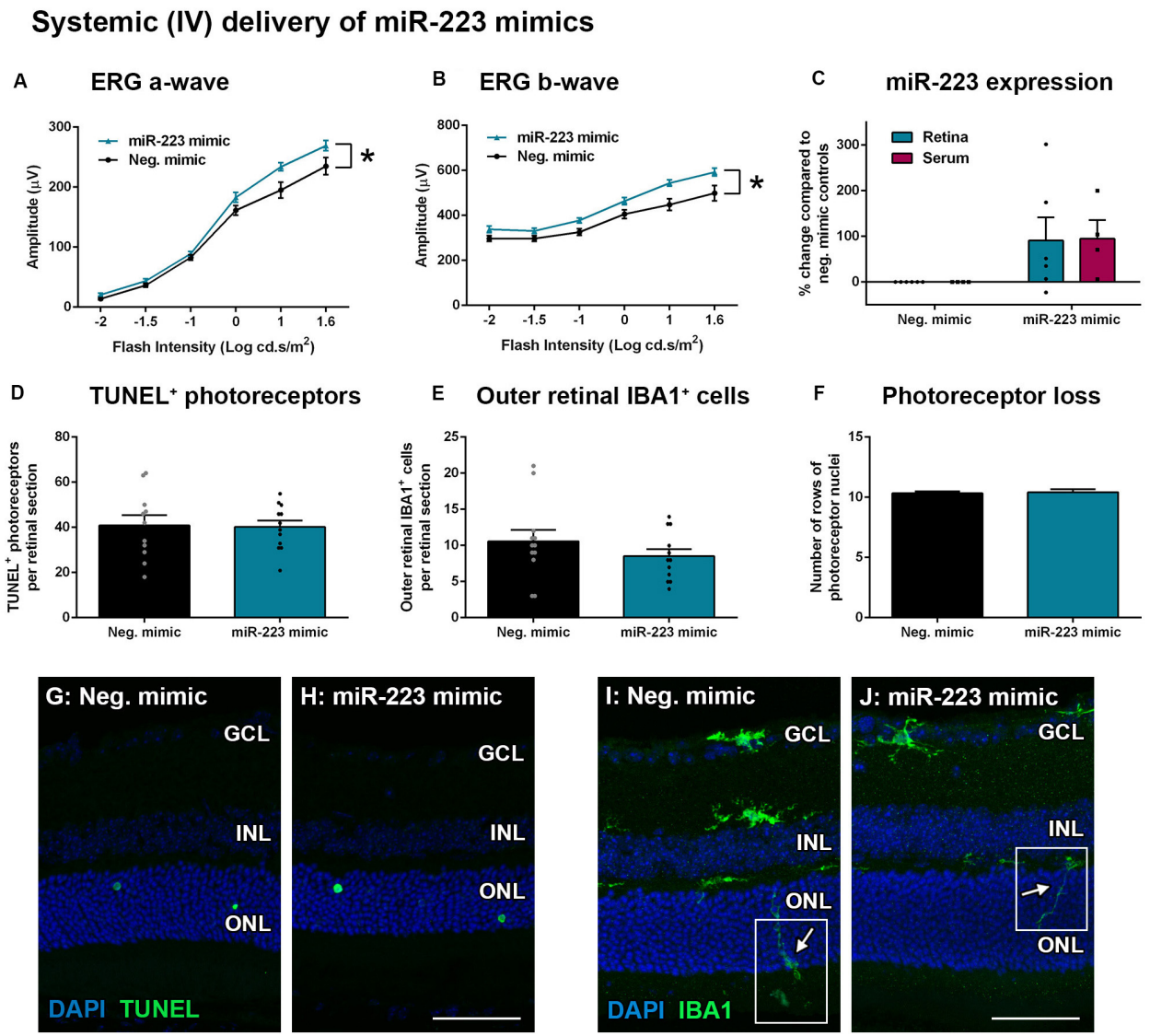

FIGURE 6 | Systemic intravenous (IV) delivery of miR-223 mimics during 5 days of photo-oxidative damage. **(A,B)** WT animals injected with miR-223 mimics again had a significantly higher a-wave **(A)** and b-wave **(B)** than negative controls ($P < 0.05$), as assessed by electroretinography (ERG). **(C)** Retinal and serum expression of miR-223 were not significantly changed after miR-223 mimic injections ($P > 0.05$). **(D,E)** There was no significant difference in TUNEL$^+$ **(D)** or IBA1$^+$ cells in the outer retina **(E)** between groups ($P > 0.05$). **(F)** No change in photoreceptor cell rows was observed between groups ($P > 0.05$). **(G,H)** Representative images depicting TUNEL$^+$ photoreceptors (green). **(I,J)** Representative images depicting IBA1 immunohistochemistry (green), with arrows pointing toward the processes of IBA1$^+$ cells in the outer retina (white boxes). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar is 50 μm. Sample size is $N = 4$–6. *Denotes significance ($P < 0.05$).

5 days photo-oxidative damage, the ERG a-wave and b-wave were significantly increased after IV injection with the miR-223 mimic, compared to negative controls (**Figures 6A,B**, $P < 0.05$). When assaying both the retina and the serum for miR-223 levels, there was no significant difference between miR-223 mimic-injected animals and negative controls (**Figure 6C**, $P > 0.05$). There was also no difference in TUNEL$^+$ photoreceptors (**Figures 6D,G,H**, $P > 0.05$), outer retinal IBA1$^+$ microglia and macrophages (**Figures 6E,I,J**, $P > 0.05$), or number of photoreceptor cell rows (**Figure 6F**, $P > 0.05$), indicating no major differences in histology following injections.

These data demonstrate that delivery of miR-223 mimics to the retina via local (IVT) or systemic (IV) injection may improve the retinal response. However, only local delivery of miR-223 may be effective in providing protection to the photoreceptor cell layer.

DISCUSSION

In this study, we identified several key roles of miR-223 in the healthy and degenerating retina. First, we found that miR-223 is

required for the maintenance of healthy retina, with deficiency in miR-223 causing aberrant retinal function. Secondly, we found that although retinal, circulating, and s-mEV miR-223 are all upregulated in response to retinal degeneration, miR-223 expression is reduced in microglia and macrophages. A deficiency in miR-223 in both retinal and circulating immune cells in degeneration may alter the expression of key inflammatory genes, including *Ctse*. Finally, we demonstrated that local delivery of a miR-223 mimic may reduce photoreceptor loss and improve retinal function. Overall, these data implicate miR-223 in the retina as having important roles in influencing both retinal function and inflammation.

miR-223 Is Required for Maintaining Retinal Function

In dim-reared miR-223$^{-/-}$ animals, our data demonstrated that absence of miR-223 had an adverse effect on the retinal function of both photoreceptors (a-wave) and the ON-bipolar and Müller cells (b-wave), as well as minor photoreceptor loss. miR-223 deficiency led to a small reduction in retinal *C1qa*, a classical complement activator produced by microglia that is thought to be critically involved in synapse elimination by phagocytosis (Stevens et al., 2007) and maintaining inner nuclear layer (INL) integrity (Mukai et al., 2018). It is possible that reduced *C1qa* in miR-223-null retinas contributes to altered retinal function in normal conditions; although as *C1qa* is not a known target of miR-223, this could be occurring through other microglial components that may activate the complement system. Other studies have suggested a role for miR-223 in the neuroprotection of photoreceptors and second-order neurons, showing that the expression of glutamate receptor (GluR) subunits may be regulated by miR-223 (Harraz et al., 2012; Morquette et al., 2019). In particular, GluR2 and NR2B have been identified as potential targets of miR-223 (Harraz et al., 2012), with GluR2 thought to be expressed by bipolar cells housed within the INL (Lin et al., 2012). miR-223 *in situ* hybridization in an EAU model has indicated that in addition to being expressed within the perivascular lesion and in subretinal inflammatory cells, miR-223 may be also expressed within the INL (Watanabe et al., 2016). In the miR-223$^{-/-}$ retina, it is possible that the absence of miR-223 may lead to dysregulation of GluR density on bipolar cells, decreasing their threshold of depolarization and inducing excitotoxicity. This may cause a reduced b-wave and increased gliosis, both of which were observed in the miR-223$^{-/-}$ mice in degenerating conditions. Further studies could investigate the interactions between miR-223 and these GluR subunits, as well as any potential expression of miR-223 by bipolar cells in the retina. This may contribute toward an understanding of whether miR-223-mediated neuroprotection plays a role in modulating retinal function and neuronal survival.

Cathepsin E Is Upregulated During miR-223 Deficiency

We identified *Ctse* [a predicted target of miR-223 (Gantier, 2013)] as a key inflammatory gene that was heavily upregulated in all miR-223-null retinas and isolated CD11b^{+} cells in this study. CTSE is an aspartic proteinase known to mediate a range of critical macrophage functions including lysosomal activity, autophagy, and recruitment (Yamamoto et al., 2012). As miR-223 deficiency would likely affect *Ctse* as it is a predicted target, its upregulation in the retina could contribute to macrophage dysregulation. Alternatively, CTSE has been previously thought to contribute to neuronal degeneration during aging and may be expressed by aging neurons during degeneration (Nakanishi et al., 1994; Nishishita et al., 1996). In human retinas, increasing neuronal expression of CTSE was evident with age, in addition to its expression in immune cells and some Müller cells (Bernstein et al., 1998). Interestingly, excessive stimulation of GluR in hippocampal neurons has been shown to induce *Ctse* gene expression in these neurons, in addition to activated microglia (Tominaga et al., 1998). It is possible that interactions between miR-223, GluR, and *Ctse* could be taking place in retinal neurons (including bipolar cells) as well, as we have indicated that miR-223 can be expressed in neuronal cell types (photoreceptors). As hypothesized above, the altered b-wave response associated with miR-223$^{-/-}$ mice may be due to GluR signaling and excitotoxicity. If high neuronal and microglial *Ctse* expression is stimulated by GluR dysregulation in miR-223$^{-/-}$ mice, it is possible that *Ctse* may be contributing to neuronal degeneration under normal conditions. Accordingly, we observed photoreceptor loss and a reduced a-wave in dim-reared miR-223$^{-/-}$ mice. However, in photo-oxidative damage, we did not observe photoreceptor degeneration in the miR-223$^{-/-}$ mice, perhaps indicating that other inflammatory functions of *Ctse* were more imperative in this scenario.

Although beyond the scope of this preliminary investigation into the relationship between miR-223 and *Ctse*, further studies are needed to investigate whether retinal levels of CTSE protein are altered in miR-223$^{-/-}$ or miR-223-supplemented mice and to determine what roles CTSE may have in influencing neuronal function or inflammation in the retina.

miR-223 Regulates the Inflammatory Profile of Microglia and Macrophages

We identify retinal microglia and macrophages as an abundant source of miR-223, and their levels decrease substantially in degenerating conditions. As miR-223 is a critical inhibitor of many inflammatory processes, including the NLRP3 inflammasome (Bauernfeind et al., 2012; Yang et al., 2015; Neudecker et al., 2017b), cathepsin activity (Gantier, 2013), chemokine signaling (Dorhoi et al., 2013), the NF-κB pathway (Zhou et al., 2018), and JAK/STAT signaling (Chen et al., 2012), a reduction in miR-223 would likely cause higher inflammation within these cells. As discussed earlier, we observed that *Ctse* was substantially increased in CD11b^{+} cells from both the retina and the blood of miR-223-null mice. We also found that in miR-223-null CD11b^{+} cells from the retina, there was increased *C1qa* which is associated with progressive retinal degeneration (Silverman et al., 2016; Jiao et al., 2018), as well as increased *Stat3*, also associated with retinal disease (Chen et al., 2016, 2019). In the blood of miR-223$^{-/-}$ mice, circulating CD11b^{+} cells showed an increase in *C3* and *Il-1*β, which are both critically linked to

macrophage-mediated retinal degeneration (Rutar et al., 2011a; Hu et al., 2015; Eandi et al., 2016; Natoli et al., 2017a,b).

Additionally, we observed that there appeared to be skewing in the circulating leukocyte population of miR-223-null animals toward a higher proportion of $CD11b^+$ cells, indicating that the cells may be producing more pro-inflammatory factors. There is also a possibility that $CD11b^-$ cells may have been lost with miR-223 deficiency. We note that a study by Johnnidis et al. (2008) indicated that miR-223-null mice have an expanded granulocytic compartment, leading to neutrophil hyperactivity. However, Galloway et al. (2019) found that although miR-223-null mice showed a similar pro-inflammatory phenotype to WT mice, an absence of miR-223 hindered anti-inflammatory functions and effective M2 ($CD206^+$) polarization. Although some differences have been observed between these studies, the commonality is that miR-223 is required for normal myeloid cell activation and differentiation and that an absence of miR-223 alters the inflammatory profile to a state that may exacerbate inflammatory conditions, including retinal degenerative diseases. Further investigation into which circulating cells of the hematopoietic lineage may be affected by miR-223 deficiency may uncover therapeutic targets for modulation.

Dual Roles for miR-223 in Retinal Degeneration

Although retinal and circulating $CD11b^+$ cells from photo-oxidative damage were more pro-inflammatory in response to miR-223 deficiency, this did not translate to a more damaging outcome for the retina in knockout mice. We demonstrated that miR-223-null mice had retained more photoreceptors, accompanied by fewer outer retinal microglia and macrophages. In addition, with the exception of *Ctse*, there were no changes in any inflammatory genes assayed in the retinas of miR-223-null mice, several of which are targets of miR-223. It is possible that some of the protective functions of *Ctse* might be at play here, such as maintaining autophagic proteolysis (Tsukuba et al., 2013). This could occur in the context of photoreceptor outer segment phagocytosis, a known function of a related molecule, cathepsin D (Rakoczy et al., 2002). It is possible that miR-223 deficiency has a role in the protection of the retina during damage through the promotion of anti-inflammatory mechanisms or protective responses. Li et al. (2019) proposed that miR-223-deficient microglia appeared to remain in a resting state and retained their autophagic functions in experimental autoimmune encephalomyelitis (EAE), reducing CNS inflammation and pathology. It is possible that autophagy, which may be influenced by photo-oxidative damage (Chen et al., 2013), is hindered with miR-223 signaling in degenerating conditions. Further studies are required to address autophagy and cathepsins as a potential mechanism involved in miR-223 interactions in the retina, including autophagic photoreceptor cell death. The concept of miR-223 having alternative damaging roles has also been explored in a rabbit glaucoma model, where miR-223 mimics induced apoptosis and inflammation in retinal ganglion cells by targeting HSP-70 (Ou-Yang et al., 2020). In another study, miR-223 targeted FOXO3, positively regulating pathogenic Th17 cells in an EAU model (Wei et al., 2019).

However, miR-223-null photo-oxidative damaged mice exhibited increased GFAP, indicating a higher level of reactive gliosis and retinal cell stress. The ERG b-wave was also dampened in miR-223-null photo-oxidative damaged mice, indicating reduced ON-bipolar and Müller cell activity. A potential loss of miR-223-mediated neuroprotection could again have contributed to both of these observations (Harraz et al., 2012; Morquette et al., 2019). These data point to miR-223 having dual protective and damaging roles in the degenerating retina.

Delivery of miR-223 Mimics Improve Retinal Function

Without miR-223, retinal function appears to be worsened, whereas both locally and systemically delivered miR-223 mimics led to an improvement in retinal function. This again demonstrates that miR-223 may be required in the preservation of the retinal response, perhaps by promoting microglial homeostatic maintenance (Galloway et al., 2019; Guo et al., 2019), or through neuroprotection and GluR signaling as discussed earlier (Harraz et al., 2012; Morquette et al., 2019). When delivered locally, miR-223 supplementation also protected mice from photoreceptor loss. The discrepancy between miR-$223^{-/-}$ and miR-223-supplemented mice regarding photoreceptor layer morphology in degeneration could be a limitation of using miR-223-null mice with the photo-oxidative damage model. It is possible that any photoreceptor degeneration in miR-223-null mice in normal conditions may lead to a pre-conditioning effect against oxidative stress and inflammation caused by retinal damage.

However, given that IVT delivery increased retinal miR-223 by ∼10,000%, it was somewhat surprising that the IV injection also led to an improved retinal response, even though no detectable increase in retinal miR-223 was observed at the time of tissue collection. It is possible that the amount of miR-223 delivered to animals through IV delivery was sufficient to see an effect on retinal function; however, more sustained delivery to increase the bioavailability in the blood might be required to attain a measurable effect in the photoreceptor layer. It is also possible that the circulating immune cells had taken up the systemic-injected miR-223 mimics and were altered prior to retinal infiltration, shifting them toward an anti-inflammatory state (Galloway et al., 2019) and potentially reducing pathogenic mechanisms that may adversely affect retinal function. As miR-223 mimics have been useful in reducing pathogenesis in CNS studies (Shi et al., 2013; Yang et al., 2015; Ding et al., 2018; Galloway et al., 2019), identifying how miR-223 mimics modulate the retinal response or inflammation (including *Ctse*) will be key to determining any therapeutic potential of manipulating miR-223 levels in the retina and circulation. In further studies investigating alternative IV doses and injection paradigms of miR-223 mimics, system-wide organ pathology and circulating immune profiles should be monitored following systemic treatments. In addition, fluorescent tracing studies of miR-223 mimics in circulation may assist in determining both retinal and systemic cell transfection.

miR-223 as a Circulating miRNA in Retinal Degeneration

We determined that retinal miR-223 levels were upregulated during degeneration in the photo-oxidative damage model, corroborating previous findings using a range of retinal disease models (Fuller-Carter et al., 2015; Saxena et al., 2015; Chung et al., 2016; Watanabe et al., 2016; Huang et al., 2017). This may have occurred either through increased photoreceptor expression of miR-223, or the accumulation of large numbers of miR-223-expressing recruited macrophages in areas of retinal degeneration (Natoli et al., 2017a). We also identified circulating serum levels of miR-223 to also be upregulated in photo-oxidative damage. miR-223 has previously been identified as a circulating biomarker in a range of chronic inflammatory conditions (Zhou et al., 2015; Wang et al., 2016; Cisilotto et al., 2020), including neurological disorders (Fenoglio et al., 2013; Wang et al., 2014). In retinal degeneration, it is possible that elevated miR-223 levels in the circulation may be an indication of disease, as occurs in neovascular AMD patient plasma (Ertekin et al., 2014) and in peripheral blood cells (Litwinska et al., 2019). An increase in systemic miR-223 could have occurred due to elevation in the retina in response to light exposure, which has been previously shown to alter retinal miRNA expression levels (Krol et al., 2010). Excess miR-223 may have leeched out of the retina into the blood. Alternatively, miR-223 may have been upregulated in circulating immune cells in response to light exposure.

Interestingly, miR-223 may also participate in exosomal communication in neurological disorders, including stroke and dementia (Chen et al., 2017; Wei et al., 2018). Shurtleff et al. (2016) demonstrated that miR-223 was a specific highly abundant exosome cargo in HEK293T cells, where Y-box protein 1 (YBX1) was required for selective sorting of specific miRNAs (including miR-223) into exosomes. YBX1 has recently been proposed as a central transcription factor involved in aging (Ma et al., 2020). In retinal degeneration, it is possible that miR-223-loaded s-mEVs (including exosomes) from the retina could leech out into the circulation, or s-mEVs could be produced systemically. In the current study, we found that s-mEVs isolated from the retina during degeneration had significantly increased miR-223 content. However, the question remains as to which cells produce these miR-223-loaded s-mEVs in the retina, and whether leeching into the circulation would have any systemic effects. In other diseases, macrophage-derived extracellular vesicles carrying miR-223 have been shown to supplement epithelial ovarian cancer cells with miR-223 (Zhu et al., 2019), while neutrophil-derived extracellular vesicles act to increase miR-223 in pulmonary epithelial cells (Neudecker et al., 2017a). Hence, further investigation into which cells produce these miR-223-loaded s-mEVs in the retina will help to elucidate mechanisms by which s-mEV-derived miR-223 exerts local and systemic responses that influence the progression of retinal degeneration.

CONCLUSION

We demonstrate in this study that miR-223 is required for maintenance of the healthy retina, through its regulation of retinal function and modulation of inflammatory molecules by retinal and circulating immune cells. We show that miR-223 mimics may be effective in improving the retinal response to photo-oxidative damage and demonstrate that local miR-223 delivery can achieve a beneficial effect in the preservation of the photoreceptor layer during retinal degeneration. As miR-223 may target hundreds of genes and several biological pathways, further understanding the interactions of miR-223 in the retina will be critical in defining the therapeutic utility of miR-223 in retinal degenerative diseases.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Australian National University (ANU) Animal Experimentation Ethics Committee (Ethics ID: A2017/41).

AUTHOR CONTRIBUTIONS

NF designed the study, conducted experiments and analysis, and wrote the manuscript. JW, SD, CDi, RA-B, AC, YW, and CN conducted experiments and/or analysis. JW, SD, RA-B, AC, YW, JC-T, US, RE, and SR revised the manuscript. CDo and SR managed the miR-223$^{-/-}$ animals and contributed to the study design. RE, SM, and JP designed the study. RN supervised and designed the study, conducted experiments and analysis, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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MicroRNAs and Autoimmune-Mediated Eye Diseases

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MicroRNAs (miRNAs) are evolutionarily conserved short non-coding RNAs that act at post-transcriptional regulation of gene expression by destroying target messenger RNA or inhibiting its translation. Recently, miRNAs have been identified as important regulators in autoimmunity. Aberrant expression and function of miRNAs can lead to dysfunction of immune system and mediate autoimmune disorders. Here, we summarize the roles of miRNAs that have been implicated in three representative ocular autoimmune disorders, including autoimmune uveitis, Grave's ophthalmopathy, and Sjögren's syndrome dry eye, and discuss the potential of miRNAs as biomarkers and therapeutic targets for the diagnosis and treatment of these diseases.

Keywords: miRNAs, autoimmune uveitis, Grave's ophthalmopathy, Sjögren's syndrome dry eye, biomarkers, therapeutic targets

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INTRODUCTION

MicroRNAs (miRNAs), a class of evolutionarily conserved, short non-coding RNAs, are potent post-transcriptional regulators of gene expression through binding to the 3' untranslated region of a target mRNA (Bartel, 2004). Since the discovery of the first miRNA in *Caenorhabditis elegans* in 1993, miRNAs have been identified as key players in a plethora of cellular processes, such as cell growth, proliferation, differentiation, and apoptosis (Lee et al., 1993; Ambros, 2004). Besides, miRNAs are critical for normal development and function of immune system, and abnormal expression and function of miRNAs can lead to immunological aberrations and autoimmunity (Mehta and Baltimore, 2016). Recently, accumulating evidence from animal models and clinical studies has revealed the critical significance of miRNAs in the pathogenesis of many autoimmune diseases (Chen et al., 2016; Garo and Murugaiyan, 2016; Long et al., 2018), including autoimmune-mediated eye diseases.

Abbreviations: miRNAs, microRNAs; EAU, experimental autoimmune uveitis; EAAU, experimental autoimmune anterior uveitis; BD, Behcet's disease; PBMCs, peripheral blood mononuclear cells; TAB2, TGF-beta activated kinase 1 binding protein 2; Ets-1, E26 transformation specific-1; SNP, single nucleotide polymorphism; VKH, Vogt–Koyanagi–Harada; OSM, oncostatin M; CCL1, C–C motif chemokine ligand 1; SO, sympathetic ophthalmia; GO, Grave's ophthalmopathy; CASQ1, calsequestrin; OF, orbital fibroblast; ICAM-1, intercellular adhesion molecule-1; FN, fibronectin; α-SMA, α-smooth muscle actin protein; ZNRF3, zinc and ring finger 3; PTEN, phosphatase and tensin homolog; PDCD4, programed cell death 4; PLZF, promyelocytic leukemia zinc finger; SS, Sjögren's syndrome; BAFF, B-cell activating factor; pSS, primary Sjögren's syndrome; IRAK1, IL-1 receptor-associated kinase 1; MSC, mesenchymal stem cell.

The eye, an immune privileged organ, has adapted several negative regulators to prevent inflammation within its tissue microenvironment (Benhar et al., 2012; de Andrade et al., 2016). These regulators suppress inflammatory activity, induce production of ant-inflammatory cytokines and mediate the activation of tolerogenic antigen-presenting cells and regulatory T cells (Taylor, 2016; Keino et al., 2018). However, in the inflammatory setting, aberrant activation of effector immune cells (e.g., T cells and B cells) and excessive expression of proinflammatory mediators contribute to the breakdown of ocular immune privilege and elicit the development of autoimmunity (Caspi, 2006; Stern et al., 2010). Here, we summarize the current knowledge on miRNAs dysregulation (**Table 1**) and their pathogenic roles in three autoimmune-mediated eye diseases, including autoimmune uveitis, Grave's ophthalmopathy, and Sjögren's syndrome dry eye (**Figure 1**), focusing on three aspects: (1) alterations in miRNAs expression profiles, (2) the effect of aberrant miRNA expression on the onset and progression of diseases, and (3) the related molecular mechanisms. Moreover, we discuss the potential of miRNAs as potent biomarkers and therapeutic targets for the diagnosis and treatment of these diseases.

MICRORNAS AND DIFFERENT TYPES OF UVEITIS

MicroRNAs and Experimental Autoimmune Uveitis

Autoimmune uveitis is an intraocular inflammatory disease characterized by immune-mediated damage in the uveal tissues and retina. The progressive irreversible photoreceptors' damage caused by autoreactive T cells eventually leads to visual impairment and even blindness (Caspi, 2010). Experimental autoimmune uveitis (EAU) is an animal model of human uveitis, and its etiology has not been fully discerned (Caspi et al., 2008).

Dysregulation of certain miRNAs has been shown to be closely associated with the progression of EAU. Upregulation of miR-223 and miR-146a and downregulation of miR-181a were observed in the retina of rats during the course of EAU, corresponding with the score of EAU and the upregulation of IL-1β/MCP-1 (Watanabe et al., 2016). Additionally, 36 upregulated miRNAs and 31 downregulated miRNAs were found in peripheral blood lymphocytes from rats with EAU (Guo et al., 2015), and targets of these differentially expressed miRNAs were implicated in some immune signaling pathways such as T-cell receptor signaling pathway and Toll-like receptor signaling pathway, both of which are closely associated with the pathogenesis of EAU (Horai et al., 2013; Xiao et al., 2016). More recently, it was shown that miR-30b-5p expression was decreased in spleen, lymph nodes, and eye tissues of EAU rats, and miR-30b-5p directly targeted IL-10 and TLR4 in T cells and reduced the level of IL-10 and TLR4 positive cells, regulating the development of EAU (Sun et al., 2018).

Th17 cells driven by IL-23 are central to the pathogenesis of EAU (Peng et al., 2007). Th17 cell program is tightly regulated by various transcription factor, such as RORγt, STAT3, and FOXO3 (Yang et al., 2007; Maddur et al., 2012; Wei et al., 2019). IL-17, the signature cytokine of Th17 cells, has been shown to promote inflammation and tissue damage (Sun et al., 2015). Recent advances have revealed the important role of miRNAs in regulating Th17 cell response. Ishida et al. identified the increased expression of miR-142-5p and miR-21 and reduced expression of miR-182 in ocular tissues from mice with EAU, which paralleled the dynamic expression of IL-17. It was implied that these dysregulated miRNAs might regulate the development of EAU by affecting the expression of IL-17 (Ishida et al., 2011). Moreover, it has been reported that STAT3 binds directly to the miR-155 locus and induces its expression, and STAT3/miR-155 axis promotes the expansion of pathogenic Th17 cells, thereby contributing to the development of EAU (Escobar et al., 2013). More recently, it was revealed that miR-21-5p might affect EAU progression by altering Th17/Treg balance (Shi et al., 2019). Our own group has verified that miR-223-3p was significantly upregulated in IRBP-specific Th17 cells, and knockdown of miR-223-3p decreased the pathogenicity of Th17 cells in a T-cell transfer model of EAU. Mechanistic studies showed that miR-223-3p directly repressed the expression of FOXO3, and FOXO3 negatively regulated pathogenic Th17 responses partially via suppression of IL-23R expression (Wei et al., 2019). Collectively, these observations indicate that miRNAs are vital regulators of Th17 responses and provide new insights into the molecular pathogenesis of EAU.

MicroRNAs and Experimental Autoimmune Anterior Uveitis

Experimental autoimmune anterior uveitis (EAAU), in which inflammation is restricted to ocular anterior segment while retinal tissues and photoreceptor cells are not involved, resembles human idiopathic anterior uveitis (Broekhuyse et al., 1991). EAAU is characterized histologically by the infiltration of lymphocytic and mononuclear cell in the anterior uvea. The etiologies are largely unknown, but epigenetic mechanisms are paving the way for a better understanding of this disease. Recently, Hsu et al. (2015) demonstrated that the expression of miR-146a-5p, miR-155-5p, miR-147b, and miR-223-3p was decreased after EAAU induction, whereas the expression of miR-9-3p, miR-182-5p, and miR-183-5p was elevated. In addition, both the secretion of IFN-γ, IL-17, IL-12A, IL-1β, and IL-6 in aqueous humor and their mRNA expression in iris and ciliary bodies were upregulated in rats with EAAU as compared to controls. Based on these observations, it was speculated these differentially expressed miRNAs might promote Th1 and Th17 specific cytokine production, thereby contributing to the pathogenesis of EAAU, but the potential roles of miRNAs in the EAAU are still in the early discovery stage and need to be fully explored in the future.

MicroRNAs and Behcet's Disease

Behcet's disease (BD) is a systemic inflammatory disease of unknown etiology, characterized by ocular lesions, oral ulcer, genital ulcer, and multiple skin lesions (Yang et al., 2008; Zeidan et al., 2016). The eye involvement mainly manifests

TABLE 1 | Differential expression of miRNAs in ocular autoimmune disorders.

Disease	Sample	Upregulated miRNA	Downregulated miRNA	Target	References
Experimental autoimmune uveitis	Retina	miR-223	miR-181a		Watanabe et al., 2016
		miR-146a			
	Spleen, lymph nodes and eye tissues		miR-30b-5p	IL-10; TLR4	Sun et al., 2018
	Ocular tissues	miR-142-5p	miR-182		Ishida et al., 2011
		miR-21			
	CD4 + T cells	miR-155			Escobar et al., 2013
	Retina and splenic lymphocytes	miR-21-5p		IL-10	Shi et al., 2019
	Th17 cells	miR-223-3p		FOXO3	Wei et al., 2019
Experimental autoimmune anterior uveitis	Iris/ciliary bodies; popliteal lymph node	miR-9-3p	miR-146a-5p		Hsu et al., 2015
		miR-182-5p	miR-155-5p		
		miR-183-5p	miR-147b		
			miR-223-3p		
Behcet's disease	PBMCs	miR-155			Kolahi et al., 2018
	PBMCs	miR-3591-3p	miR-638		Woo et al., 2016
			miR-4488		
	PBMCs	miR-326	miR-21		Jadideslam et al., 2019
	PBMCs and dendritic cells		miR-155	TAB2	Zhou et al., 2012
	CD4 + T cells	miR-155		Ets-1	Na et al., 2016
	CD4 + T cells		miR-23b		Qi et al., 2014
	Serum	miR-146a			Ibrahim et al., 2019
Vogt–Koyanagi–Harada syndrome	CD4 + T cells		miR-20a-5p	OSM; CCL1	Chang et al., 2018
Sympathetic ophthalmia	globes		miR-9	TNF-α; NF-κB1	Kaneko et al., 2012
			miR-let-7e		
			miR-1		
			miR-182		
Grave's ophthalmopathy	Orbital adipose tissue	miR-146a			Jang et al., 2016, 2018
	CD4 + T cells		miR-146a	NUMB	Hu et al., 2017; Yang et al., 2017
	Orbital fibroblast	miR-146a		ZNRF3	Woeller et al., 2019
		miR-155		PTEN	
	Orbital fibroblast	miR-21			Tong et al., 2015
	Orbital fibroblast	miR-21		PDCD4	Lee et al., 2016
	Orbital fat tissue		miR-27a		Jang et al., 2019
			miR-27b		
	CD4 + T cells	miR-183		EGR-1	Thiel et al., 2019
		miR-96			
	Serum		miR-146a		Wei et al., 2014
Sjögren's syndrome dry eye	Tears	miR-16-5p	miR-30b-5p		Kim et al., 2019
		miR-34a-5p	miR-30c-5p		
		miR-142-3p	miR-30d-5p		
		miR-223-3p	miR-92a-3p		
			miR-134-5p		
			miR-137		
			miR-302d-5p		

(Continued)

TABLE 1 | Continued

Disease	Sample	Upregulated miRNA	Downregulated miRNA	Target	References
			miR-365b-3p		
			miR-374c-5p		
			miR-487b-3p		
	PBMCs		miR-150-5p		Chen et al., 2017a
	CD4 + T cell	miR-155-5p	miR-let-7d-3p		Wang-Renault et al., 2018
		miR-222-3p	miR-30c-5p		
		miR-146a-5p	miR-378a-3p		
		miR-28-5p			
	CD19 + B cell		miR-30b-5p	BAFF	
		miR-222-3p	miR-378a-3p		
			miR-26a-5p		
			miR-19b-3p		
	PBMCs	miR-146a			Pauley et al., 2011; Shi et al., 2014
	PBMCs	miR-146a/b		IRAK-1	Zilahi et al., 2012
	PBMCs		miR-155		Shi et al., 2014
	PBMCs	miR-155			Pauley et al., 2011; Chen et al., 2017b
	PBMCs	miR-181a			Peng et al., 2014

with chronic, recurrent bilateral non-granulomatous uveitis with necrotizing retinal vasculitis (Park et al., 2014). The pathogenesis of BD is highly complex, with immunological aberrations, environmental factors, genetic predisposition, and epigenetic alterations involved, but remains largely unknown (Greco et al., 2018). In recent years, studies have paid much attention to the critical implication of miRNAs in the development of BD.

The disturbed miRNA expression in peripheral blood mononuclear cells (PBMCs) from patients with BD has been unveiled, and certain miRNAs have been deemed as potent diagnostic biomarkers. MiR-155 expression was significantly increased in the PBMCs of BD patients with uveitis, whereas miR-146a expression had no significant difference between BD patients and controls (Kolahi et al., 2018). In addition, altered expression of miR-638, miR-4488, and miR-3591-3p in the PBMCs of BD patients was also reported (Woo et al., 2016), which is associated with the production of IL-6, an inflammatory cytokine involved in the pathogenesis of BD (Lin, 2015). More recently, Jadideslam and colleagues compared the expression of miR-21, miR-146b, and miR-326 in PBMCs from Iranian Azari BD patients with that in healthy controls, and revealed the downregulation of miR-21 and upregulation of miR-326. Furthermore, they suggested the great potential of miR-326 as a biomarker for predicting the uveitis and severe eye involvement in BD (Jadideslam et al., 2019).

Th17 cells can facilitate neutrophil inflammatory response that underlies the pathogenesis of BD (Leccese and Alpsoy, 2019). Increased frequencies of Th17 cells have been reported in BD patients with active uveitis (Chi et al., 2008). Recent evidence from clinical studies implicates miRNAs as key regulators of Th17 responses in BD. Zhou et al. (2012) found that miR-155 inhibited dendritic cell-driven Th17 responses by targeting TGF-beta-activated kinase 1 binding protein 2 (TAB2), while Na et al. (2016) demonstrated upregulated miR-155 in CD4+ T cells promoted Th17 responses via suppression of E26 transformation-specific-1 (Ets-1) in BD. The difference in cells types and disease stage may account for the discrepancy in these studies. Besides, Qi et al. (2014) revealed that downregulation of miR-23b was closely associated with activation of the Notch pathway and expansion of Th1/Th17 cells, hinting the important role of miR-23b in the pathogenesis of BD.

Genome-wide association studies have identified that single nucleotide polymorphisms (SNPs) in miRNAs are involved in many autoimmune diseases, including BD. For example, there is a strong correlation between rs2910164 in the pre-miR-146a gene and development of BD (Zhou et al., 2014; Oner et al., 2015; Ibrahim et al., 2019). Decreased miR-146a expression, as well as reduced proinflammatory cytokine production (including IL-17, TNF-α, and IL-1β), was found in individuals carrying rs2910164 CC genotype and C allele. It was implied that this SNP might protect against BD possibly via suppression of miR-146a expression and proinflammatory cytokine production. Moreover, miR-196a2/rs11614913 confers susceptibility to BD by regulating miR-196a expression and proinflammatory cytokine (IL-1β and MCP-1) secretion (Qi et al., 2013). Another extensively investigated variant involved in autoimmune diseases is the pre-miR-499 rs3746444 polymorphism. This SNP has been shown to be associated with an increased risk of BD in a Turkish population (Oner et al., 2015). In addition, reduced CC genotype and C allele frequencies of miR-182/rs76481776 in BD patients were also revealed (Yu et al., 2014).

MiRNAs and their SNPs affect cells and molecules involved in BD, which deepens our understanding of BD pathogenesis and sheds new light on the diagnosis and treatment of BD. More

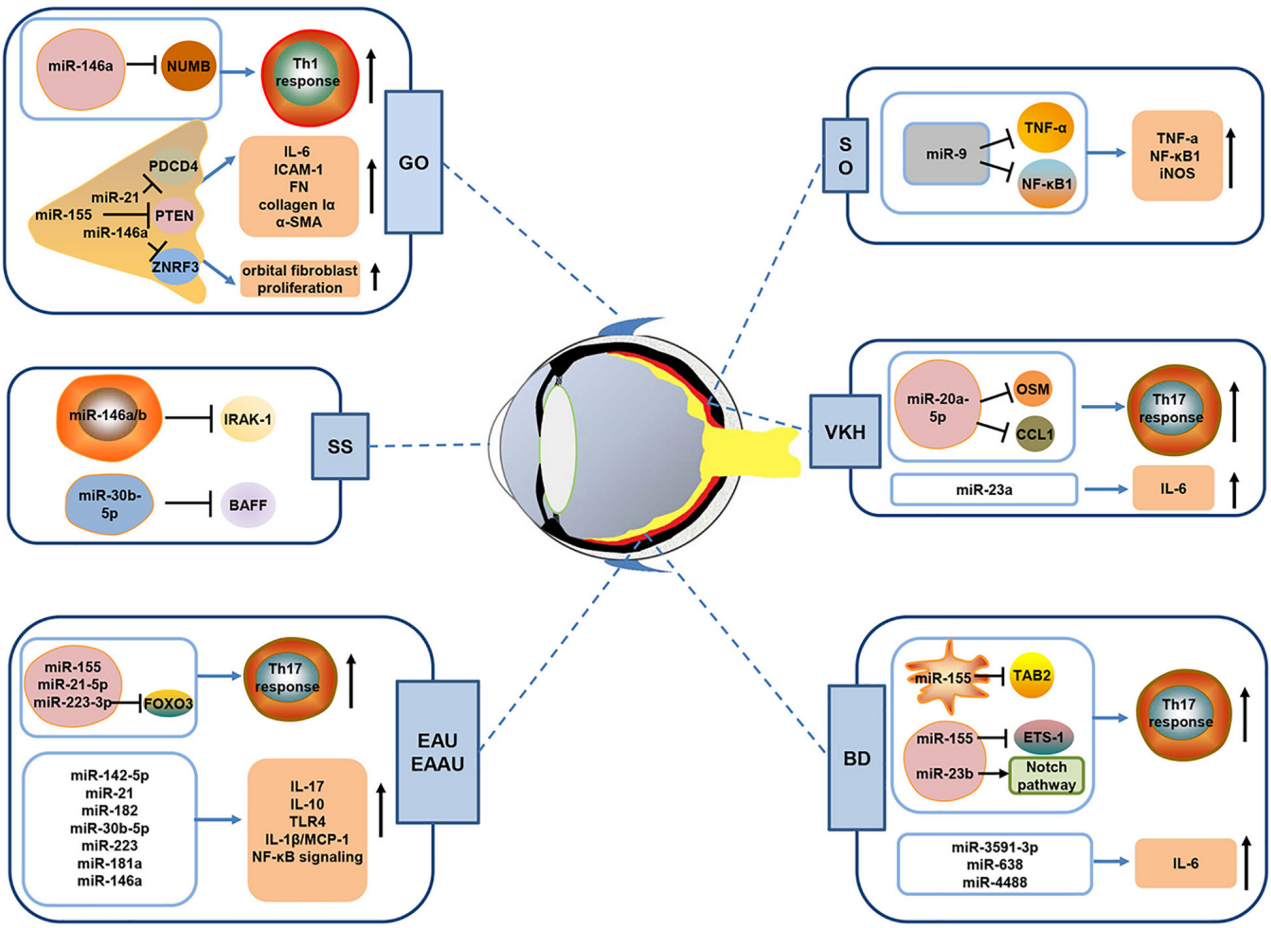

FIGURE 1 | Schematic diagram showing the possible mechanisms of miRNAs in autoimmune-mediated eye diseases. A growing body of evidence has shown that miRNAs are implicated in the pathogenesis of autoimmune-mediated eye diseases including autoimmune uveitis (EAU, EAAU, BD, VKH and SO), Grave's ophthalmopathy (GO), and Sjögren's syndrome (SS) dry eye. As shown in Figure, dysregulated miRNAs can promote the production of pro-inflammatory cytokine and enhance the activity of inflammatory pathways in autoimmune uveitis. Besides, some miRNAs (including miR-223-3p, miR-155, miR-23b, and miR-20a-5p) have been demonstrated to promote Th17 cells response via suppression of their target gene expression. In GO, aberrantly expressed miRNA, such as miR-146a, miR-155, and miR-21, could affect Th1 cell response or orbital fibroblasts proliferation to regulate disease development. In addition, miR-146a/b and miR-30b-5p in SS patients may negatively regulate IRAK-1 and BAFF, which are key molecules that initiate SS development. EAU, experimental autoimmune uveitis; EAAU, experimental autoimmune anterior uveitis; BD, Behcet's disease; VKH, Vogt–Koyanagi–Harada; SO, sympathetic ophthalmia.

in-depth studies are warranted to explore the miRNAs-mediated mechanisms in the development of BD.

MicroRNAs and Vogt–Koyanagi–Harada Syndrome

Vogt–Koyanagi–Harada (VKH) syndrome, one of the sight-threatening diseases, is characterized by bilateral granulomatous uveitis associated with neurological, auditory, and dermatological manifestations, which are presumably caused by T-cell-mediated autoimmune response against melanocyte-associated antigens in multiple organs (Du et al., 2016; Silpa-Archa et al., 2016). Active VKH patients express enhanced Th17 cell responses (Yang et al., 2013). Most recently, Chang et al. reported that the expression of miR-20a-5p was decreased in the CD4 + T cells of active VKH patients, and miR-20a-5p negatively regulated IL-17 production via suppression of the expression of oncostatinM (OSM) and C–C motif chemokine ligand 1 (CCL1) as well as the activity of the PI3K-AKT pathway (Chang et al., 2018), highlighting the involvement of miR-20a-5p in Th17 cell responses during VKH. Moreover, copy number variations of miR-23a, miR-146a, and miR-301a as well as genetic variants of miR-182 have been revealed to confer risk for VKH disease (Yu et al., 2014; Hou et al., 2016). Further functional studies indicated that miR-23a might contribute to the development of VKH syndrome by promoting the production of IL-6 (Hou et al., 2016). miRNA research in the VKH syndrome remains in its infancy, and more profound research is warranted and will facilitate the delineation of novel diagnostic biomarkers and therapeutic targets in VKH.

MicroRNAs and Sympathetic Ophthalmia

Sympathetic ophthalmia (SO) is a rare, granulomatous uveitis found in bilateral eyes, occurring after ocular trauma and

intraocular surgery. It is mainly characterized by acute or chronic uveitis, accompanied by mutton-fat KP in anterior segments and yellow-white lesions of choroid in the posterior segment (Damico et al., 2005; Chang and Young, 2011). The etiology is not clearly understood, but a vast majority of studies indicate that T-cell-mediated autoimmune responses against ocular self-antigens is critically implicated in the pathogenesis of SO (Chan and Mochizuki, 1999).

Although the accumulating data have demonstrated the crucial roles of miRNAs in immune cell function, the role of miRNAs in SO pathogenesis is still largely unknown and only one research group reported this topic. It was shown that four miRNAs including miR-1, let-7e, miR-9, and miR-182, which are associated with T-cell-mediated inflammatory pathway, were downregulated in globes of patients with SO compared to those in controls. Among them, hsa-miR-9 directly targeted proinflammatory TNF-α and NF-κB1, both of which are crucial factors in the pathogenesis of SO (Kaneko et al., 2012). Therefore, the specific role of miRNAs in SO is calling for extensive research.

MICRORNAS AND GRAVE'S OPHTHALMOPATHY

Grave's ophthalmopathy (GO), an extra-thyroidal complication of Graves' disease, is characterized by the inflammation and extensive remodeling of orbital adipose/connective tissues. Its clinical manifestations include exophthalmos, eyelid retraction, strabismus, and exposure keratitis, which may cause cosmetic and functional deficits (Dik et al., 2016). Although the precise pathophysiology of GO remains unclear, increasing evidence has shown this disease may result from the autoimmune reactions in which the sensitive T cells as well as autoantibodies against common antigens [including thyrotropin receptor (TSHR), insulin-like growth factor-1 receptor, thyroglobulin, calsequestrin (CASQ1) and collagen XIII] contribute to the activation and proliferation of orbital fibroblasts (OFs), resulting in extraocular tissues edema and fibrosis (Lahooti et al., 2010; Bahn, 2015; Shanmuganathan et al., 2015; Lacheta et al., 2019). Recently, particular attention has been paid to the role of miRNAs in the pathogenesis of GO.

MiR-146a is a key regulator of orbital tissue fibrosis and GO development. Upregulation of miR-146a in OFs suppressed the production of inflammatory protein [including IL-6 and intercellular adhesion molecule-1 (ICAM-1)] and TGF-β-induced fibrotic markers [fibronectin (FN), collagen Iα and α-smooth muscle actin protein (α-SMA)], suggesting a key role for miR-146a in anti-inflammatory and anti-fibrotic process (Jang et al., 2016, 2018). Conversely, downregulation of miR-146a in CD4 + T cells has been shown to contribute to the development of GO by promoting pro-inflammatory Th1 cytokine production and human T cells proliferation or via targeting NUMB (Hu et al., 2017; Yang et al., 2017). Most recently, Collynn and colleagues revealed that the TSHR signaling in GO patients can enhance OFs proliferation partially via induction of miR-146a and miR-155, and the effects of miR-146a and miR-155 may be due to their suppression on zinc and ring finger 3 (ZNRF3) and phosphatase and tensin homolog (PTEN) that normally limit cell proliferation (Woeller et al., 2019).

In addition to miR-146a, miR-21 also plays a pivotal role in the regulation of fibrosis. Tong et al. (2015) demonstrated that the expression of miR-21 was increased in OFs from patients with GO compared to that in control group. MiR-21 promoted OF proliferation and differentiation and suppressed the apoptosis, which contribute to the fibrosis of extraocular muscles. Moreover, another study revealed that platelet-derived growth factor-BB increased the expression level of miR-21 in OFs, and miR-21 mediated platelet-derived growth factor-BB induced downregulation of programed cell death 4 (PDCD4) in OFs, thereby contributing to cell proliferation and GO development (Lee et al., 2016).

With regard to other miRNAs, decreased miR-27a and miR-27b have been observed in orbital fat tissue from patients with GO. The overexpression of miR-27a and miR-27b leads to a significant reduction in the expression of adipogenesis-related genes such as PPARγ, C/EBPα, and C/EBPβ, suggesting a possible role of miR-27a and miR-27b in adipocyte development (Jang et al., 2019). Additionally, a recent study found that miR-183 and miR-96 were upregulated in CD4 + T cells from peripheral blood of GO patients. miR-183 and miR-96 targeted early growth response protein 1 (EGR-1) to regulate PTEN/Akt signaling, contributing to the activation of CD4 + T cells (Thiel et al., 2019). Besides, let-7b, which was upregulated in PBMCs, serum, and thyroid tissue of patients with Graves' disease, was verified to directly suppress promyelocytic leukemia zinc finger (PLZF) expression and enhance the expression of TSHR in thyroid cells *in vitro* (Chen et al., 2018). Further studies including primary human orbital tissue or animal models are needed to determine the interaction of let-7b and TSHR signaling in GO.

Recently, investigators have linked polymorphisms in thyroid-specific and immune-modulating genes to the susceptibility to GO, which open novel avenues on understanding this disease. For example, Beata et al. found that rs179247 TSHR polymorphism was correlated with lower risk of GO in young GD patients (Jurecka-Lubieniecka et al., 2014). Moreover, the results from Lahooti et al. revealed an association between the CASQ1 SNP rs74123279, rs3838216, and rs2275703 and the development of GO (Lahooti et al., 2015). However, the roles of SNPs in miRNA genes in GO remain unexplored, awaiting further investigation.

MICRORNAS AND SJÖGREN'S SYNDROME DRY EYE

Sjögren's syndrome (SS) is a chronic systemic autoimmune disease, mainly characterized by lymphocytic infiltration of lacrimal and salivary glands, which results in ocular and oral dryness (de Paiva and Rocha, 2015). The pathogenesis of SS is multi-facetted and largely unknown. It has been reported that activation of innate and adaptive immune pathways, including type I IFN pathway, TGF-β/SMAD/Snail signaling pathway, and B cell activating factor (BAFF)/BAFF receptor axis, plays a crucial role in the pathogenesis of SS (Mavragani, 2017; Sisto et al., 2018).

Recently, studies have established a close relationship between miRNAs dysregulation and the pathogenesis of SS.

The altered miRNA expression in immune cells from patients with SS was recently reported. Chen et al. (2017a) indicated 26 miRNAs with aberrant expression pattern in PBMCs from primary Sjögren's syndrome (pSS) patients. Among them, the downregulation of miR-150-5p is a novel finding. In addition, Wang-Renault et al. found that in CD4 + T cells from patients with pSS, miR-let-7d-3p, miR-30c-5p, and miR-378a-3p were significantly downregulated, while miR-155-5p, miR-222-3p, miR-146a-5p, and miR-28-5p were upregulated. In CD19 + B cells, the expression of miR-378a-3p, miR-26a-5p, miR-30b-5p, and miR-19b-3p was reduced in pSS, while miR-222-3p expression was increased (Wang-Renault et al., 2018). Of note, miR-30b-5p was further identified as a negative regulator of BAFF, one of the key molecules that initiate SS development (Nocturne and Mariette, 2013). Moreover, a SS-specific miRNA profile in CD14 + monocytes was also displayed (Williams et al., 2016). Determining the functional contribution of these miRNAs to SS may clarify previously unknown cellular processes and unveil new potential therapeutic targets.

The dysregulation of miR-146a may be associated with the pathogenesis of SS. A previous study reported increased miR-146a expression in PBMCs from both SS patients and Sjs-prone mouse, which may affect innate immunity and contribute to the initiation and progression of SS (Pauley et al., 2011). In addition, Zilahi et al. (2012) found that miR-146a/b expression was upregulated in PBMCs from SS patients compared to that in healthy controls, while IL-1 receptor-associated kinase 1 (IRAK1) expression was downregulated, implying the existence of transcriptional repression of IRAK1 by miR-146a in SS patients. Another study verified the over-expression of miR-146a in PBMCs from SS patients and demonstrated that there was a positive correlation between the expression level of miR-146a and the VAS scores for dry eyes (Shi et al., 2014). All these studies suggest that upregulation of miR-146a may contribute to the development of SS, albeit the underlying mechanism need to be further determined.

Regarding other miRNAs, Shi et al. (2014) found that the expression of miR-155 was reduced in PBMCs from untreated Asian pSS patients. On the contrary, another two studies found that the expression of miR-155 was elevated in PBMCs from European and American pSS patients (Pauley et al., 2011; Chen et al., 2017b). The opposite findings may be due to different inclusion criteria for patients and distinct sample size applied in the studies. Additionally, Peng et al. (2014) revealed the upregulation of miR-181a and multiple virus-derived miRNAs in PBMCs from Chinese patients with pSS, indicating the possible role of miR-181a and virus infection in pSS. Therefore, the changes of miRNA expression in pSS patients indicate the potential clinical implications of miRNAs in this disease. However, the underlying mechanisms that regulate miRNA expression and the roles of deregulated miRNAs in SS pathogenesis remain to be defined.

CIRCULATING MICRORNAS AS BIOMARKERS FOR AUTOIMMUNE-MEDIATED EYE DISEASES

The potential value of miRNAs as biomarkers has gained tremendous interests in recent years. The disease-specific expression pattern makes some of them suitable biomarker candidates. Owing to their encapsulation in extracellular vesicles (EVs) or association with RNA-binding proteins, miRNAs found in various body fluids are highly stable (Schwarzenbach et al., 2014). Moreover, they are easily measured with the advances in detection technology such as microarray and deep sequencing (Schwarzenbach et al., 2014). It has been found that some miRNAs present in biofluids exhibit altered levels in ocular autoimmune disorders. As evidenced by Wei et al. (2014), miR-146a was downregulated in the serum of patients with active GO, with a negative correlation between the miR-146a level and clinical activity score. Ibrahim et al. (2019) found that elevated serum levels of miR-146a were closely associated with eye activity of BD patients, implying its diagnostic value. The discrepancy in the expression patterns of serum miR-146a in active GO and BD patients may be attributed to the complex mechanisms of the different ocular autoimmune diseases. In addition to serum, tear is also applicable as a source for circulating miRNAs. Recently, Kim et al. (2019) revealed four miRNAs being upregulated and 10 miRNAs being downregulated in tear samples of SS patients, indicating that tear miRNAs may provide clues to the pathogenesis of lacrimal gland dysfunction in SS patients. Hence, it seems that finding of new circulating miRNAs shows significant promise for the diagnosis or understanding of biology processes in autoimmune-mediated eye diseases. As a variety of issue, with respect to miRNAs, are addressed, circulating miRNAs may be applied as non-invasive biomarkers for clinical practice in the near future (Zhang et al., 2020).

MICRORNAS AS THERAPEUTIC TARGETS FOR AUTOIMMUNE-MEDIATED EYE DISEASES

MicroRNAs are emerging as potential molecular targets for the treatment of a variety of diseases owing to their unique expression profiles, crucial regulatory functions, and target specificity (Lu et al., 2019). Currently, in the cancer, heart disease and diabetes field, miRNA-based therapeutics have entered clinical trials stage (Li and Rana, 2014; Takahashi et al., 2019). To use miRNAs as therapeutic agents for ocular autoimmune diseases, it is required to maintain their stability and deliver them to ocular tissues efficiently. Adenovirus-mediated gene expression has efficiently transduced foreign genes into ocular tissues (Mallam et al., 2004), making adenovirus vectors attractive for delivery of miRNAs in the treatment of uveitis. A recent study by Shi et al. (2019) found that subretinal

injection of anti-miR-21-5p adenovirus alleviated retinal injury and apoptosis in EAU mice by increasing IL-10 and decreasing IL-17, TNF-α and IFN-γ production. Moreover, Hsu et al. (2017) showed that administration of locked nucleic acid miR-146a mimics via intravitreal injection effectively dampened intraocular inflammation in EAAU. The therapeutic effects may partly be ascribed to increased miR-146a mimics stability by locked nucleic acid modification and good intraocular concentration provided by intravitreal injection. More recently, exosomes have been shown to be potential therapeutics through RNA transfer mechanisms (Tran et al., 2019), leading to their emergence as promising vehicles for delivering therapeutic miRNAs. Studies have demonstrated that mesenchymal stem cell (MSC)-derived exosomes could be manipulated to deliver miRNAs to exhibit their therapeutic potential (Che et al., 2019; Lou et al., 2020). However, the research on MSC-derived exosomes as carriers of miRNAs to treat autoimmune-mediated eye diseases remains in the early stage, calling for more extensive investigations.

CONCLUSION

MicroRNA alterations are closely associated with the pathogenesis of ocular autoimmune disorders. Due to the important roles of miRNAs in regulating inflammation and immune response, miRNAs can be potentially therapeutic targets for autoimmune-mediated eye diseases. Nevertheless, the exact roles of most miRNAs and the underlying mechanisms have not been clarified, requiring further investigation. Additionally, for successful translation to clinical therapies, it is necessary to develop safe and effective delivery system that can transport therapeutic miRNAs specifically to target sites.

AUTHOR CONTRIBUTIONS

YW read the literature related to the topic and participated in drafting the manuscript. NL, LZ, and CY participated in searching and archiving the literature related to the topic and discussed the contents of the manuscript. BM, XL, and RW revised the manuscript. HN participated in the design, revision, and final approval of the manuscript. All authors read and approved the final manuscript.

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Abundant Neural circRNA Cdr1as Is Not Indispensable for Retina Maintenance

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Cdr1as is the abundant circular RNA (circRNA) in human and vertebrate retinas. However, the role of Cdr1as in the retina remains unknown. In this study, we aimed to generate a Cdr1as knockout (KO) mouse model and investigate the retinal consequences of Cdr1as loss of function. Through *in situ* hybridization (ISH), we demonstrated that Cdr1as is mainly expressed in the inner retina. Using CRISPR/Cas9 targeting Cdr1as, we successfully generated KO mice. We carried out ocular examinations in the KO mice until postnatal day 500. Compared with the age-matched wild-type (WT) siblings, the KO mice displayed increased b-wave amplitude of photopic electrophysiological response and reduced vision contrast sensitivity. Through small RNA profiling of the retinas, we determined that miR-7 was downregulated, while its target genes were upregulated. Taken together, our results demonstrated for the first time that Cdr1as ablation led to a mild retinal consequence in mice, indicating that Cdr1as abundance is not indispensable for retinal development and maintenance.

Keywords: retina, circular RNA, CDR1as, retinal function, knockout

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INTRODUCTION

Circular RNAs (circRNAs) are a class of stable, covalently closed RNA molecules produced from precursor mRNAs (pre-mRNAs) through back-splicing reactions (Jeck et al., 2013; Lasda and Parker, 2014; Ebbesen et al., 2017). Recently, a large number of circRNAs with complex tissue- and stage-specific expression patterns have been identified in eukaryotes (Salzman et al., 2013; Guo et al., 2014; Veno et al., 2015). Recent studies have demonstrated the biological functions of circRNAs at the molecular level (Chen, 2016; Li et al., 2018), including sequestration of microRNAs or associated proteins (Hansen et al., 2013; Li et al., 2017), splicing interference of their linear cognates (Ashwal-Fluss et al., 2014), modulation of transcription of parent genes (Li et al., 2015), and translation to produce peptides (Wang and Wang, 2015). However, considering the low efficiency of back-splicing and special structure of circRNAs, the function of most individual circRNAs remains elusive.

Circular RNAs are reported to be highly enriched in the central nervous system (CNS) and regulate synaptic function (Rybak-Wolf et al., 2015; You et al., 2015). As the most extensively

characterized circRNA, Cdr1as is highly abundant in neurons and acts as a post-transcriptional regulator with many conserved binding sites for miR-7 and miR-671 (Hansen et al., 2013; Memczak et al., 2013). Expression of human Cdr1as in zebrafish caused midbrain defects, similar to miR-7 knockdown, and Cdr1as was once considered to possess important regulatory function (Memczak et al., 2013). However, Cdr1as knockout (KO) mouse, the first and sole circRNA KO mouse model, displayed a mild neuropsychiatric phenotype reflected in sensorimotor gating deficit and dysfunctional synaptic transmission (Piwecka et al., 2017).

The retina is a key part of the CNS and is responsible for vision production. As the abundant circRNA in the retina, Cdr1as is upregulated during retinal development (Chen et al., 2020), indicating the regulatory potential of Cdr1as in the retina. Whether and how Cdr1as affects retinal function remains unknown. To address this question, we generated a Cdr1as KO model using the CRISPR/Cas9 strategy. We found that Cdr1as deletion in mice caused mild alterations both in retinal phenotypic and vision functions as late as P300. In addition, miR-7, its target genes, and immediate early genes (IEGs) were deregulated in the Cdr1as KO retina. Our results demonstrated for the first time that Cdr1as ablation led to a mild retinal consequence in mice, indicating that Cdr1as abundance is not indispensable for retinal development and maintenance.

RESULTS

Spatial Expression Pattern of Cdr1as in Retina and Other Tissues

It has been reported that Cdr1as is highly abundant in neurons, but is scarce in other tissues (Piwecka et al., 2017). To validate its spatial expression pattern, we investigated the expression of Cdr1as in different tissues by quantitative RT-PCR (qRT-PCR). As expected, Cdr1as was highly expressed in the brain and retina, and was expressed at low levels in other tissues, such as the lung, heart, liver, spleen, kidney, and muscle (**Supplementary Figure 1A**). To evaluate the localization of Cdr1as, we performed *in situ* hybridization (ISH) in the mouse retina at P120 using the BaseScope assay (**Supplementary Figure 1B**). We found Cdr1as located in the inner retina, predominantly in the inner nuclear layer (INL) near the inner plexiform layer (IPL). ISH and immunofluorescence (IF) staining demonstrated a portion of Cdr1as expressed in TH+ amacrine cells, which could release dopamine neurotransmitter (**Supplementary Figure 1C**). Together with our previous findings that Cdr1as increased sharply from P1 to P14 (**Supplementary Figure 1D**), these results indicated the potential role of Cdr1as during retinal development. However, we could not find any obvious abnormal phenotype during the retinal development, these may due to the compensation mechanisms in organisms.

Successful Knockout of Cdr1as in Mice

To investigate the role of Cdr1as in the retina, we attempted to generate KO mice by using CRISPR/Cas9. Because the linear transcript of Cdr1as cannot be detected, it is feasible to explore the function of Cdr1as using a KO mouse model without concerning the phenotype caused by linear transcript interruption. In this study, we used the CRISPR/Cas9 strategy to generate the Cdr1as KO mouse model. Two sgRNAs were designed to bind upstream and downstream of Cdr1as splice sites (**Figure 1A** and **Supplementary Table 1**). The F1/R1 and F1/R5 primer pairs capable of distinguishing homozygotes from the heterozygotes and wild-type (WT) were designed for genotyping (**Figure 1B** and **Supplementary Table 2**). The head-to-tail junction sequence was confirmed by Sanger sequencing (**Supplementary Figure 2**). qRT-PCR experiments showed that Cdr1as disappeared in retinal and brain tissues of KO mice (**Figure 1C**). Additionally, ISH further validated the successful deletion of Cdr1as in the retina of KO mice (**Figure 1D**). The overall survival and life span of mice revealed no difference between Cdr1as KO and WT mice.

Cdr1as Ablation Does Not Alter Retinal Structure

To assess the retinal structure in Cdr1as KO mice, fundus photography and high-resolution spectral-domain optical coherence tomography (SD-OCT) were performed to detect the retinal morphologies and organization in adult and aged mice. Fundus photographs showed normal fundus appearances in both WT and KO mice at P70, P360, and P500 (**Figure 2A** and **Supplementary Figure 3A**). *In vivo* OCT imaging displayed that the inner retinal layer was unaltered obviously, even though a few changes happened at certain locations in Cdr1as KO mice (**Figure 2B** and **Supplementary Figures 3B–D**). We further examined the photoreceptor, retinal neurons, and synaptic structure by IF staining in adult mice. Photoreceptor cells, immunolabeled for recoverin and cone-arrestin, showed a preserved pattern in Cdr1as KO as in WT retinas. Consistent with this, immunostaining for calbindin, pkcα, pax6, and rbpms to label horizontal cells, bipolar cells, amacrine cells, and ganglion cells were also unchanged with the Cdr1as deletion. Additionally, immunostaining patterns for synaptic vGlut1, α-synuclein, and ctbp2 demonstrated similar patterns in the plexiform layer (**Figure 2C** and **Supplementary Figures 4A–C**).

Cdr1as Knockout Resulted in Slight Changes of Retinal Function

To investigate the impact of Cdr1as deletion on visional function, we first monitored electroretinography (ERG) responses in adult and aged mice. The b wave amplitudes of both scotopic and photopic ERG response were unchanged at P150 and P300 (**Figures 3A,B**). However, ERG results of elder mice (P500) showed that the photopic b wave amplitude increased significantly (**Figure 3C**). This phenotype is similar to the effects mediated by dopamine D2 receptor (D2R) KO in mice (Lavoie et al., 2014; Tian et al., 2015) and D2R antagonist in goldfish (Kim and Jung, 2012) and cats (Schneider and Zrenner, 1991). Indicating the function of dopamine impaired in Cdr1as KO mice. Due to partial Cdr1as expressed in certain amacrine cells, which could release dopamine. Cdr1as deletion

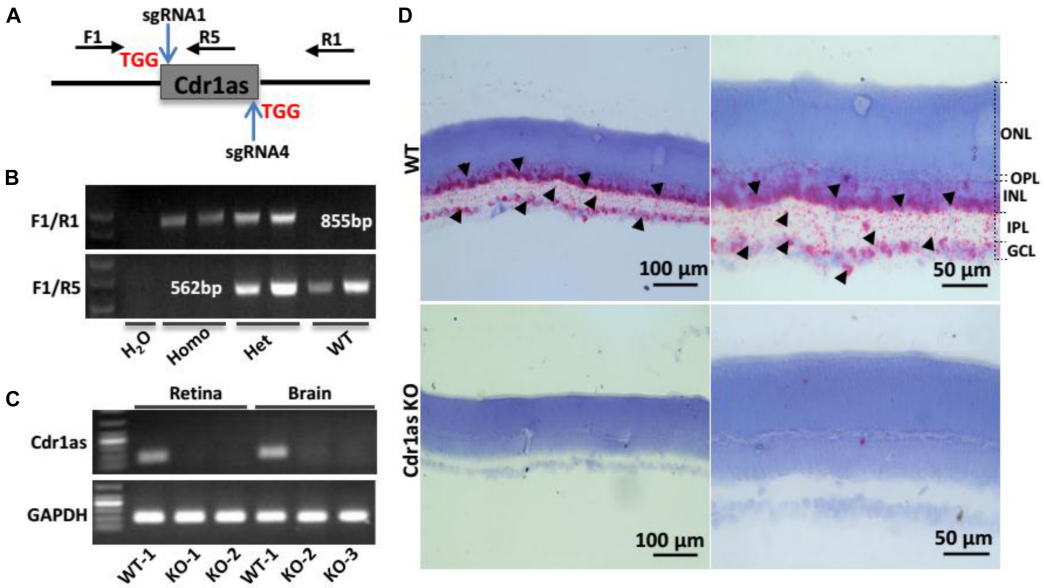

FIGURE 1 | Generation and identification of Cdr1as KO mice. **(A)** Cdr1as was removing using CRISPR/Cas9 strategy, and two sgRNAs targeting different regions of Cdr1as were designed. PAM sequences were labeled in red. **(B)** Genotyping strategy for the WT, Hete, and Homo strains using F1/R1 and F1/R5, which are shown in **(A)**. **(C)** QPCR analysis of Cdr1as expression in the retinas and brains of WT and Cdr1as KO mice at P30. **(D)** BaseScope assay showing the expression of Cdr1as in WT and Cdr1as KO retinas at P30; red dots show the sense of ISH image, which has been labeled by black arrowhead. ONL, outer nuclear layer; OPL, outer plexiform layer, INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

results in abnormal function of amacrine and then further affects dopamine function. The major ERG wave components, oscillatory potentials (OPS), which are displayed by certain amacrine cells in the retina were also analyzed at P240. As a result, there was no significantly difference between WT and KO mice, even though the slight lower amplitude of OPS was observed in Cdr1as KO mice (**Supplementary Figure 3E**). Considering that Cdr1as is expressed in the inner retina, the visual evoked potential (VEP) was applied to assess the electric conduction from the retina to visual cortices at P240 and P500. The amplitudes from the N1 to P1 peak in Cdr1as KO mice were slightly decreased, but no difference was observed in two groups (**Supplementary Figure 3F**).

Additionally, an optomotor response (OMR) assay was performed to assess the animal photopic response to a visual stimulus at P360 and P500. We found that both Cdr1as KO and WT mice responded to the rotating grating through head tracking, the vision contrasts sensitivity showed no significant alteration at P360 but decreased significantly at P500 (**Figure 3D**). The reduced vision acuity may be due to the reduction of dopamine in the retina, which has been reported previously (Witkovsky, 2004). Taken together, these results indicated that the deletion of Cdr1as has a very mild effect on visual function.

Retinal miRNAs and Their Target Genes Are Deregulated in Cdr1as KO Retinas

Following the assessment of the retinal structure and function in Cdr1as KO mice, molecular phenotypes were further investigated. Considering the strong sponge efficiency of Cdr1as for miRNAs, we evaluated the expression pattern of retinal miRNAs in Cdr1as KO mice. Whole-retinal small RNA-seq from adult mice showed that the expression of 25 miRNAs, such as miR-7, miR-344c, miR-322, miR-326, miR-122, and miR-7048 was significantly changed (fold change $>$ 1.5, $p < 0.05$) between the Cdr1as KO and WT groups (**Figure 4A**), miR-7, the highly expressed miRNA, was confirmed by qRT-PCR and was downregulated in Cdr1as KO retinas (**Figure 4B**). The miR-7 target genes we tested, including Nr4a3, Klf4, Irs2, α-synuclein, and c-fos, displayed an increasing expression tendency (**Figure 4C**). Among the target genes, Nr4a3 and c-fos are IEGs, which have been linked to neural activity and could respond to different stimuli (Caputto and Guido, 2000; Piwecka et al., 2017). Other IEGs, such as Egr1, Egr4, and Arc, were also upregulated in KO retinas (**Supplementary Figure 5**). These results suggested that degeneration in Cdr1as KO retinas was more likely to occur under certain stimuli.

In addition, we performed the GO and KEGG to analyze the 1686 target genes of 25 miRNAs that changed significantly. As was shown in **Figure 5A**, several GO terms were found to be significantly enriched, including nervous system development, regulation of axon regeneration, and neuron projection development, suggesting that some biological processes of the neuron system may be affected in the KO mice. The subsequent KEGG pathway analysis showed that PI3K-Akt, MAPK, Hif-1, Wnt, mTOR, and other important signaling pathways were significantly enriched (**Figure 5B**).

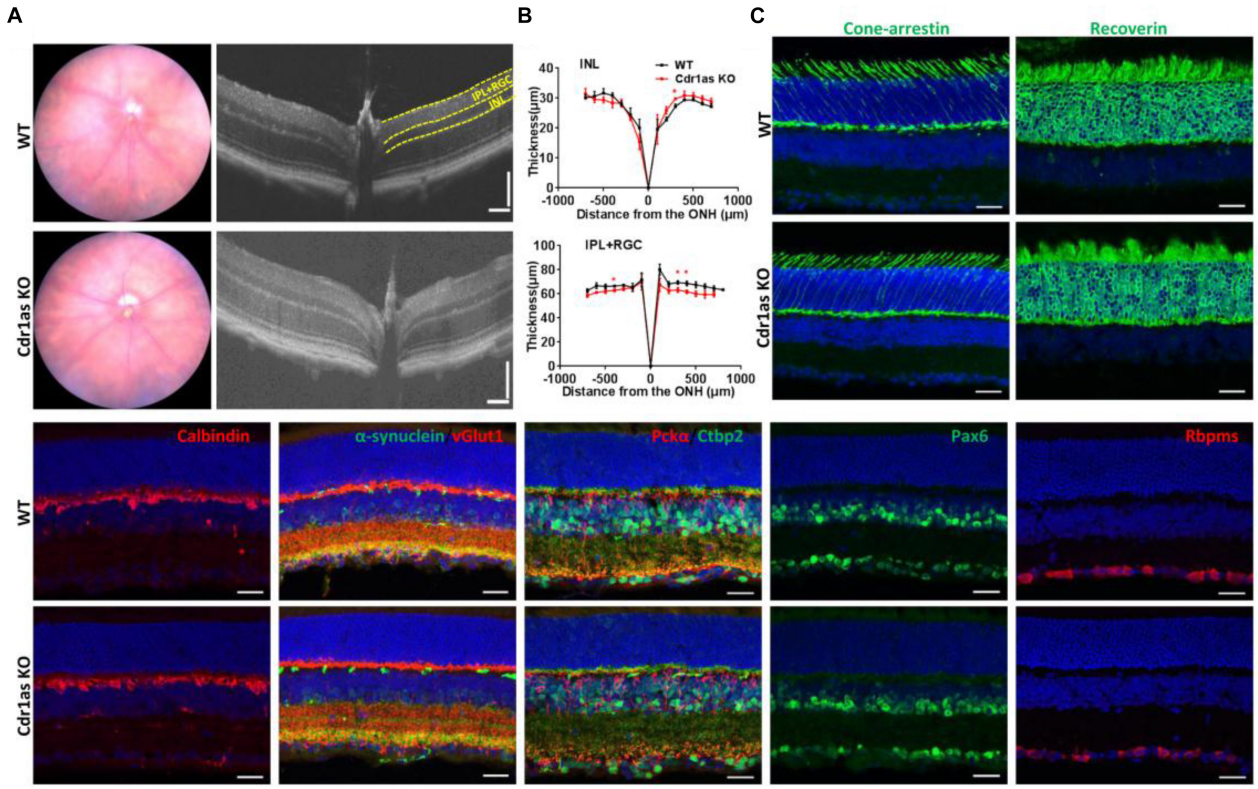

FIGURE 2 | Structural phenotypes of retina in the adult Cdr1as KO mice. **(A)** Fundus photographs of WT and Cdr1as KO retinas in adult (n = 4) (left), OCT examination of WT and Cdr1as KO retinas at P70 (right). **(B)** Quantification of the thickness of the INL and IPL+RGC from the SD-OCT image in **(A)** (n = 5 for WT, n = 4 for KO). **(C)** Immunostaining of retinal neurons and synapses in WT and Cdr1as retinas (n = 3, scale bar: 25 μm).

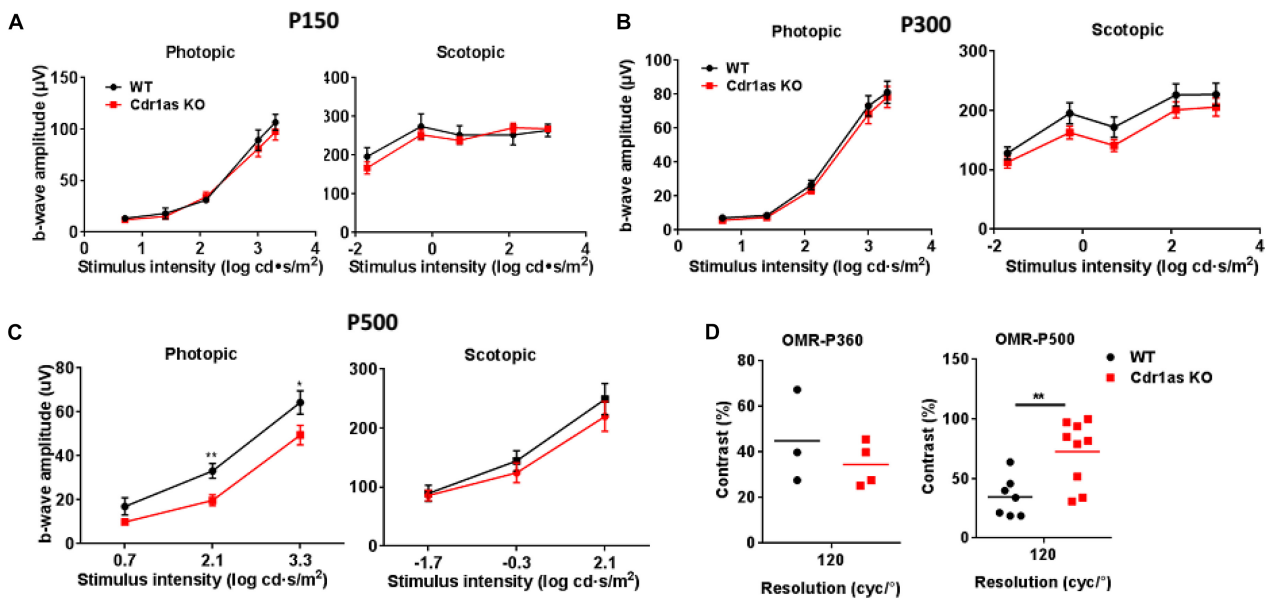

FIGURE 3 | Assessment of retinal function in the adult and aged Cdr1as KO mice. **(A)** b-Wave amplitudes of scotopic and photopic for the Cdr1as KO and WT retinas at P150 (n = 5 for WT, n = 6 for KO). **(B)** b-Wave amplitudes of scotopic and photopic for the Cdr1as KO and WT retinas at P300 (n = 13 for WT, n = 13 for KO). **(C)** b-Wave amplitudes of scotopic and photopic for the Cdr1as KO and WT retinas at P500 (n = 10 for WT, n = 10 for KO). **(D)** OMR examination of Cdr1as KO and WT mice at P360 (n = 3 for WT, n = 4 for KO) and P500 (n = 7 for WT, n = 9 for KO). The normalized values represent means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, Student's t-test.

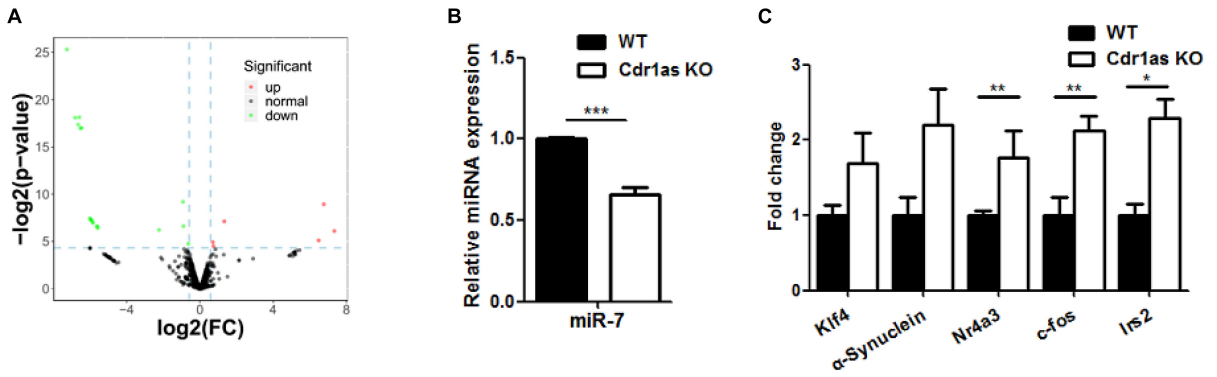

FIGURE 4 | miRNAs and genes expression changes in Cdr1as KO retina. **(A)** Differentially expressed genes between Cdr1as and WT retinas in adult mouse (n = 3). The red dots represent the upregulated genes, and green dots represent the downregulated genes. **(B)** miR-7 expression changes in Cdr1as and WT retinas (n = 3). **(C)** Changes in the expression of target genes of miR-7 in Cdr1as and WT retinas (n = 3). The normalized values represent means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's t-test.

DISCUSSION

In this study, we successfully generated a Cdr1as KO mouse model and first explored the function of Cdr1as in the retina. Despite of the seemingly normal appearance, Cdr1as KO mice had mild retinal phenotypes. We found that miR-7, which is highly sponged by Cdr1as, was downregulated and IEGs were upregulated (**Supplementary Figure 5**). These data suggested that Cdr1as KO retinas might be more vulnerable to degenerative alterations. The function of Cdr1as retina could be studied under different stimuli, such as light and circadian regulation.

The localization of Cdr1as was partially similar to that of α-synuclein (Surguchov et al., 2001; Chandra et al., 2005), which is aggregated in Parkinson's disease retinas. miR-7 was reported to repress α-synuclein expression and protect cells against oxidative stress (Junn et al., 2009). We also found phospho-α-synuclein increased in Cdr1as KO retina (**Supplementary Figure 6**). Whether α-synuclein aggregation induced by miR-7 downregulation could cause the retina to be more vulnerable to stress needs further study.

Among the downregulated miRNAs in Cdr1as KO retinas, miR-326, miR-322, miR-344, miR-7048, and miR-122 should be mentioned here. CDC42 which is a member of the Rho GTPase family is a target of miR-326, and CDC42 dysregulation is linked to neuronal diseases such as Alzheimer's and Parkinson's disease (Zhu et al., 2000; Mitchell et al., 2007). CDC42 was upregulated in hippocampal neurons of Alzheimer's patients (Zhu et al., 2000). Abnormal CDC42 may lead to altered retinal function. RNA helicase Ddx3x was found to be the direct target of miR-322 (Che et al., 2019). Ddx3x was reported to be essential for the even distribution of cells across layers, and Ddx3x was shown to be involved in the network that could control miR-183C accumulation in the photoreceptor layer architecture (Krol et al., 2015). Dysregulation of Ddx3x may contribute to the abnormalities in retinal function. miR-344 directly targets glycogen synthase kinase 3 beta (GSK3β) (Chen et al., 2014), which was reported to regulate Wnt signaling via phosphorylation of β-catenin (Cohen and Frame, 2001). Additionally, inactivation of GSK3β led to β-catenin stabilization and MG proliferation without retinal injury (Yao et al., 2016). miR-7048 and its target Ascl1 were involved in enhanced neuronal regeneration after injury (Lisi et al., 2017). miR-122 was downregulated in canine models of retinal degeneration (Kok et al., 2015). miR-122 was also reported to be downregulated in extracellular vesicles from ARPE19 cells under oxidative conditions (Genini et al., 2014). We speculate that dysregulated miRNA-induced target genes alteration could have some effects on the retina. We also predicted the targets of 25 miRNA that changed significantly using seven tools and further performed bioinformatics analysis based on these 1686 target genes. GO and KEGG pathway analysis showed that these genes were related to neuron system and several important signaling pathway. These results broadly reflected the biological effect of Cdr1as in the retina.

There are several unknowns in the present study. First, since the location of Cdr1as is mainly in the inner retina while the ERG response is derived from photoreceptors to bipolar and amacrine cells, it seems that ERG is not a reasonable method to assess the retinal function of Cdr1as KO retinas. In contrast, the VEP results in our study seemed to be more believable compared to the ERG data. Second, we do not know if any ultrastructural changes in the retina, which could be done by electron microscopy analysis in the future. Third, the abundant expression of Cdr1as in retina seems to be mismatch with the mild retinal phenotype in the Cdr1as KO mice.

As mentioned above, Cdr1as is the most abundant in the mammalian brain, followed by the retina and is expressed at least level or absent in other tissues. It has been reported that loss-of-function Cdr1as *in vivo* causes miRNA deregulation, mild physiological consequences, and impaired sensorimotor gating (Piwecka et al., 2017). In contrast, the investigation of Cdr1as in zebrafish experiments showed the potential regulatory role of Cdr1as (Memczak et al., 2013); these differences might be due to the different experimental approaches (Oltra et al., 2019). Additionally, the slight effect of Cdr1as on the brain and retina

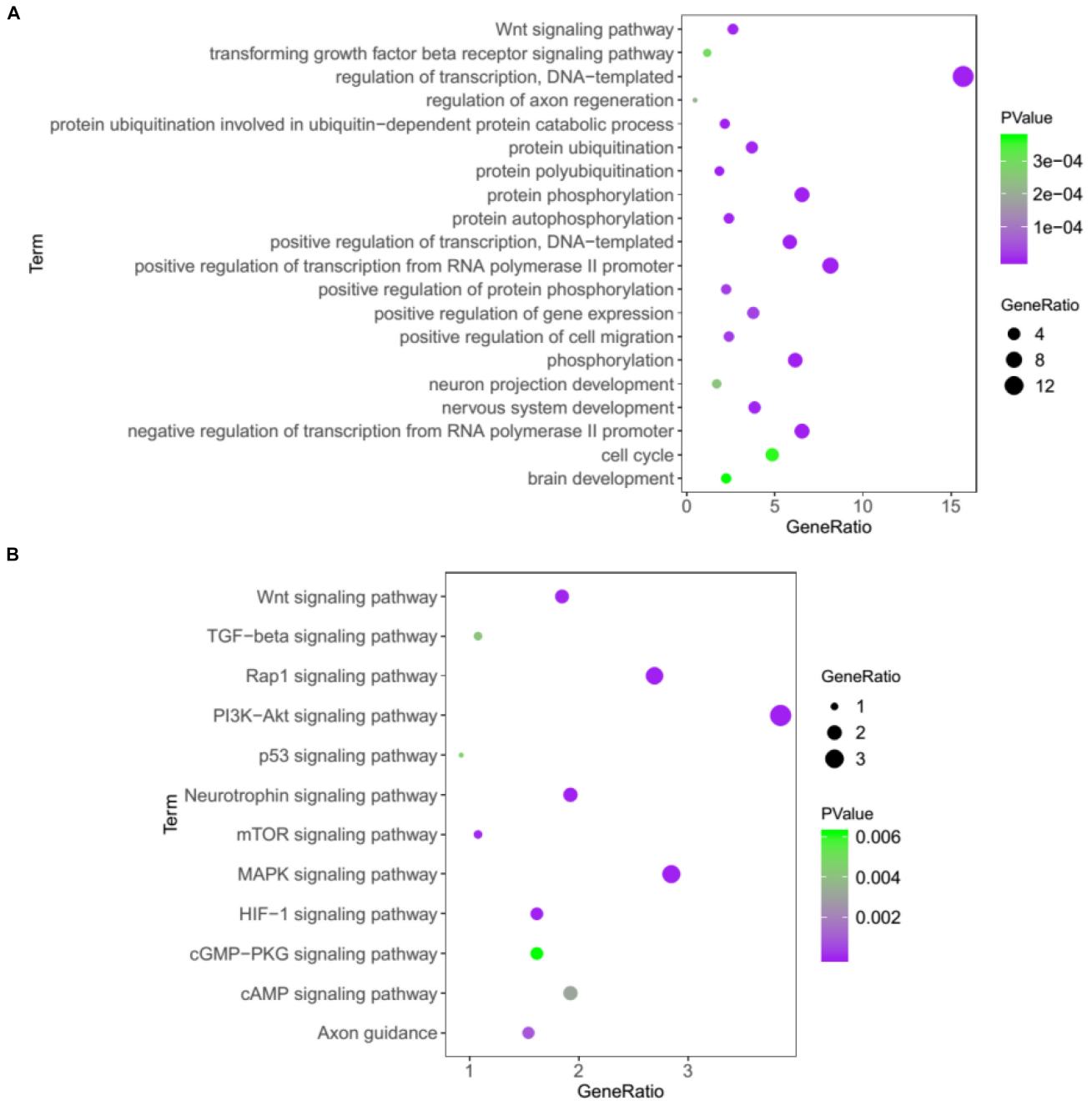

FIGURE 5 | Bioinformatics analysis of the target genes of 25 miRNAs that changed significantly in Cdr1as KO retina using GO **(A)** and KEGG pathway analysis **(B)**.

further confirmed that as epigenetic factors, the regulation of some circRNAs or miRNAs is typically relatively minor, even though their high expression in eukaryotes (Jin et al., 2009; Piwecka et al., 2017; Kleaveland et al., 2018).

Taken together, we demonstrated for the first time that Cdr1as is abundantly expressed in the inner retina and that its KO altered retinal miRNA expression patterns as well as the expression of their target genes but had a slight influence on retinal morphogenesis and function.

MATERIALS AND METHODS

Animals

The Cdr1as KO mice and C57BL/6J mice were bred and maintained in the animal facility of Wenzhou Medical University with a 12-h light/12-h dark cycle and had free access to food and water. All experiments and procedures about mice were approved by the Institutional Animal Care and Use Committee.

Cdr1as KO Mice

The Cdr1as KO mice were generated and maintained on the C57BL/6J background with CRISPR/Cas9-mediated genome editing technology. The sgRNAs (sgRNA1 and sgRNA4) to mouse Cdr1as and Cas9 mRNA were co-injected into fertilized mouse eggs to generate targeted KO offspring. F0 founders were identified by PCR followed by sequence analysis, which were bred to WT mice to test germline transmission and F1 animal generation.

Fundus Photography and High-Resolution Spectral-Domain Optical Coherence Tomography (SD-OCT)

Cdr1as KO and C57BL/6J mice were anesthetized intraperitoneally with pentobarbital sodium. Before the examination, 2.5% hydroxypropyl methylcellulose was dropped into the eyes to improve the connection with the machine (Micron IV, Phoenix Research Labs), and then fundus photography was performed. For SD-OCT measurements, images crossing through the optic nerve were obtained and collected for each eye. The thicknesses of the different retinal layers were measured using Insight software (Pleasanton, CA, United States).

Electroretinography (ERG)

Electroretinography responses in both eyes of mice were carried out as described in the instrument manual (Phoenix Research Laboratories) and as previously described (Jin et al., 2014; Xiang et al., 2017). In brief, mice were dark-adapted overnight and then anesthetized intraperitoneally with pentobarbital sodium. Pupils were dilated with 0.5% tropicamide. A drop of 1% methylcellulose was applied on the cornea to improve the conjunction with the gold wire loop electrode. Ground electrodes and referential needles were punctured into the tail and scalp, respectively. Scotopic ERG was recorded at −2.2 and 0.3 log cd-s/m^2 stimulus intensity with a 30-s interstimulus interval. Photopic ERG was measured at 0.65 log cd-s/m^2 with a 0.4-s interstimulus interval after 10 min of light adaptation with a background illumination of 30 cd/m^2.

Immunofluorescence Staining

Whole eyeballs of mice were extracted immediately after euthanasia. After removing the cornea and lens, the eyecups were fixed in 4% paraformaldehyde for 2 h. Then, retinas were dehydrated in 30% (wt/vol) sucrose and then embedded in embedding medium (Neg-50, Thermo). Sections with 12-μm-thick cryosection slides were cut and washed with PBS, blocked in blocking buffer [4% bovine serum albumin (BSA), 0.5% Triton X-100 in PBS] for 1 h, treated with primary antibody at 4°C overnight, and then incubated with secondary antibody at room temperature for 1 h. The following primary and secondary antibodies were used: mouse anti-Rhodopsin (1:500, Sigma), rabbit anti-Cone-arrestin (1:50, Millipore), mouse anti-Calbindin (1:200, BD), rabbit anti-Recoverin (1:500, Millipore), mouse anti-α-synuclein (1:200), guinea pig anti-Vglut1 (1:200, Millipore), rabbit anti-Pkcα (1:100, Sigma), mouse anti-Ctbp2 (1:100, BD Biosciences), mouse anti-Ctbp2 (1:200, BD), rabbit anti-Pax6 (1:200, Sigma), mouse anti-Rbpms(1:50, Santa Cruz) and donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (1:200, Life Technologies), donkey anti-rabbit IgG conjugated to Alexa Fluor 594 (1:200, Life Technologies), donkey anti-mouse IgG conjugated to Alexa Fluor 594 (1:200, Li-cor Biosciences), and goat anti-guinea pig IgG conjugated to Alexa Fluor 568 (1:200, Abcam). Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, 1:3000, Invitrogen, Carlsbad, CA, United States), Morphologies of the stained retina were imaged using a Leica SP8 laser scanning confocal microscope (Leica, Wetzlar, Germany).

In situ Hybridization

BaseScope is an efficient method of ISH. ACdr1as probe targeting the junction site was designed and BaseScope™ Reagent Kit—RED supplied by Advanced Cell Diagnostics (ACD) was used. Slides of mouse retinas were dried completely at RT and treated with hydrogen peroxide for 10 min and then protease plus for another 30 min at room temperature; then, probe hybridization was performed strictly according to the manufacturer's protocol. Images were acquired using a microscope (Nikon Eclipse).

Visual Evoked Potential (VEP)

Visual evoked potential was recorded using the same equipment as ERG. After 5 min of dark adaptation in cages, the mice were anesthetized as previously described. Pupils were dilated with 0.5% tropicamide. A drop of 1% methylcellulose was applied on the cornea to avoid eye drying. During each VEP session, body temperature was maintained at 37°C using a heating pad. The active electrode was positioned on the back of the head at the location of the visual cortex; the reference electrode was put into the cheek, and the ground electrode was placed into the tail. VEPs were evoked by continuousflash of 1.4 and 2.0 Hz, 5 cds/m^2 white light. VEP signals were recorded by a commercial system (RETIport, Roland Consult GmbH, Germany).

Optomotor Response (OMR)

Mice were placed on a raised platform surrounded by a motorized drum with vertical black and white stripes; the drum could rotate clockwise or anticlockwise. The stripe pattern slowly rotated around the animal at a speed of 12°/s, and triggered the optomotor reflex. Animal behavior was monitored by camera, and the behavior was automatically detected and then analyzed by OptoDrum software (Striatech, Germany). The stimulus pattern was continuously and automatically adjusted during the experiment to find the Cdr1as KO and WT mouse visual thresholds (visual acuity or contrast sensitivity).

RNA Isolation, qRT-PCR, and Small RNA Sequencing

The retinas were isolated from mice and collected in TRIzol (Invitrogen, United States). RNAs were extracted using the RNeasy Kit (Qiagen). For miRNA, qRT-PCR was carried out using the Bulge-Loop miRNA qRT-PCR Starter Kit according

to the manufacturer's protocol. For mRNAs, complementary DNA (cDNA) was synthesized using random primers (Promega) and quantified by FastStart Universal SYBR Green Master Mix (Roche). GAPDH was used as the reference gene. Primers for genes were listed in **Supplementary Table 3**. For small RNA sequencing, retinas were isolated from 2M WT and Cdr1as KO mice (n = 3); the integrity and quantity of RNA was assessed using Agilent 2200 TapeStation and Qubit2.0, respectively. Small RNA libraries were constructed and sequenced by HiSeq 2500 (Illumina, United States) at Ribobio Co. Ltd. (Ribobio, China). miRDeep2 was used to identify known mature miRNA based on miRBase21 and predict novel miRNAs. The expression levels of miRNAs were normalized by RPM, RPM = (number of reads mapping to miRNA/number of reads in clean data) $\times$ 10^6. Differential expression between WT and Cdr1as KO retina was calculated by edge R algorithm according to the criteria of $|$ Fold Change$|$ $\geq$ 1.5 and P-value $<$ 0.05.

GO and KEGG Pathway Analysis

GO and KEGG pathway analysis was based on the targets of different expressed 25 miRNAs in Cdr1as KO retina. The target genes of miRNAs were predicted using seven tools, including PITA[1], RNA22[2], miRNAmap[3], microT[4], miRanda[5], PicTar[6], and TargetScan[7]. Target genes predicted by at least three tools and verified by CLIP assay (Zhou et al.; Li et al., 2014) were retained for further GO and KEGG pathway analysis[8].

Western Blots

Retinas (P300) were isolated, collected, and lyzed in lysis buffer containing 1× PMSF. Protein was then extracted and quantified using a BCA protein assay kit (Invitrogen). Proteins were separated using SDS-PAGE and then analyzed by anti-α-synuclein (1:2000, Abcam), anti-phospho-α-synuclein (1:2000, Wako), and anti-GAPDH (1:1000, KangChen Biotech).

[1] http://genie.weizmann.ac.il/pubs/mir07/mir07data.html

[2] http://cbcsrv.watson.ibm.com/rna22.html

[3] http://mirnamap.mbc.nctu.edu.tw/

[4] http://www.microrna.gr/microT

[5] http://www.microrna.org/microrna/home.do

[6] http://www.pictar.org/

[7] http://www.targetscan.org/

[8] https://david.ncifcrf.gov/summary.jsp

Statistical Analysis

The values shown in the graphs represent averages of several independent experiments and the actual number of samples for each experiment stated in the figure legends. The results are represented as mean ± SEM. The statistical significance was assessed by a two-tailed student's t-test. $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee WMU.

AUTHOR CONTRIBUTIONS

Z-BJ conceived and supervised the whole study. X-JC, M-LL, Y-HW, HM, ZW, X-YW, and C-JZ performed the experiments. XX participated in the data interpretation. SB, Z-HX, and HZ performed bioinformatics statistical analysis. X-JC wrote the manuscript. Z-BJ revised the manuscript. All authors contributed to the article and approved the submitted version.

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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.2020.565543/full#supplementary-material

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Mutation Screening in the miR-183/96/182 Cluster in Patients With Inherited Retinal Dystrophy

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Inherited retinal dystrophy (IRD) is a heterogenous blinding eye disease and affects more than 200,000 Americans and millions worldwide. By far, 270 protein-coding genes have been identified to cause IRD when defective. However, only one microRNA (miRNA), miR-204, has been reported to be responsible for IRD when a point-mutation occurs in its seed sequence. Previously, we identified that a conserved, polycistronic, paralogous miRNA cluster, the miR-183/96/182 cluster, is highly specifically expressed in all photoreceptors and other sensory organs; inactivation of this cluster in mice resulted in syndromic IRD with multi-sensory defects. We hypothesized that mutations in the miR-183/96/182 cluster in human cause IRD. To test this hypothesis, we perform mutation screening in the pre-miR-183, -96, -182 in >1000 peripheral blood DNA samples of patients with various forms of IRD. We identified six sequence variants, three in pre-miR-182 and three in pre-miR-96. These variants are in the pre-miRNA-182 or -96, but not in the mature miRNAs, and are unlikely to be the cause of the IRD in these patients. In spite of this, the nature and location of these sequence variants in the pre-miRNAs suggest that some may have impact on the biogenesis and maturation of miR-182 or miR-96 and potential roles in the susceptibility to diseases. Although reporting on negative results so far, our study established a system for mutation screening in the miR-183/96/182 cluster in human for a continued effort to unravel and provides deeper insight into the potential roles of miR-183/96/182 cluster in human diseases.

Keywords: microRNA, miR-183/96/182 cluster, inherited retinal dystrophy, sequence variants, susceptibility

INTRODUCTION

Inherited retinal dystrophy (IRD) is a heterogeneous blinding eye disease and affects more than 200,000 Americans and millions worldwide (Daiger et al., 2013). Identification of genes that are responsible for IRD when defective is of great importance to the basic understanding as well as development of efficient gene diagnosis and treatment of the disease (Xu, 2015). By far, 307 genetic loci are linked to various forms of IRD; among these, 270 protein-coding genes have

been identified to cause IRD when defective (RetNet[1]. Last updated April 29, 2015). However, the roles of non-coding elements of the genome in IRDs are still not fully investigated, in spite that increasing evidences suggest that non-coding elements are critically important in the regulation of genome structures and expression of protein-coding genes, and that species-specific non-coding elements shape species-specific functions (Lander, 2011; Ulitsky and Bartel, 2013; Ransohoff et al., 2018). microRNAs (miRNAs) are endogenous, small, non-coding, regulatory RNAs (Bartel, 2004). miRNAs quantitatively regulate gene expression at posttranscriptional levels (Bartel, 2004). Since its discovery in 1993 (Lee et al., 1993; Wightman et al., 1993), miRNAs have been proven to play important roles in normal functions, as well as in diseases when defective (Ambros, 2004; Bartel, 2004). Based on miRBase Release 22.1 (October, 2018)[2], at least 2654 mature miRNAs (1917 precursors) have been identified in humans. In spite of their importance in gene-expression regulation, by far, only one miRNA, miR-204, been identified to cause syndromic IRD with ocular coloboma when a dominant point mutation nt37 C > T in the seed sequence of miR-204 occurred in a large 5-generation family (Conte et al., 2015). The seed sequence of a miRNA is one of the most important determinants of the downstream target genes and, therefore, functions of the miRNA (Lewis et al., 2003). The nt37 C > T mutation results in both gain-of-function and loss-of-function effects, because this mutation in the seed sequence creates abnormal target genes that wild type miR-204 does not regulate, meanwhile loses control of many downstream target genes that miR-204 normally targets (Conte et al., 2015). However, no mutations in other miRNAs have been identified to cause IRD in human, although increasing evidences suggest that miRNAs are required for the normal development and functions of photoreceptors and the retina as a whole (Sanuki et al., 2011; Lumayag et al., 2013; Busskamp et al., 2014; Sundermeier et al., 2014; Aldunate et al., 2019).

Previously, we and others identified a conserved, paralogous, polycistronic miRNA cluster, the miR-183/96/182 cluster (hereafter referred to as miR-183/96/182), which is contained within 4 kilo bases (kb) on mouse chromosome 6qA3 with conservation of synteny to human chromosome 7q32.2 and is highly specifically expressed in all sensory organs (Lagos-Quintana et al., 2003; Wienholds et al., 2005; Kloosterman et al., 2006; Weston et al., 2006; Xu et al., 2007; Lumayag et al., 2013). Member of miR-183/96/182 share high sequence homology and overlapping downstream genes and, therefore, functions (Xu et al., 2007). Consistent with their high-level specific expression in sensory organs, point mutations of miR-96 result in progressive, non-syndromic hearing loss in both human (Mencia et al., 2009) and mouse (Lewis et al., 2009), with no apparent retinal phenotype, suggesting that miR-96 plays a predominant role in the inner ear, but not in retina (Lewis et al., 2009; Mencia et al., 2009; Li et al., 2010; Kuhn et al., 2011) and that members of miR-183/96/182 have distinct roles in different tissues/organs. In adult mouse retina, miR-183/96/182 is mainly expressed in all photoreceptors and a subgroup of ganglion cells. Members of miR-183/96/182 are among the highest expressed miRNAs in the retina (Xu et al., 2007; Lumayag et al., 2013). miR-182 accounts for ∼64%, miR-183 another ∼4% of all miRNAs in cone photoreceptors (Busskamp et al., 2014). Developmentally, miR-183/96/182 is minimally expressed in embryonic retina; but significantly upregulated soon after birth, suggesting that miR-183/96/182 play important roles in the postnatal functional differentiation of photoreceptors (Xu et al., 2007; Lumayag et al., 2013). Expression of miR-183/96/182 in the retina follows a diurnal rhythmic pattern – lowest around noon, highest in the early evening, suggesting a role in circadian function in the retina (Xu et al., 2007; Xu, 2009; Ko, 2020). In addition, miR-183/96/182 is responsive to light (Krol et al., 2010) – induced by light, and downregulated by darkness with 30 minutes (Krol et al., 2010). Targeted deletion of miR-182 alone in mouse did not result in a discernible phenotype, suggesting functional compensation by miR-183 and miR-96 (Jin et al., 2009). However, knockdown of miR-183/96/182 in postmitotic rod photoreceptors in a miRNA-sponge transgenic mouse model resulted in increased susceptibility to light damage in the retina (Zhu et al., 2011), with no discernible histological or functional defects in the retina under normal lighting conditions (Zhu et al., 2011), suggesting that miR-183/96/182 plays an important role in protecting the retina from light damage. We demonstrated that complete inactivation of miR-183/96/182 in mice results in syndromic IRD with multi-sensory defect (Lumayag et al., 2013), suggesting that miR-183/96/182 is required for the normal development and physiological functions of the retina and other sensory organs (Lumayag et al., 2013), providing one of the first evidences that mutation of an individual miRNA gene results in retinal dysfunction and degeneration in mammals. Our report (Lumayag et al., 2013) has been further validated by several other groups (Busskamp et al., 2014; Fan et al., 2017; Xiang et al., 2017) and our follow-up studies (Geng et al., 2018). Recent reports further substantiated critical roles of miR-183/96/182, especially miR-182/miR-183, in functional differentiation and maturation of photoreceptors (Busskamp et al., 2014; Mahmoudian-Sani et al., 2019; Peskova et al., 2020). Based on these evidences, we hypothesize that mutations in miR-183/96/182 in human also cause IRD, possibly syndromic IRD. To test this hypothesis, we established a collaboration with the National Ophthalmic Genotyping and Phenotyping Network (eyeGENE®)[3], which was created by the National Eye Institute (NEI), National Institutes of Health (NIH) to enhance the study of inherited eye diseases. We obtained >1000 IRD patient DNA samples. Here we report our results in the mutation screening in these patients by far.

MATERIALS AND METHODS

Human Genomic DNA Samples

This study was performed in accordance with the tenets of Declaration of Helsinki, and informed consent was obtained from all participants. The research was approved by the

[1] sph.uth.edu/Retnet

[2] www.mirbase.org

[3] https://eyegene.nih.gov/

Institutional Review Board (IRB) of Wayne State University, National Institutes of Health, and Committee for Protection of Human Subjects, University of Texas Southwestern Medical Center. The eyeGENE® Network constructed a framework to collect DNA samples from patients with various forms of IRD and linked them with clinical information and genetic testing data. To discover potential disease-causing mutations in miR-183/96/182 responsible for IRDs, we obtained DNA samples of IRD patients, in which no disease-causing mutations have been found in any protein-coding genes that have been screened by eyeGENE. Most testing on protein-coding genes at the eyeGENE was performed by standard commercial, fee for service panel testing, but did not go through CNV detection and evaluation of deep intronic variants.

Although the phenotypes in the miR-183/96/182 knockout mice do not completely match any human IRD, they resemble various aspects of several human IRD. The following rationales in reference to the phenotypes of the miR-183/96/182 knockout (ko) mice (Lumayag et al., 2013) were considered for sample selection: (1) since miR-183/96/182 is located at human chromosome 7q32.2, cases with X-linked IRD are excluded; (2) the most prominent ERG abnormalities in miR-183/96/182 ko mice is a decreased *b*-wave amplitude in both scotopic and photopic ERG (Lumayag et al., 2013). This phenotype resembles those observed in human incomplete Schubert-Bornschein type of stationary congenital night blindness (iSCNB) (Miyake et al., 1986); (3) the sensory syndromic feature are clinically reminiscent of Usher syndrome (Yan and Liu, 2010); (4) the cone photoreceptor system is more affected than rod photoreceptors in the ko mice; (5) it has been shown that different mutations in genes associated with IRD can cause different forms of IRD; same mutations in the orthologous genes in mouse can cause different phenotypes compared to patients (Riazuddin et al., 2010; Naeem et al., 2012; Daiger et al., 2013). Based on these considerations, we hypothesized that mutations in human miR-183/96/182 may cause one or more forms of IRD, with increased likelihood to cause SCNB, Usher syndrome and cone-rod dystrophy (CRD), and other type of cone diseases. Therefore, we first collected DNA samples of patients with SCNB (n = 16), Usher syndrome (n = 74), CRD (n = 248), Stargardt disease (n = 249), Achromatopsia/Blue Cone Monochromacy (A/BCM) (n = 4), Occult Macular Dystrophy (OMD. n = 6), Pattern Dystrophy and Adult Onset Foveomacular Dystrophy (PD and AOFD. n = 105) (**Table 1**). In addition, 509 samples with Retinitis Pigmentosa (RP) were also collected considering that miR-183/96/182 plays important roles in both rod and cones; rod system is also affected in the miR-183/96/182 ko mice. As we could not predict the inheritance pattern of a potential IRD caused by mutations in miR-183/96/182, samples with both autosomal dominant and recessive inheritance were included. DNA samples of 80 unaffected individuals were included as controls.

Amplification of Pre-miR-183/96/182 and Sanger Sequencing

The miR-183/96/182 is located on human chromosome 7q32.2 and clustered with 5 kb (Xu et al., 2007). Since pre-miR-183 and pre-miR-96 are only 135 nucleotides (nt) apart, while pre-miR-182 is ∼4.2 kb 3′ of pre-miR-96 (**Figure 1A**), to screen miR-183/96/182, we amplified pre-miR-183, pre-miR-96 and pre-miR-182 in two amplicons (**Figure 1A**). pre-miR-183 and pre-miR-96 were amplified together using primers: 183/96-F1 (forward primer) and 183/96-R1 (reverse primer) (**Figure 1A**), which will produce a 754-bp amplification product. Pre-miR-182 was amplified separately using primers, 182-F0/182-R3 with a 681-bp amplicon.

The PCR reaction was carried out with 100 ng genomic DNA, 3 μM forward and reverse primers, 1xPCR buffer and 5 units of Taq polymerase (MIDSCI) on a S1000 thermal cycler (Bio-Rad) with the following program: 95°C, 5 min; then 95°C, 30 s; 62°C, 1 min; 72°C, 2 min for 30 cycles, followed 72°C, 10 min.

The sequences of the PCR primers are as the following:

183/96-F1: 5′-GAAGGTCATCTTGGGCTGAT-3′;
183/96-R1: 5′- CCTACAGATGGTTTCAGACTC-3′.
182-F0: 5′-TCTGGCCTGGCTTGTGCTG-3′;
182-R3: 5′-GGCTTCCCAGCTGACTTGAG-3′.

The amplification product was run on a 1.5% agarose gel to ensure the specificity (**Figure 1B**), before purified using the gel/PCR DNA fragment extraction kit (IBI Scientific). 50 ng of purified PCR product was sent for Sanger sequencing by the Genome Sciences Core, Wayne State University or Genewiz with the following nested sequencing primers:

For pre-miR-183/96 amplicon: 183/96-F2: 5′-GTGGATC TTGTGAAGAGGTG-3′; 183/96-R2: 5′- AGGCAGTGT AAGGCGATCTG -3′.
For pre-miR-182 amplicon: 182-F1: 5′-ACAGGAACT GCAGGTTACAGA -3′; 182-R2: 5′- CTTGAGGACCTGT GACCTCA-3′.

Sequences obtained from sequencing using the forward primers (183/96-F2 or 182-F1) were aligned with the reference sequence downloaded from the UCSC Genome Browser [Human Dec. 2013 (GRCh38/hg38) Assembly] (**Supplementary Material**) using Vector NTI 11.0 (Invitrogen). All sequence variants identified by the sequence alignment were confirmed by the sequencing using the reverse primers (183/96-R2 or 182-R2).

Copy Number Assay

Patient genomic DNA was extracted from blood using the IBI gMAX mini genomic kit. DNA was diluted to 5 ng/μL with nuclease–free water. 4 μl of diluted DNA was added per well in a 96 well plate. Custom designed TaqMan® Copy Number Assay (4400294. Assay ID: MIR182_CDYMJXM) for pre-miR-182 was added along with TaqMan® Copy Number Reference Assay *RNase P*. The reactions were run on an Applied Biosystems 7500 Fast Real-Time PCR System using the following cycle parameters: 95°C, 10 min; then 95°C, 15 s; 60°C, 60 s for 40 cycles. Data generated was analyzed using Applied Biosystems CopyCaller® Software v2.0.

TABLE 1 | DNA samples of various inherited retinal dystrophy and unaffected controls.

Disease	SCNB	Usher Syn.	Cone-rod dys.	Stargardt Disease	A/BCM	OMD	PD and AOFD	RP	Subtotal	Unaffected	Total
Number of samples	16	74	239	242	4	6	105	509	1179	80	1259

SCNB, stationary congenital night blindness; Usher syn., Usher syndrome, Cone-rod dys., cone-rod dystrophy; A/BCM, Achromatopsia/Blue Cone Monochromacy; OMD, Occult Macular Dystrophy; PD and AOFD, Pattern Dystrophy and Adult Onset Foveomacular Dystrophy; RP, retinitis pigmentosa.

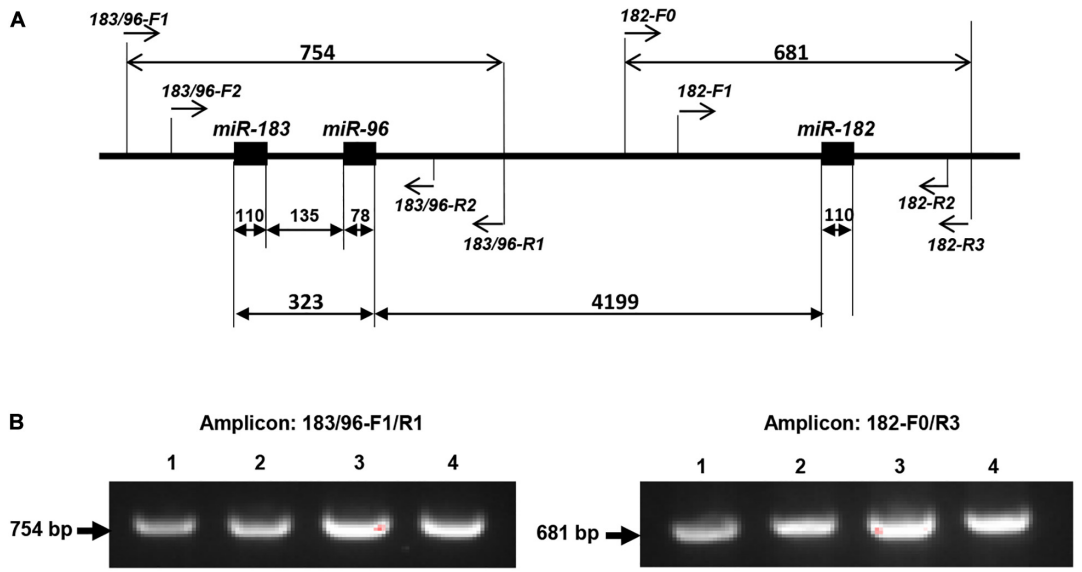

FIGURE 1 | PCR-sequencing strategy for mutation screening in the miR-183/96/182 cluster. **(A)** Schematic illustration of the miR-183/96/182 cluster in relation to the PCR and sequencing primers. Pre-miR-183/96 is amplified by 183/96-F1/R1 and sequenced by primers of 183/96-F2 and 183/96-R2. pre-miR-182 was amplified with primers 182-F0/R3, and sequenced by 182-F1 and 182-R2. The numbers above the double arrows indicate the sizes of the marked fragments in base pairs (bp). **(B)** Examples of amplification products.

Isolation of Mononuclear Cells From Peripheral Blood

Peripheral blood mononuclear cells (PBMNC) were isolated from human peripheral blood (with EDTA or heparin as anti-coagulant) using the Histopaque-1077 (Sigma-Aldrich. Cat No. 10771). Manufacturer's instruction was followed. Briefly, 3 ml of peripheral blood was carefully layered on 3 ml Histopaque-1077 in a 15-ml conical tube and centrifuged at 400 $\times$ *g* for 30 min at room temperature. After centrifugation, the upper plasma layer was aspirated off. Then the PBMNC layer was carefully removed and washed in cold PBS. The cells are divided into two tubes for total RNA isolation using the RNeasy RNA isolation kit (Qiagen) and miRNA isolation using the miRVana miRNA isolation kit (Ambion). RNA concentration and quality were analyzed using a NanoDrop 2000 (Thermo Fisher Scientific).

Genomic DNA Preparation

Two milliliters of peripheral blood was used for genomic DNA preparation using the gMAX DNA mini kit (IBI Scientific) following manufacturer's instruction. Subsequently, DNA concentration and quality were assayed on the NanoDrop 2000 (Thermo Fisher Scientific).

Quantitative (q)RT-PCR of miR-182

qRT-PCR was performed using TaqMan miRNA primers and RT-PCR kit (Life Technologies) on a CFX Connect Real-time System (Bio-Rad, Hercules, CA, United States) with snRNA U6 as an endogenous control as described before.

Secondary Structure Prediction of Hsa-pre-miR-182 and Hsa-pre-miR-96

The sequences of hsa-pre-miR-182 and hsa-pre-miR-96 were downloaded from miRBase[4]. The residues of sequence variants identified in this study were modified to obtain the sequences of the mutant forms of hsa-pre-miR-182 and hsa-pre-miR-96. Then these sequences were uploaded to the RNAfold web server[5] to obtain predicted secondary structures with minimum free energy (MFE) and partition function (Mathews et al., 2004; Gruber et al., 2008; Lorenz et al., 2011).

Statistical Analysis

When the comparison was made among more than two conditions, One-way ANOVA with Bonferroni's multiple

[4]http://www.mirbase.org/

[5]http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi

comparison test was employed (GraphPad Prism); adjusted $p < 0.05$ was considered significant. Otherwise, a two-tailed Student's t test was used to determine the significance; $p < 0.05$ was considered significant. The significance of the association of sequence variants to various disease categories in this cohort was compared to general population by Fisher's exact test, or Chi square test for the tri-allelic SNP rs80041074 (pre-miR-182 nt94 G > A).

RESULTS

Among the 1179 IRD patient samples (**Table 1**), we identified three sequence variants in the pre-miR-182: nt106 G > A, nt105 C > T and nt94 G > A (**Figure 2**), and three sequence variants in pre-miR-94: nt36 T > C, nt39 C > T and nt42 C > T (**Figure 3**).

Sequence Variants in Hsa-pre-miR-182

Pre-miR-182 nt106 G > A

This variant was found in 32 IRD patients, including two in CSNB patients, nine CRD, one Usher syndrome, four PD and AOFD, three Stargardt disease patients, and 13 patients with RP (**Table 2** and **Figures 2A,B**). Among these, we found homozygosity for the less common A allele in five patients including one CRD, two Stargardt, and two RP patients (**Table 2**). Copy-number assay confirmed that these cases are true homozygosity for this variant instead of a micro-deletion in one allele. In 80 unaffected individuals, we identified one individual heterozygous for pre-miR-182 nt106 G/A. The A allele frequency in our samples is as the following: overall frequency in IRD patients: 1.569%; in CSNB patients: 6.250%; Usher syndrome: 0.676%; CRD: 2.092%; Stargardt dis.: 1.033%; PD and AOFD: 1.905%; other RP: 1.473% (**Table 2**). In unaffected individual: 0.625%.

The pre-miR-182 nt106 G > A variant is a known single nucleotide polymorphism (SNP) documented in the dbSNP as rs76481776 with the allele frequencies of 5.952% (7211/121160) for the A allele, while 94.048% (113949/121160) for the G allele based on data from UCSC genome browser (dbSNP build 151). Statistical analysis suggest that the pre-miR-182 A allele appears to be significantly lower in all groups of patients and overall IRD population in this cohort, except in CSNB patients (**Table 2**).

The pre-miR-182 nt106 G > A is located at the 5th nucleotide from the 3′ end of the hairpin structure of the human pre-miR-182 (**Figure 2E**). It is predicted that nt106 G > A change results in a transition of G•U wobble base-pairing to a classical Watson-Crick base pair A•U, suggesting potential functional significance

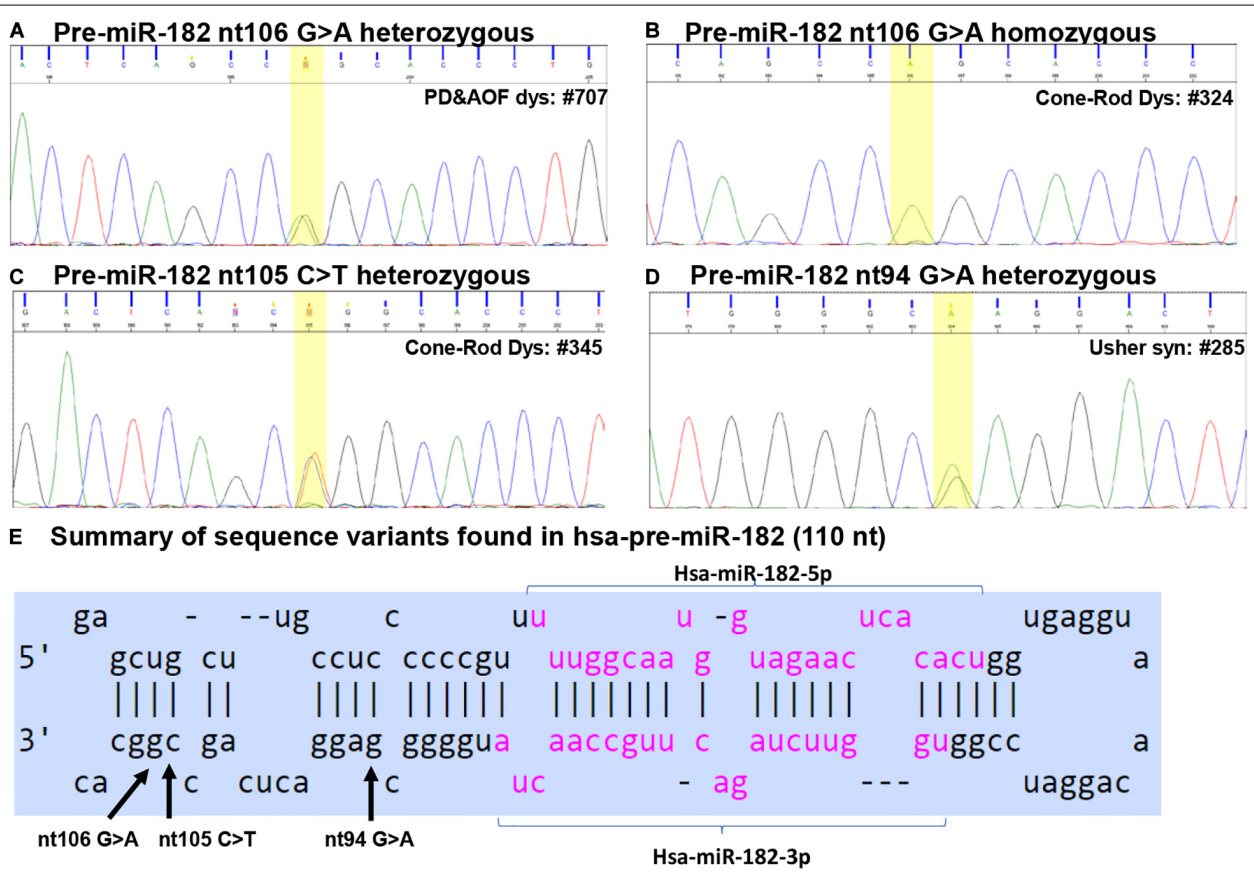

FIGURE 2 | Sequence variants identified in pre-miR-182. **(A–D)** Example of Sanger sequencing chromatography of patient DNA samples which are heterozygous for pre-miR-182 nt106 G/A alleles **(A)** and homozygous for pre-miR-182 nt106 A/A allele **(B)**, heterozygous for pre-miR-182 nt105 C > T **(C)** and nt94 G > A variants **(D)**. **(E)** Summary of the sequence variants of pre-miR-182 in the context of the hairpin structures of hsa-miR-182. Reference: miRbase.org.

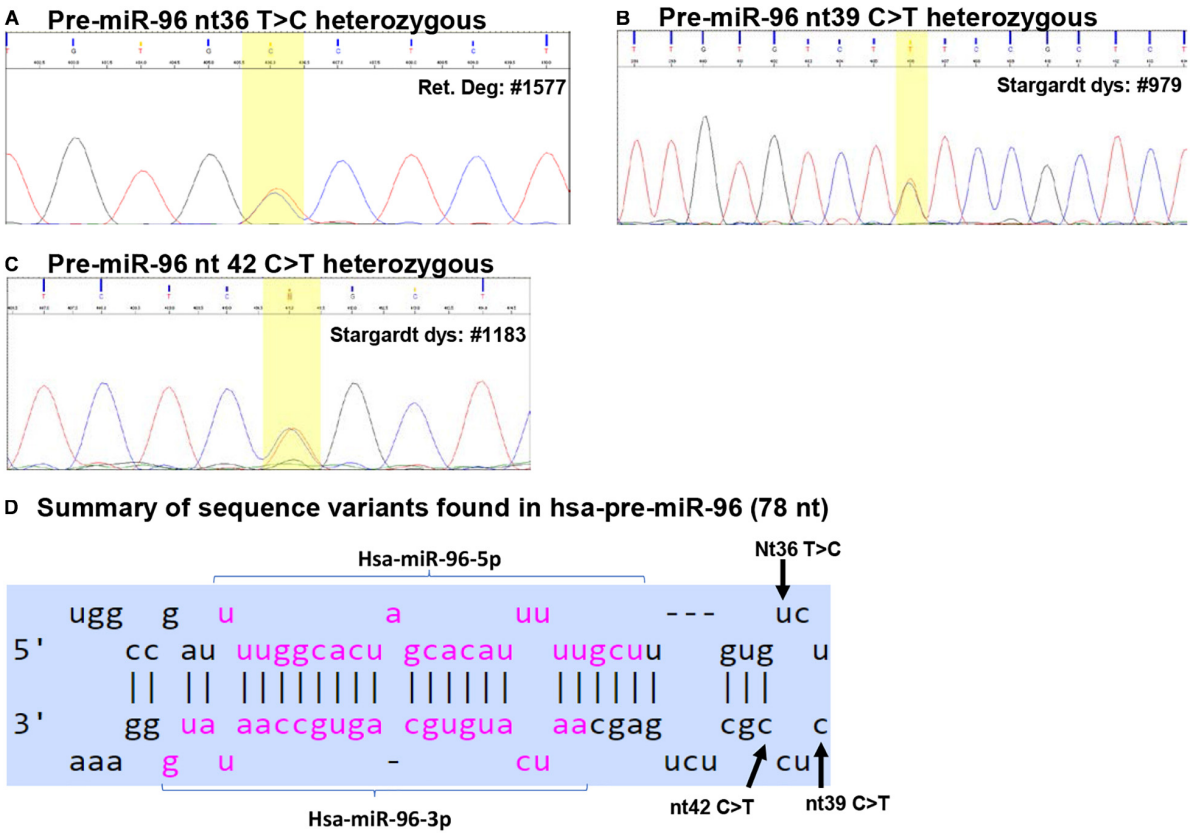

FIGURE 3 | Sequence variants identified in pre-miR-96. **(A–C)** Example of Sanger sequencing chromatography of patient DNA samples which are heterozygous for pre-miR-96 nt36 T > C **(A)**, nt39 C > T **(B)**, and nt42 C > T variants **(C)**. **(D)** Summary of the sequence variants of pre-miR-96 in the context of the hairpin structures of pre-miR-96. Reference: miRbase.org.

(Varani and McClain, 2000). However, a secondary structure prediction using the RNAfold algorithm (Mathews et al., 2004; Gruber et al., 2008; Lorenz et al., 2011)[6] showed little changes in the secondary structure and the minimum free energy of the hair-pin structure of pre-miR-182 (**Figure 4A** and **Table 3**).

Pre-miR-182 nt105 C > T

Pre-miR-182 nt105 C > T was identified in one CSNB and one CRD patient (**Table 2** and **Figure 2C**), which are heterozygous for this allele. The overall frequency of the T allele in our IRD cohort: 0.085%. In SCNB: 3.125%; in CRD: 0.209% (**Table 2**). This variant was not identified in any unaffected individuals. This variant is documented in dbSNP as a rare SNP rs77586312: with frequencies of the T allele 0.152% (184/121378), while the C allele 99.848% (121194/121378). Comparing to the general population data compiled at dbSNP, the rare pre-miR-183 nt105 T appears to be significantly enriched in CSNB patients in this cohort.

This variant is also near the 3′ end of the hsa-pre-miR-182, located at the 6th nucleotide from the 3′ end (**Figure 2E**). It results in a change of the classical Watson-Crick base pair G•C to a G•U wobble base pair. Secondary structure prediction by RNAfold suggest that nt105 C > T variant may induce changes in the basal segment of the stem of pre-miR-182 (the bracketed area in **Figure 4A**), although it did not cause drastic changes of the minimum free energy (**Table 3**).

Pre-miR-182 nt94 G > A

Pre-miR-182 nt94 G > A was identified in one Usher syndrome, two unrelated Stargardt disease, one PD and AOFD and three unrelated other RP patients, as well as in one unaffected individual (**Table 2** and **Figure 2D**). All are heterozygous for this allele. The overall frequency of this variant in all IRD patients in this cohort was 0.297%. It has a frequency of 0.676% in Usher syndrome; 0.413% in Stargardt disease; 0.476% in PD and AOFD; 0.295% in other RP patients. In unaffected individuals: 0.625% (**Table 2**).

Pre-miR-182 t94 G > A is documented as a rare tri-allelic SNP rs80041074 with minor allele frequency as A 0.389% (475/121998), T allele 0.001% (1/121998), while the common allele G has a frequency of 99.61% (121522/121998) in the dbSNP (dbSNP build 151).

This SNP is located at the 17th nucleotide from the 3′ end of the hsa-pre-miR-182. It disrupts a C•G in the predicted hair-pin structure (**Figure 2E**). Secondary structure prediction by RNAfold showed that nt94 G > A change may induce changes in the size of the bulge (bracketed area in **Figure 4A**) of the

[6]http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi

TABLE 2 | Allele frequency of the sequence variants among the samples.

Disease	Variant	nt106 G > A			nt105 C > T		nt94 G > A		nt36 T > C		nt39 C > T		nt42 C > T	
		pre-miR-182 (rs76481776)			pre-miR-182 (rs77586312)		pre-miR-182 (rs80041074)		pre-miR-96 (rs41274239)		pre-miR-96 (rs73159662)		pre-miR-96 (rs73159662)	
	Freq in dbSNP	G: 94.048%; A: 5.952%			C: 99.848%; T: 0.152%		G: 99.61%; A: 0.389%; T: 0.001%		T: 99.752%; C: 0.249%		C:99.99%; T: 0.01%		C:99.713%; T: 0.287%	
	No	No	No of homo	freq (%)	No	freq (%)	No	freq (%)	No	freq (%)	No	freq (%)	No	freq (%)
SCNB	16	2		6.250	1	3.125*								
Usher syn.	74	1		0.676**			1	0.676						
Cone-rod dys.	239	9	1	2.092***	1	0.209								
Stargardt disease	242	3	2	1.033***			2	0.413			1	0.207	2	0.413
A/BCM	4													
OMD	6													
PD and AOFD	105	4		1.905**			1	0.476						
Other RP	509	13	2	1.473***			3	0.295	3	0.295				
Subtotal	1179	32	5	1.569***	2	0.085	7	0.297	3	0.127	1	0.042	2	0.085
Un-affected	80	1		0.625			1	0.625						
Total	1261	65		1.507	4	0.079	15	0.317	6	0.119	2	0.04	4	0.079

*Freq, frequency; No, number; homo, homozygote; SCNB, stationary congenital night blindness; Usher syn., Usher syndrome; Cone-rod dys., cone-rod dystrophy; A/BCM, Achromatopsia/Blue Cone Monochromacy; OMD, Occult Macular Dystrophy; PD and AOFD, Pattern Dystrophy and Adult Onset Foveomacular Dystrophy; RP, retinitis pigmentosa. *, **, ***: $p < 0.05$, 0.01, and 0.001 when compared to general population data compiled by dbSNP by Fisher's exact test.*

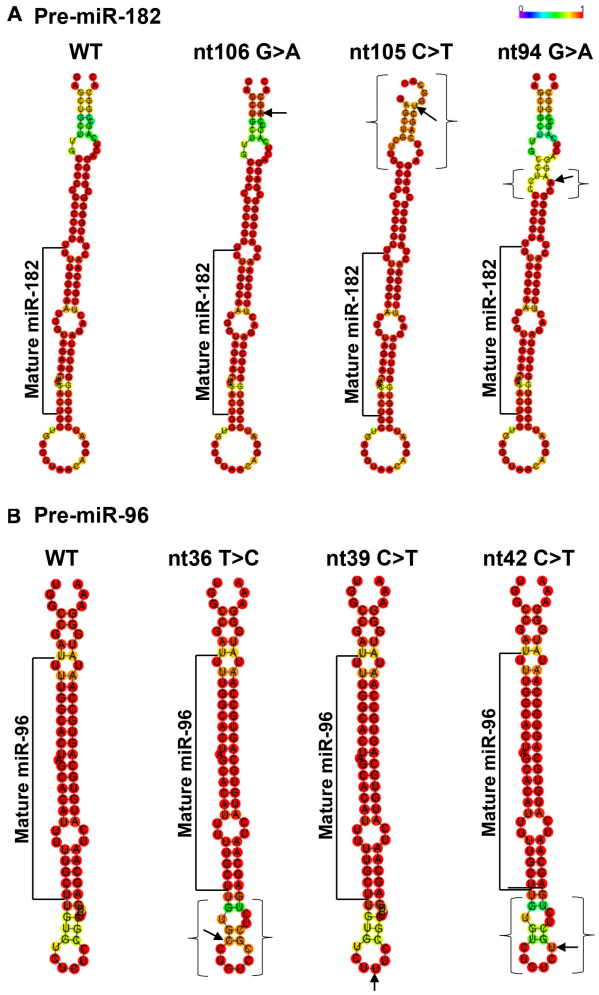

FIGURE 4 | Minimum free energy secondary structure prediction with base-pair probability on wild type (WT) hsa-pre-miR-182 **(A)** and hsa-pre-miR-96 **(B)** and their mutant forms with sequence variants. Data was compiled using publicly available software *RNAfold* (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi). Arrows indicate the sequence variant nucleotide. The brackets mark the areas with predicted changes of the secondary structure because of the sequence variants.

secondary structure of pre-miR-182, resulting in an increase of the minimum free energy level (**Table 3**. from −47.10 to −43.80) at its thermodynamic ground state. However, whether these changes impose any impact on miR-182 maturation need to be test experimentally.

Sequence Variants in Hsa-pre-miR-96

We identified three sequence variants in pre-miR-94: nt36 T > C, nt39 C > T and nt42 C > T (**Figure 3**).

Pre-miR-96 nt36 T > C

The pre-miR-96 nt36 T > C was identified in three unrelated RP patients heterozygous for this allele, but not in any other IRD patients and unaffected controls (**Table 2** and **Figure 3A**). The minor C allele has a frequency of 0.295% in RP patients, 0.127% in all IRD patients. This variant is located in the loop region of hsa-pre-miR-96 (**Figure 3D**).

The pre-miR-96 nt36 T > C variant is known as a rare SNP rs41274239 with allele frequencies of 0.249% (65/26162) for the minor C allele; 99.752% (26097/26162) for the common T allele based on data from UCSC genome browser (dbSNP build 151).

Secondary structure prediction using RNAfold showed that pre-miR-96 nt36 T > C may change the landscape of the terminal loop of pre-miR-96 (the bracketed area in **Figure 4B**) at its thermodynamic ground state, although this change did not induce drastic changes in the free energy of the secondary structure of pre-miR-96 (**Table 3**).

Pre-miR-96 nt39 C > T

The pre-miR-96 nt39 C > T variant was identified in one Stargardt disease patient who is heterozygous for this allele (**Table 2** and **Figure 3B**). In the study cohort, the minor T allele has a frequency of 0.207% in Stargardt disease patients, and 0.042% in all IRD patients. None was detected in the unaffected samples (**Table 2**). This variant is documented as rare SNP rs73159662: C: 99.990% (10181/10182); T: 0.010% (1/10182) in dbSNP (build 151).

This variant is also located in the stem loop region of the hsa-miR-96 (**Figure 3D**). Secondary structure prediction by RNAfold program did not expect significant changes in the thermodynamic status of the secondary structure of pre-miR-96 (**Figure 4B** and **Table 3**).

Pre-miR-96 nt42 C > T

This variant was identified in two unrelated Stargardt disease patients heterozygous for this allele. In the study cohort, the minor T allele in Stargardt disease patient is 0.413%, while the overall frequency in all IRD is 0.085% (**Table 2**).

Pre-miR-96 nt42 C > T variant is documented as a rare SNP rs73159662 with the minor allele T frequency as: 0.287% (75/26094), the common C allele 99.713% (26019/26095) in the dbSNP (build 151).

This variant is predicted to disrupt the classical Watson-Crick base pair G•C at the first residue after the terminal loop of the predicted hsa-pre-miR-96 structure (miRbase) (**Figure 3D**), suggesting potential impact on the structures around the terminal loop. Secondary structure prediction by RNAfold algorithm suggested that nt42 C > T may induce similar changes in the terminal loop of pre-miR-96 at its thermodynamic ground state, as did the pre-miR-96 nt36 T > C variant (the bracketed area in **Figure 4B**), although it did not induce drastic changes in the free energy of the secondary structure of pre-miR-96 (**Table 3**). Since the terminal loops of pre-miRNAs can play important roles in miRNA biogenesis (Castilla-Llorente et al., 2013; Treiber et al., 2019), whether these variants impose significant impact on miR-96 maturation should be further studied.

Segregation Study in a Family, Family 4100, of a Proband Homozygous for Pre-miR-182 nt106 G > A Variant

In spite that the pre-miR-182 nt106 G > A is common variant with a frequency of 5.952% in general population (dbSNP),

TABLE 3 | Changes in the predicted secondary structures and their free energy caused by the variants of pre-miR-182 (A) and pre-miR-96 (B).

Pre-miR-182	**WT**	**nt106 G > A**	**nt94 G > A**	**nt105 C > T**
Minimum free energy (MFE)	−47.10	−47.80	−43.80	−48.60
Free energy of the thermodynamic ensemble	−48.41	−49.03	−45.26	−49.19
Frequency of the MFE structure (%)	11.89	13.55	9.41	38.11
Ensemble diversity	7.40	6.27	9.30	4.64
Pre-miR-96	**WT**	**nt36 T > C**	**nt39 C > T**	**nt42 C > T**
Minimum free energy (MFE)	−34.80	−35.60	34.80	−33.60
Free energy of the thermodynamic ensemble	−35.26	−36.19	35.26	−34.37
Frequency of the MFE structure	47.49	38.16	47.51	28.84
Ensemble diversity	2.90	2.42	2.90	2.97

it was reported that minor A allele is associated with higher risk of late insomnia in major depression patients (Saus et al., 2010). *In vitro* expression studies showed that the A allele resulted in an increased production of mature miR-182 and enhanced inhibition of its target genes (Saus et al., 2010). In addition, pre-miR-182 nt106 G > A is also reported to be associated with high tension glaucoma (HTG), but not normal tension glaucoma (NTG); the A allele gives 30% higher expression of mature miR-182 *in vitro* transfection/expression experiment (Liu et al., 2016). Furthermore, this variant is also shown to be associated with genetic susceptibility to Behcet's disease and Vogt-Koyanagi-Harada (VKH) syndrome, both of which have autoimmune uveitis; mature miR-182 showed an increased expression level in activated $CD4^+$ T cells of the patients with nt106 A allele than normal controls (Yu et al., 2014). These reports suggest that pre-miR-182 nt106 G > A variant may have significant impact on the maturation of miR-182, which play important roles in the differentiation of photoreceptors and the function of the retina and other sensory systems (Lumayag et al., 2013; Busskamp et al., 2014; Fan et al., 2017; Mahmoudian-Sani et al., 2019; Peskova et al., 2020). Consistent with this report, recent reports from us and others have shown that miR-183/96/182, including miR-182, is also expressed and plays important roles in the immune systems and autoimmune diseases (Stittrich et al., 2010; Donatelli et al., 2014; Ichiyama et al., 2016; Li et al., 2016; Muraleedharan et al., 2016, 2019; Wan et al., 2016; Wurm et al., 2017; Wang et al., 2019).

If the A allele does affect the miR-182 maturation and contribute to various diseases, we hypothesized that homozygosity of pre-miR-182 nt106 G > A may have significant functional impact on mature miR-182 expression and contribute to syndromic IRD. To test this hypothesis, we collected DNA samples from several members of a family – Family 4100, in which multiple members have Stargardt disease and the proband, II-2, is homozygous for the less common, pre-miR-182 nt106 A allele (A/A) (**Figure 5A**). To test whether the Stargardt disease is associated with syndromic immune defects, we expanded our clinical observation to include both retinal and immune/inflammatory symptoms. The proband was diagnosed to have Stargardt disease at age of 65 in 2019 with clear vitreous, normal optic nerve, normal retinal vessels, pigmentary changes throughout macula, GA OU (both eyes), mfERG results in 2013 were abnormal surrounding the fovea, visual fields showed central scotoma and general reduction of sensitivity. However, his vision acuity has stayed remarkably good (20/20 and 20/50 in 2019). Patient reports unknown autoimmune ear disease leading to a sudden and permanent hearing loss in the left ear in 2004, and thought to be attributed to psoriatic arthritis. Patient also reports mild flares of arthritis in thumbs and knee. One sister of the proband, II-3, was diagnosed with Stargardt disease in 2016 with pigmentary changes throughout macula (flecks). She has no reported auto-immune disease diagnosed. Another sister of the proband, II-5, is also diagnosed with Stargardt disease and was reported to have Lupus-like symptoms, although they have not been accurately diagnosed.

To test whether the Stargardt disease and/or the autoimmune symptoms segregate with the A/A homozygosity, blood samples were collected from individual II2, 4, 5 and III-1,2,3. Unfortunately, genotyping on the pre-miR-182 showed that neither the Stargardt disease nor the autoimmune symptoms segregated with pre-miR-182 nt106 A/A homozygosity (**Figure 5A**). II-4, homozygous to A/A, is phenotypically normal; while II-5 and III-3, who are heterozygous (G/A) for the pre-miR-182 nt106 allele, are affected with Stargardt disease, as well as autoimmune symptom (for II-5) (**Figure 5A**). Therefore, homozygosity of pre-miR-182 nt106 A/A is unlikely to be the cause of the Stargardt disease and autoimmune symptoms in this family.

To test whether the pre-miR-182 nt106 G > A variant has any impact on the expression levels of mature miR-182, we isolated RNA from PBMNC in these family members. qRT-PCR result showed no clear correlation between the expression levels of mature miR-182 and the genotype of pre-miR-182 nt106 alleles (A/A or G/A) (**Figures 5B,C**). Homozygosity for the pre-miR-182 nt106 A allele (A/A) did not show increased expression of mature miR-182, as described by previous reports (Saus et al., 2010; Yu et al., 2014; Liu et al., 2016). On the contrary, mature miR-182 in PBMNC from individuals with G/A allele had an increasing trend than the ones with A/A allele (**Figures 5B,C**), although the difference did not pass statistical significance threshold, because of limited number of samples.

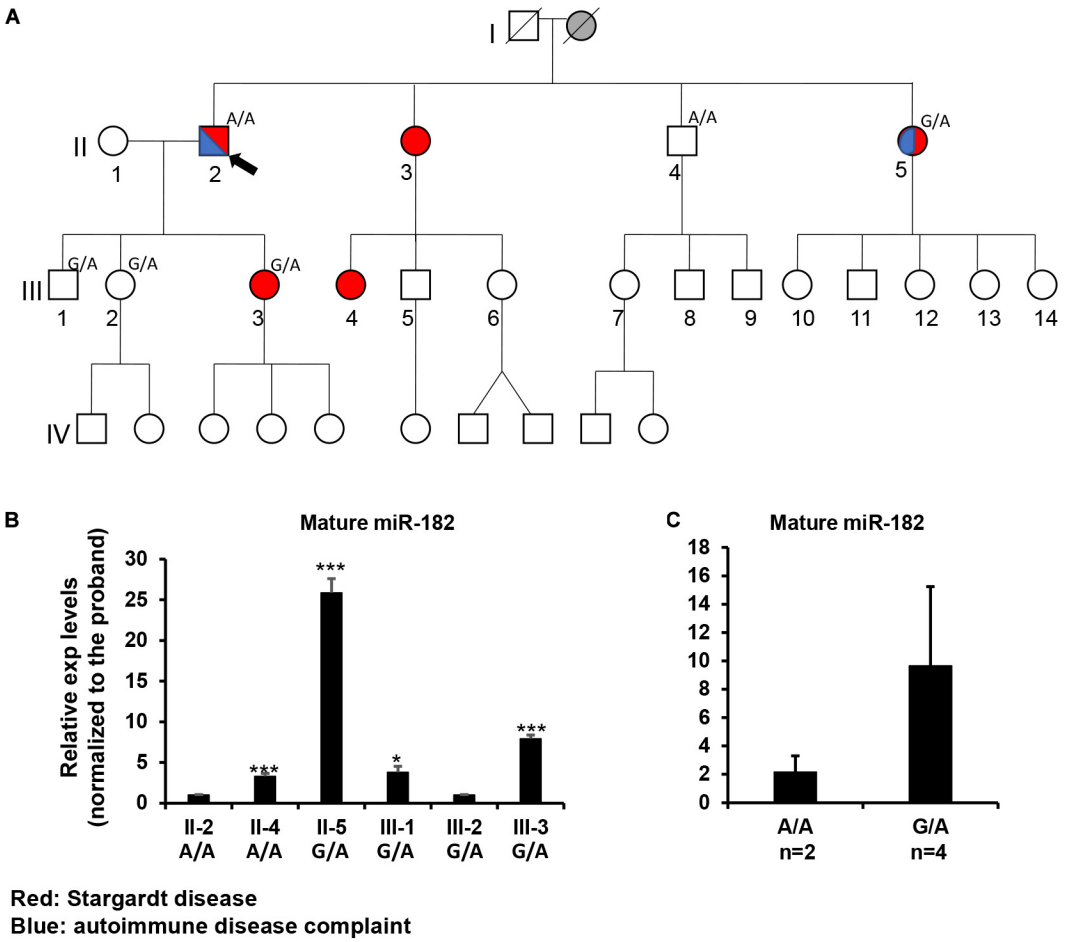

FIGURE 5 | Inheritance patterns of the pre-miR-182 nt106 G/A allele and the expression levels of mature miR-182 in peripheral blood mononuclear cells (PBMNC) in a family with multiple cases of Stargardt disease -Family 4100. **(A)** the family pedigree. Proband II-2 and his brother II-4 are homozygous for the less common pre-miR-182 nt106 A allele (A/A); while II-5, III-1, 2, 3 are heterozygous (G/A) for this allele. Red: with Stargardt disease. Blue: with Complaint of autoimmune disease(s). **(B,C)** qRT-PCR on pre-miR-182 in the PBMNC of selected family members. $^{*}p < 0.05$; $^{***}p < 0.001$.

DISCUSSION

By far, only three miRNAs, miR-96, miR-184 and miR-204, have been identified to cause inherited diseases in human when mutated. Point mutations in the seed region of the mature miR-96 (nt13 G > A and nt14 C > A) cause autosomal dominant non-syndromic hearing loss (Mencia et al., 2009). A point mutation in the seed sequence of miR-184 (nt57 C > U) is reported to cause autosomal dominant, familial keratoconus with early onset anterior polar cataract (Hughes et al., 2011) and an autosomal dominant syndromic anterior segment dysgenesis – the endothelial dystrophy, iris hypoplasia, congenital cataract, and stromal thinning (EDICT) syndrome (Iliff et al., 2012). A dominant mutation in miR-204, nt37 C > T, causes an autosomal dominant IRD associated with bilateral ocular coloboma (Conte et al., 2015) – the first and only report that a point mutation in an individual miRNA results in significant functional consequence and causes IRD in human. In these three cases, the disease-causing mutations are all in the seed sequences of mature miRNAs. The seed sequence of a miRNA is one of the most important determinants of its downstream target genes and hence functions (Lewis et al., 2003). These mutations in the seed sequences of miR-96, miR-184, and miR-204 resulted in both loss and gain of function effects – loss of control of normal target genes while creating new, abnormal target genes, leading to global dysregulation of gene expression and dominant inherited diseases (Mencia et al., 2009; Hughes et al., 2011; Iliff et al., 2012; Conte et al., 2015). Here we identified three sequence variants in pre-miR-182; three sequence variants in pre-miR-96 among 1179 patients with various forms of IRD. All of these sequence variants are located in the pre-miR-182 or pre-miR-96, but not in the mature miR-182 or miR-96. Therefore, they won't have any impact on the spectrum of downstream targets of miR-182 or miR-96. In addition, these variants are all known SNPs which have been identified in the general population and reported in the dbSNP. Therefore, the sequence variants that we identified so far are less likely to be the disease-causing mutations responsible for

the IRD symptoms in these patients. In the CSNB group, the rare pre-miR-182 nt105 T appears to be significantly enriched in CSNB patients when compared to the general population data compiled at dbSNP; however, we only have a small number of CSNB patients in this cohort; whether this "enrichment" is of biological significance or a random bias because of the small number of samples needs to be further investigated in future studies with more CSNB patient samples. In our cohort of a total of 1179 patients, five probands (0.42%) were found homozygous for the pre-miR-182 nt106 G > A (rs76481776). Although there is no data on the homozygosity of this variant in the general population, it is reasonable to predict a ∼0.35% frequency in the general population based on its 5.952% allele frequency, which is similar to the frequency in our cohort (0.42%). In Family 4100, in which multiple members are diagnosed with Stargardt disease and the proband is homozygous for the minor pre-miR-182 nt106 A allele, homozygosity of the A allele did not segregate with either the Stargardt disease or autoimmune symptoms in members of this family, suggesting that it is unlikely to be the cause for these diseases. While we were investigating on mutations in miR-183/96/182, eyeGENE continued on the screening in protein-coding genes and identified that the proband of Family 4100 was heterozygous for ABCA4 c.5461-10T > C VUS (variant of uncertain significance). This variant is located in intron 38 of the ABCA4 gene. It was first discovered in 1999 in Stargardt disease patients (Maugeri et al., 1999), and has been described as the third most frequent gene variant associated with Stargardt disease in individuals with European or African descent (Zernant et al., 2011; Roberts et al., 2012; Miraldi Utz et al., 2014). Although it was initially identified as a sequence variant without significant functional consequence (Rivera et al., 2000), recent reports suggest that this mutation results in a decreased expression level of wild type ABCA4 and splicing defects with exons 39/40 skipped, leading to frameshift and premature stop, playing a causative and a pathological role in Stargardt disease (Sangermano et al., 2016; Aukrust et al., 2017; Jonsson et al., 2018). We predict that the intronic mutation, c.5461-10T > C in ABCA4, contributes to the Stargardt disease in Family 4100. Further investigation is required to determine whether the disease in this family is solely caused by the ABCA4 c.5461-10T > C variant or whether mutations in other genes are involved.

Therefore, we haven't yet identified disease-causing mutations in the miR-183/96/182 cluster in human. However, this is the first stage of a long-term study. The negative results so far cannot exclude miR-183/96/182 as a disease-causing gene in these IRD patients yet. In current study, the mutation screening is purely focused on the sequences of pre-miR-183/-96/-182. In the next stage of our study, we plan to sequence the entire genomic region of the miR-183/96/182 gene to test whether there are mutations in the promoter/enhancer and other regions of the miR-183/96/182 cluster gene which may affect the transcription and post-transcriptional regulation of the miR-183/96/182 cluster and contribute to or cause IRD in these patients. We are aware of the challenges to the identification of disease-causing mutations in this cohort. First, most IRD patients are sporadic cases without family history, inheritance pattern and DNA samples from other members of the family. Second, no RNA and other tissue samples from the same patients are available to test the expression levels of miR-183/96/182 in the retina or other tissues. To meet these challenges, as we showed in Family 4100, we extended our collaboration with dedicated physician scientists and candidate family members to obtain additional family history and samples to make conclusive studies, once clues are identified in the initial screening. In addition, future studies including Crispr-Cas9-mediated mutagenesis in iPS cells in combination of *in vitro* organogenesis will further help meet these challenges.

In spite that we have not yet identified disease-causing mutations, studies on the rare sequence variants have stimulated greater insights into the potential of mutations of miR-183/96/182 in human diseases. Although these sequence variants are not expected to change the spectrum of downstream target genes of miR-182 or miR-96, we do not exclude the possibility that they have significant impact on miRNA biogenesis and maturation of the miR-182 or miR-96. miRNA biogenesis involves two RNase III-type enzymes, DROSHA in the nucleus (Lee et al., 2003; Denli et al., 2004; Han et al., 2004; Landthaler et al., 2004) and DICER in the cytosol (Grishok et al., 2001; Hutvagner et al., 2001). Drosha and a dimer of its cofactor DGCR8 form the core of a heterotrimeric Microprocessor (Nguyen et al., 2015), which essentially determines the mature miRNA sequence, as Drosha generates the termini of pre-miRNA, from which ∼22 nt are measured by DICER (Auyeung et al., 2013; Fang and Bartel, 2015; Kim et al., 2017). The mammalian Microprocessor prefers a stem of 35 ± 1 bp, a terminal unstructured loop and single-stranded regions flanking the base of hairpin (**Figure 6**; Fang and Bartel, 2015; Bartel, 2018). Throughout the stem, pairing is preferred, although a few mismatches or small bulges are tolerated. Several features of the hairpin structure of a pre-miRNA enhances the processing and help specify the sites of cleavage by Drosha (**Figure 6**; Auyeung et al., 2013; Fang and Bartel, 2015; Bartel, 2018). Drosha recognizes the base of the hairpin, including the basal UG motif and the mismatched GHG motif (H = A, C or U), while DGCR8 dimer recognizes the apical region, including the apical UGU motif. Drosha and DGCR8 dimer act as a molecular caliper to measure the length of the stem (Auyeung et al., 2013; Fang and Bartel, 2015; Nguyen et al., 2015; Bartel, 2018; **Figure 6**). A 3′ flanking CNNC motif, ∼17–18 nt downstream of the Drosha cleavage site (Auyeung et al., 2013), is recognized by auxiliary factors, e.g., SRp20 or p72, to enhance the processing (Auyeung et al., 2013; Mori et al., 2014; Bartel, 2018; **Figure 6**). The pre-miR-182 nt94 G > A variant is located in the vicinity of the GHG motif; while pre-miR-182 nt106 G > A and nt105 C > T are located in the CNNC motif of the pri-miR-182 hairpin structure. These sequence variants may modulate the processing by the Microprocessor and result in changes in the production of mature miR-182. Our preliminary study on the expression of mature miR-182 in PBMNCs of the family with Stargardt disease (**Figure 5C**) showed a trend consistent with this hypothesis. The average expression levels of mature miR-182 in the individuals with homozygous nt106 A/A allele showed a decreased trend when compared to the ones heterozygous for this allele (nt106 G/A). However, we only obtained two

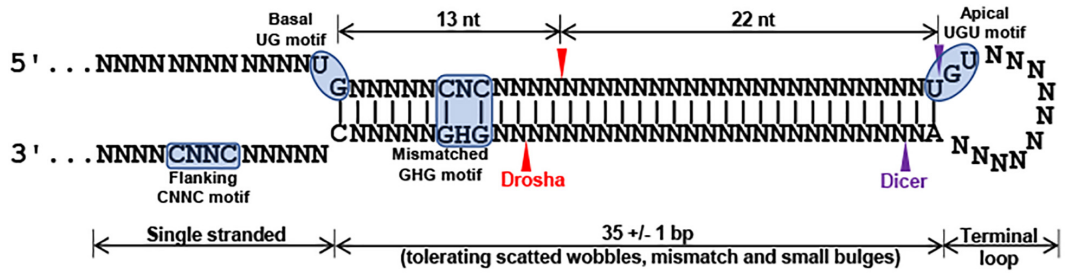

FIGURE 6 | Structural and primary sequence features of pri-miRNA stem of canonical miRNA hairpin (modified from Fang and Bartel, 2015; Nguyen et al., 2015; Bartel, 2018). A canonical miRNA hairpin includes a 35 +/− 1 bp stem, an unstructured terminal loop and singled stranded flanking regions. A basal UG motif, a mismatched GHG motif (H = A, C or U), an apical UGU motif, and flanking CNNC (N = any of the four nucleotides) motif enhance the processing and the precision of Drosha cleavage (red arrowheads). Purple arrowheads mark the sites for DICER cleavage.

samples with homozygous nt106 A/A allele because of its rarity, therefore, cannot make statistically significant conclusion yet. Further larger-scale study is needed to validate this result and prove our hypothesis. In addition, we'd clarify that the current study serves as the discovery stage of a long-term project. We will perform downstream experiments to evaluate the functional consequences and potential roles in diseases of all other variants in the future.

Intriguingly, all three sequence variants identified in pre-miR-96 are in the terminal loop of pri-miR-96 hairpin structure. Recent reports demonstrate that increasing number of RNA binding proteins (RBPs) recognize the terminal loops of selective pri-miRNA hairpin structures, e.g., Lin28a (Viswanathan et al., 2008; Nam et al., 2011), hnRNPA1 (Guil and Caceres, 2007; Michlewski et al., 2008; Kooshapur et al., 2018), KSRP (Trabucchi et al., 2009; Michlewski and Caceres, 2010), to modulate the processing of pri-miRNAs by the Microprocessor and/or pre-miRNA by Dicer (Castilla-Llorente et al., 2013; Treiber et al., 2019). The temporal and tissue specificity of the expression of these RBPs may play a role in defining the expression pattern and function of selective miRNAs at a given time and in a given cell type (Castilla-Llorente et al., 2013). It is reasonable to speculate that the sequence variants in pre-miR-96 may have consequential impact on the maturation and expression levels of mature miR-96. Since miR-182 and miR-96 are among the highest expressed miRNAs and play important roles in the functional differentiation of photoreceptors, hair cells and other sensory neurons (Weston et al., 2006, 2011; Xu et al., 2007; Lumayag et al., 2013; Busskamp et al., 2014; Fan et al., 2017; Geng et al., 2018), if these sequence variants have significant influence on the production of mature miR-182 or miR-96, we further hypothesize that these changes could impose quantitative modulation on the functions of photoreceptors and other sensory neurons and confer susceptibility to various types of IRDs and syndromic diseases in other sensory organs.

As we mentioned earlier, recent discoveries from us and other groups have greatly expanded our knowledge on the miR-183/96/182 cluster. In addition to the sensory organs, miR-183/96/182 cluster plays important roles in both innate and adaptive immune systems. In innate immune system, we demonstrated that miR-183/96/182 cluster is expressed in macrophages and neutrophils and modulates their phagocytosis and intracellular bacterial killing capacity and their inflammatory response to bacterial infection through targeting Nox2 and DAP12 (Muraleedharan et al., 2016, 2019). Inactivation of miR-183/96/182 cluster in mice resulted in increased phagocytosis and bactericidal capacity and decreased production of pro-inflammatory cytokines in response to *Pseudomonas aeruginosa* (PA) infection and decreased overall severity of PA keratitis (Muraleedharan et al., 2016, 2019). Wurm et al. further discovered that miR-182 modulates granulocytic differentiation through a negative regulatory loop with targeting transcription factor C/EBPα (Wurm et al., 2017). C/EBPα is a master regulator of myelopoiesis. It blocks miR-182 expression by direct promoter binding during myeloid differentiation; while miR-182 targets C/EBPα and impairs myelopoiesis and granulocytic differentiation (Wurm et al., 2017). miR-182 expression is highly elevated in acute myeloid leukemia (AML) patients with C/EBPα mutations disrupting its C-terminal DNA binding domain and is recommended as a prognostic marker in cytogenetically high-risk AML patients (Wurm et al., 2017). In natural killer (NK) cells, miR-183/96/182 cluster is induced by TGFβ and mediate TGFβ-induced inhibition of NK cell functions, e.g., tumor cytolysis, through targeting DAP12 (Donatelli et al., 2014).

In adaptive immune system, we showed that miR-183/96/182 is involved in invariant NKT cell development, maturation and effector functions (Wang et al., 2019). miR-182 and miR-183, but not miR-96, are shown to be drastically induced in activated helper T (Th) cells through the IL-2/CD25/STAT5 pathway (Stittrich et al., 2010). miR-182 promotes clonal expansion of activated Th cells through targeting Foxo1 (Stittrich et al., 2010). Furthermore, we and our collaborators further showed that in Th17 cells, induced by IL-6-STAT3 signaling, the miR-183/96/182 cluster promotes Th17 pathogenicity by targeting Foxo1-IL1r1 pathway (Ichiyama et al., 2016). Inactivation of miR-183/96/182 cluster in mice results in decreased production of pathogenic cytokines and decreased severity of experimental autoimmune encephalomyelitis (EAE) (Ichiyama et al., 2016). Another study suggested that miR-182 inhibits $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cell differentiation (Wan et al., 2016); knockdown of miR-182 results in increased Tregs in peripheral lymph nodes and spleen, contributing to decreased severity of EAE in mice

(Wan et al., 2016). In addition, miR-183/96/182 cluster is highly induced during B cell activation as well (Pucella et al., 2015, 2019; Li et al., 2016). In spite of its highly responsiveness to B cell activation, miR-183/96/182 ko or miR-182 ko mice have intact B-cell and T-cell development and follicular helper (Tfh) cell and germinal center (GC) B-cell population (Pucella et al., 2015, 2019; Li et al., 2016). However, miR-182 ko mice showed a delayed generation of antigen-specific antibody production at an early stage in the immunization regimen when challenged with a T-cell-dependent antigen and a complete impairment of antigen response in a T-cell-independent type II antigen challenge, suggesting that miR-182 is required for the extrafollicular antigen response (Li et al., 2016). Consistent with the functions of miR-183/96/182 cluster in immune systems, in an association study, Yu et al. (2014) reported that the pre-miR-182 nt106 A allele is associated with genetic susceptibility to Behcet's disease and Vogt-Koyanagi-Harada (VKH) syndrome, both of which are characterized with autoimmune uveitis (Yu et al., 2014).

Collectively, these reports suggest that miR-183/96/182 cluster plays important roles in different aspects of the functions of various types of innate and adaptive immune cells and tumorigenesis, in addition to its essential roles in photoreceptors and other sensory neurons. Therefore, we predict that mutations in the miR-183/96/182 cluster and dysregulation of its expression may result in defects or phenotypic changes in different aspects of the immune system and the inflammatory/immune responses to antigen invasion and other external and internal pathological insults. Diseases caused by defects in the miR-183/96/182 are most possibly syndromic in nature with constellations of symptoms beyond either sensory or immune systems.

Considering that miRNAs are quantitative regulators of gene expression and that immune/inflammation processes play important roles in normal aging and many multi-factorial retinal diseases, e.g., autoimmune uveitis, age-related macular degeneration, diabetic retinopathy, and glaucoma (Chen et al., 2019), we predict that sequence variants and other mutations in the miR-183/96/182 cluster may contribute to pathogenesis and enhance the susceptibility to these diseases. Studies on the functional consequence of the sequence variants or other mutations of miR-183/96/182 must be conducted in a broader context including both sensory and immune system as well as neuroimmune interactions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/snp/, rs76481776 https://www.ncbi.nlm.nih.gov/snp/, rs77586312 https://www.ncbi.nlm.nih.gov/snp/, rs80041074 https://www.ncbi.nlm.nih.gov/snp/, rs41274239 https://www.ncbi.nlm.nih.gov/snp/, and rs73159662 https://www.ncbi.nlm.nih.gov/snp/, rs73159662.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the IRB of Wayne State University, National Institutes of Health, and Committee for Protection of Human Subjects, University of Texas Southwestern Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SX: project conception, experimental design, data analysis, and manuscript preparation. AC, CM, AH, EM, CD, WL, JC, and WG: conducting the experiments and data analysis. KW and DB: sample collection and clinical data of members of Family 4100. KG: sample collection from eyeGENE. All authors contributed to the article and approved the submitted version.

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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.2020.619641/full#supplementary-material

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REVIEW
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The Impact of miRNAs in Health and Disease of Retinal Pigment Epithelium

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MicroRNAs (miRNAs), a class of non-coding RNAs, are essential key players in the control of biological processes in both physiological and pathological conditions. miRNAs play important roles in fine tuning the expression of many genes, which often have roles in common molecular networks. miRNA dysregulation thus renders cells vulnerable to aberrant fluctuations in genes, resulting in degenerative diseases. The retinal pigment epithelium (RPE) is a monolayer of polarized pigmented epithelial cells that resides between the light-sensitive photoreceptors (PR) and the choriocapillaris. The demanding physiological functions of RPE cells require precise gene regulation for the maintenance of retinal homeostasis under stress conditions and the preservation of vision. Thus far, our understanding of how miRNAs function in the homeostasis and maintenance of the RPE has been poorly addressed, and advancing our knowledge is central to harnessing their potential as therapeutic agents to counteract visual impairment. This review focuses on the emerging roles of miRNAs in the function and health of the RPE and on the future exploration of miRNA-based therapeutic approaches to counteract blinding diseases.

Keywords: miRNAs, retinal pigment epithelium, RPE differentiation, miR-204, miR-211, AMD

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INTRODUCTION

In vertebrates, the RPE originates from the dorsal portion of the optic cup, while the retina and the optic stalk develop from the distal/ventral portion (Amram et al., 2017). Once specified, the RPE cells begin to differentiate, changing in size and shape. With the folding of the optic cup, RPE progenitor cells constitute a ciliated and pseudostratified epithelium, which is committed into a cuboidal epithelium following the formation of interphotoreceptor matrix. As eye morphogenesis proceeds, the presumptive RPE tissue assumes an apical to basolateral polarity and forms both microvilli and tight junctions. On further differentiation, the RPE cells adopts a hexagonal shape, with elongated microvilli and a smooth basal surface which makes continuous contact with its basement membrane (Marmorstein et al., 1998). In adults, RPE tissue comprises a monolayer of polarized and pigmented epithelial cells that resides at the interface between the light-sensitive outer segments of the PR and vessels of choriocapillaris (Strauss, 1995, 2005). The RPE layer is a critical constituent tissue capable of absorbing the light energy focused by the lens on the retina, thus mitigating photo-oxidative stress (Bok, 1993). In addition, the RPE represents part of the tight

retinal–blood barrier (Simo et al., 2010); supports the isomerization of all-*trans*-retinal to 11-*cis*-retinal, the visual chromophore required for photoreceptor excitability (Strauss, 2005); protects from oxidative stress (Strauss, 2005); secretes growth factors that help to maintain the structural integrity of photoreceptors and choriocapillaris endothelium (Strauss, 2005; Mazzoni et al., 2014); establishes ocular immune privilege by expressing immunosuppressive factors (Ao et al., 2018); and finally is critical in the continuous renewal of the photoreceptors outer segments (POS), which are regularly shed by phagocytosis, to rebuild light-sensitive outer segments from the base of the photoreceptors (Mazzoni et al., 2014). To realize these complex functions, intricate molecular networks, activated by extracellular and intracellular signals, are simultaneously employed to maintain RPE homeostasis and function. Due to the significant activity of microRNAs (miRNAs) in modulating essential biological processes by targeting networks of functionally correlated genes, it is unsurprising that miRNAs have emerged as indispensable components of these molecular networks in the RPE (Soundara Pandi et al., 2013; Greene et al., 2014). Importantly, alterations to gene regulatory networks controlling any of the above activities of the RPE can lead to degeneration of the retina and loss of visual function. This in turn gives rise to diseases including retinitis pigmentosa, age-related macular degeneration (AMD), and other blindness conditions in humans (Strauss, 2005; Amram et al., 2017).

Abbreviations: TNRC6, trinucleotide repeat-containing gene 6A; OTX2, orthodenticle homeobox 2; MITF, melanocyte inducing transcription factor; SOX9, SRY-box 9; TARBP2, TAR RNA-binding protein 2; CTNNBIP1, catenin beta interacting protein 1; Wnt, wingless-type MMTV integration site family member; AKT, protein kinase B; mTOR, mammalian target of rapamycin; PAX6, paired box protein; TGF-βR2, transforming growth factor beta receptor II; CDKN1A, cyclin-dependent kinase inhibitor 1A; RPE65, retinal pigment epithelium-specific 65 kDa; HGF/SF, hepatocyte growth factor/scatter factor; TTR, transthyretin; ZO-1, zonula occludens-1; KIR7.1, inward-rectifier potassium channels; TRPM3, transient receptor potential cation channel subfamily M member 3; TRPML1, mammalian mucolipin transient receptor potential; KEAP1, Kelch-like ECH-associated protein 1; NRF2, nuclear factor erythroid 2-related factor 2; SRPK1, serine–arginine protein kinase 1; VEGF165, vascular endothelial growth factor 165; LRAT, lecithin retinol acyltransferase; ATG, autophagy-related; ATG16l1, autophagy-related 16-like 1; BECN1, Beclin 1; PIK3C3/VPS34, phosphatidylinositol 3-kinase, catalytic subunit type 3; ULK1, Unc-51-like autophagy activating kinase 1; FIP200, FAK family kinase-interacting protein of 200 kDa; STAT3, signal transducer and activator of transcription 3; IGTAV, integrin αV; PEDF, pigment epithelium-derived factor; RAB22A, Ras-related protein Rab-22A; CHI3L1, chitinase-3-like protein 1; ERK, extracellular signal-regulated kinase; TFEB, transcription factor EB; IL-6, interleukin 6; VEGF-A, vascular endothelial growth factor A; NF-κβ, nuclear factor kappa-light-chain-enhancer of activated B cells; TRPM1, transient receptor potential cation channel subfamily M member 1; RUNX2, Runt-related transcription factor 2; SIX3, six homeobox 3; LHX2, LIM/homeobox 2; CHX10, Ceh-10 homeodomain-containing homolog; NRL, neural retina leucine zipper; PDC, phosducin; DCT, dopachrome tautomerase; NOX2, NADPH oxidase 2; LGR4, leucine-rich repeat containing G protein-coupled receptor 4; ATF2, activating transcription factor 2; SOCS3, suppressor of cytokine signaling 3; ZEB, zinc finger E-box-binding homeobox; RhoG, Ras homology growth-related; TGFβ, transforming growth factor beta; ABCA1, ATP-binding cassette transporter A1; Cdc25A, cell division cycle 25 A; DAXX, death domain associated protein; PPARα, peroxisome proliferator activated receptor α; NR3C1, nuclear receptor subfamily 3 group C member 1; BCL-2, B-cell lymphoma 2; SIRT1, NAD-dependent deacetylase sirtuin-1; HSP70, heat shock protein 70 kDa; CUL3, Cullin-3; NKILA, NF-kappaB interacting LncRNA; HK2, hexokinase 2; 15-LOX, 15-lipoxygenase; SYN-2, synapsin II; KLH17, Kelch-like family member 7; RDH11, retinol dehydrogenase 11; CERK1, chitin elicitor receptor kinase 1; AIPL1, aryl hydrocarbon receptor interacting protein-like 1; USH1G, Usher syndrome type-1G; MnSOD, manganese superoxide dismutase; $TRXR_2$, thioredoxin reductase 2; GLS1, glutaminase 1; PDGFβ, platelet-derived growth factor β; iASPP, inhibitor of apoptosis-stimulating protein of p53; HIF1A, hypoxia-inducible factor 1-alpha; ASM, acid sphingomyelinase; TLR4, Toll-like receptor 4; NLRP3, NOD–, LRR–, and pyrin domain-containing protein 3; MC5R, melanocortin 5; BACH1, BTB domain and CNC homolog 1; SHIP1, SH-2 containing inositol 5′ polyphosphatase 1; CEBPB, CCAAT enhancer binding protein beta; IKK, inhibitor of upstream IκB kinase; IL-8, interleukin 8; TRAF6, TNF-receptor-associated factor 6; IRAK1, interleukin 1 receptor-associated kinase 1; SRPK1, SRSF protein kinase 1; CEBP, CCAAT enhancer binding protein; FLT1, Fms-related receptor tyrosine kinase 1; BDNF, brain-derived neurotrophic factor; LAMTOR, late endosomal/lysosomal adaptor and MAPK and mTOR activator 1; MMP2, matrix metalloproteinase-2; MMP9, matrix metalloproteinase-9; TNF-α, tumor necrosis factor-alpha; MYO7A, myosin VIIA; TREM2, triggering receptor expressed on myeloid cells 2; ITGB3, integrin subunit beta 3; CRP: C-reactive protein; PON2, paraoxonase 2; RB1, RB transcriptional corepressor 1; RPGR, retinitis pigmentosa GTPase regulator; EDNR, endothelin 3; CFH, complement factor H; VEGFR1, vascular endothelial growth factor receptor 1; CCL3, C–C motif chemokine ligand 3.

miRNAs Biogenesis and Function

Identified in the early 1990s in *Caenorhabditis elegans* (Lee et al., 1993; Wightman et al., 1993), miRNAs are a class of small (typically 20–26 nucleotides) non-coding RNA molecules that regulate gene expression at the posttranscriptional level, in a well-characterized process in which the miRNAs bind to target sites in the messenger RNA. miRNA biogenesis [reviewed by Ha and Kim (2014) and Treiber et al. (2019)] is a complex process that begins with a long primary transcript comprising a stem-loop hairpin structure (pri-miRNA), which is transcribed by RNA polymerase II, capped, and polyadenylated (Treiber et al., 2019). Pri-miRNA subsequently undergoes stepwise processing to produce single hairpins (typically 60–70 nucleotides) referred to as precursor miRNAs (pre-miRNAs) by the RNase III enzyme Drosha and its cofactor DiGeorge syndrome critical region gene 8 (Dgcr8), which compose the "Drosha microprocessor" complex (Hata and Kashima, 2016). Following Drosha processing, pre-miRNA is then exported to the cytoplasm by exportin 5 (Xpo5), where maturation is completed through a second round of stepwise processing by the RNase III enzyme Dicer and its cofactor transactivation-responsive RNA-binding protein (TRBP or PACT). This gives rise to a small RNA duplex (Ha and Kim, 2014). The miRNA duplex is then handed over to a member of the Argonaute (AGO) protein family, which selects one of the strands of this duplex (the guide strand) and discards the other strand (the passenger strand) (Treiber et al., 2019). Finally, the miRNA/AGO complex and the GW182/TNRC6 family of proteins form the active miRNA-induced silencing complex (miRISC), which is recruited to target mRNAs by pairing the "seed" region of miRNA, with a partially complementary sequence in the 3′-untranslated region (3′-UTR) of target mRNAs (Ha and Kim, 2014). Thus, miRNA-guided gene silencing promotes translational inhibition and mRNA decay (Treiber et al., 2019). Most miRNAs are expressed in a highly tissue-specific manner (Wienholds et al., 2005). Importantly, miRNAs have been shown to display either protective or pathological-promoting effects in several tissues. Although the number of miRNAs known to be involved in eye development has increased significantly, a more complete understanding of how miRNAs regulate cellular processes and how their own expression is regulated is yet to be achieved. In the RPE, miRNAs were found

to be key regulators of tissue differentiation, homeostasis, and function. In this review, we discuss some of the known functions of the most relevant miRNAs involved in the RPE development, maintenance, and function. A more comprehensive dissection of the roles of miRNAs in the RPE will not only improve our understanding of the molecular networks controlling RPE homeostasis and our diagnostic abilities but will also lay the foundations for studying how miRNAs could act as therapeutic tools for the treatment of ocular diseases.

miRNAs AS EMERGING REGULATORS OF RPE DEVELOPMENT AND MAINTENANCE

The gene regulatory networks involved in establishing the RPE development and differentiation have been extensively studied in a variety of model organisms reviewed in Amram et al. (2017), but the critical roles of miRNAs in these processes have only recently been explored. Recent studies have reported miRNA expression profiles during RPE development and differentiation in a variety of species, using multiple approaches including next generation sequencing (NGS) technology and *in situ* hybridization (ISH) analysis (Deo et al., 2006; Xu et al., 2007; Damiani et al., 2008; Decembrini et al., 2009; Arora et al., 2010; Georgi and Reh, 2010; Hackler et al., 2010; Karali et al., 2010; La Torre et al., 2013; Wohl and Reh, 2016). Importantly, NGS analyses showed dynamic miRNA expression profiles during RPE differentiation, indicating a strict correlation between miRNA expression levels and the stages of iPSC-RPE differentiation (Wang et al., 2014). To characterize the roles of miRNAs in the RPE development, depletion of the enzymes essential for their maturation and processing were examined in several animal models. Conditional knockout (cKO) mice, for Dicer1, Drosha, or Dgcr8 genes in the RPE, demonstrated that this class of proteins is relevant for RPE differentiation and maintenance. Dicer1 cKO in the ocular pigmented cell lineage around postnatal (PN) day 9.5, generated by crossing $Dgcr8^{LoxP/LoxP}$ animals with *Dct-Cre*, *Tyrp2-Cre*, and α-*Cre* mice, provided the earliest evidence of the impact of simultaneous loss of miRNAs in the generation and survival of non-retinal cell types (Davis et al., 2011). Consistent with these findings, Dicer1 cKO at the optic vesicle stage (E9.5), using *Dct-Cre* mice, revealed alterations to RPE differentiation. At PN11 days, $Dicer1^{loxP/loxP}$/*Dct-Cre* mice showed RPE cells that were smaller than normal, depigmented, and failed to express enzymes required for the visual cycle (Ohana et al., 2015; Amram et al., 2017). However, RPE cell fate and hexagonal cell morphology were preserved, as was the conserved expression of transcription factors (i.e., OTX2, MITF, and SOX9), which participate in RPE specification and maintenance (Amram et al., 2017). Similar phenotypes were also observed in both RPE-specific Drosha and Dgcr8 cKO mouse models (Ohana et al., 2015; Amram et al., 2017). Importantly, the maintenance of RPE cell fate was further corroborated by the transcription profile of Dicer1-deficient RPE cells. These data gave rise to the hypothesis that RPE fate might be acquired and maintained through miRNA-independent mechanisms. Together, these findings supported the notion that miRNAs were necessary for the execution of proper differentiation programs during RPE development (Ohana et al., 2015; Amram et al., 2017). In contrast to the findings on the role of Dicer1 in miRNA biogenesis during RPE differentiation, a non-canonical Dicer1 activity was demonstrated to participate in the adult RPE from $Dicer1^{loxP/loxP}$/*Best1-Cre* mice, with *Cre* recombinase expression beginning at P10 and peaking at P28 (Iacovelli et al., 2011). In differentiated RPE cells, a primary role for Dicer1 was documented in the degradation of toxic transcripts of Alu transposable elements, rather than miRNA biogenesis (Iacovelli et al., 2011). Supporting this notion, single miRNA-binding protein knockout mice, for Ago1, Ago3, Ago4, or Tarbp2, did not reveal RPE morphological alterations (Sundermeier and Palczewski, 2016). Consistent with these findings, AAV-mediated delivery of a Best1-Cre recombinase expression cassette into mice carrying conditional Drosha, Dgcr8, or Ago2 alleles did not show any defects in RPE morphology (Sundermeier and Palczewski, 2016). However, lack of miRNA biogenesis in both differentiating and adult RPE commonly affected the homeostasis and survival of the adjacent photoreceptor cells. Nevertheless, to gain deeper phenotypic understanding, additional studies should analyze alternative miRNA biogenesis mechanisms and the compensatory molecular networks that counteract lack of enzymes essential for miRNA maturation and processing in the RPE (Yang and Lai, 2011).

The Impact of miRNAs on RPE Differentiation, Proliferation, and Migration

Notably, in a few cases, the roles of individual miRNAs have been dissected in fish, frogs, and mice, demonstrating the relevance of these small non-coding RNAs in RPE development. The members of miR-204/211 family are arguably the most extensively studied miRNAs in the RPE. These miRNAs are identifiable from their seed regions and are highly enriched in the developing and differentiated RPE. Overexpression of miR-204/211 in human fetal RPE (hfRPE) cells has been shown to effectively counteract a lack of MITF, a key regulator of RPE differentiation, and has been reported to rescue RPE de-differentiation phenotype (Adijanto et al., 2012). Similarly, studies in human parthenogenetic embryonic stem cells (hPESCs) and hfRPE demonstrated that miR-204 upregulation contributes to RPE differentiation program by repressing the target gene *CTNNBIP1*, an inhibitor of the Wnt/β-catenin pathway (Li et al., 2012). Interestingly, although both miR-204 and miR-211 were required for RPE differentiation, deletion of only one member of this miRNA family resulted in no visible alterations to RPE differentiation, suggesting possible redundancy of function, at least during RPE development *in vivo*. The role of miRNAs in RPE differentiation was also confirmed through studies on miR-184 in hiPSC-RPE and zebrafish, demonstrating the role of miR-184 in controlling RPE differentiation. Downregulation of dre-miR-184 suppressed the expression levels of RPE markers, while its overexpression stimulates RPE development by inhibiting the AKT2/mTOR pathway (Jiang et al., 2016). Further supportive evidence for

the role of miRNAs in RPE differentiation programs has been reported in additional studies. miR-20b/106a and miR-222/221 families have been described to regulate RPE differentiation by modulating the expression levels of several transcription factors including Sox9, Otx1, Pax6, and Meis2 (Ohana et al., 2015). Among the miRNAs downregulated during RPE differentiation, both miR-302d-3p and miR-410 are prominently repressed during RPE development. Importantly, overexpression of miR-302d-3p induced hiPSC-RPE de-differentiation and impairment of cellular phagocytosis and promoted cell-cycle progression, via repression of both *TGF-βR2* and $p21^{Waf1/Cip1}$, a cyclin-dependent kinase inhibitor, encoded by *CDKN1A* (Li et al., 2012; Jiang et al., 2018). Importantly, the inhibition of miR-410 facilitated RPE differentiation in both AESCs and umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) by derepression multiple RPE development-relevant genes, such as *RPE65* and *OTX2* (Choi et al., 2015, 2017). Moreover, multiple studies have reported that inhibition of RPE proliferation and migration were induced by increased expression miR-451a (Shao et al., 2019) and miR-218 (Yao et al., 2019). On the other hand, an overproliferative effect of miR-182 (**Figure 1**) was reported to downregulate the p-Akt pathway in RPE via repression of *c-Met* expression (Wang et al., 2016a). Similarly, overexpression of miR-34a in cultured subconfluent ARPE-19 cells displayed remarkable inhibition of *c-Met*, in turn reducing *HGF/SF* expression and preventing cell proliferation and migration (Hou et al., 2013). Moreover, besides these well characterized miRNAs, additional RPE-enriched miRNAs have recently emerged as regulators of RPE development (**Table 1**). However, their capacities to alter RPE cell differentiation, proliferation, and migration and the identification of their targets and regulatory networks remain to be solved.

RPE HOMEOSTASIS AND FUNCTION

The RPE exhibits common features of an epithelium tissue, preserving the structural and physiological integrities of adjacent tissues. The RPE plays crucial roles in retinal homeostasis, including the formation of the outer blood–retinal barrier (BRB), through junctional complexes, which prevents the entry of toxic molecules and plasma components into the retina (Rizzolo, 2007). Alterations to RPE physiology and homeostasis result in photoreceptor death and blindness. As part of the BRB, the RPE transports water, ions, and metabolic waste from the subretinal space to the choriocapillaris (Marmor, 1990; Hamann, 2002), where it takes up nutrients such as glucose or vitamin A, retinol, and fatty acids to the photoreceptors (Ban and Rizzolo, 2000; Marmorstein, 2001). The RPE secretes different growth factors according to variable physiological and pathological conditions. During eye development, growth factors released from RPE play key roles in choroidal neovascularization. To regulate transport across the RPE, numerous pumps, channels, and carriers are specifically distributed to either the apical or the basolateral membrane. Transporters and channels with roles in the selective transport to and from the choroid vasculature are located in the basal RPE. The apical side of the RPE projects long thin

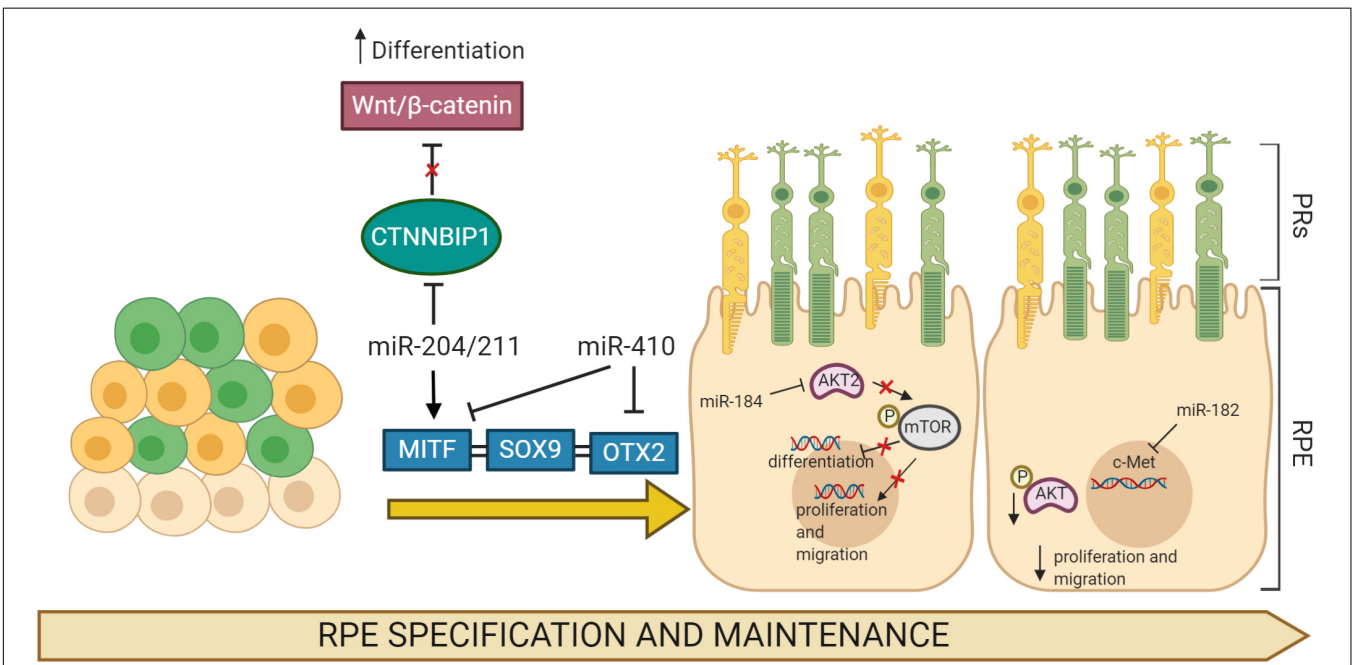

FIGURE 1 | MicroRNA (miRNA)-regulated gene networks involved in RPE specification and maintenance. Simplified schematic of a gene network regulating development, differentiation, and migration in RPE cells. Key genes in these processes, *MITF*, *SOX9*, and *OTX2*, are all regulated by specific miRNAs that modulate their expression profiles. The expression of multiple miRNAs increases and/or affects the processes of RPE differentiation, proliferation, and migration through the regulation of target gene mRNAs.

TABLE 1 | Selected miRNAs involved in the regulation of RPE development, differentiation, and migration.

miRNA	Target	Function	References
miR-204	PAX6, MITF, CTNNBIP1	Positive regulation of RPE development and differentiation	Conte et al. (2010); Adijanto et al. (2012), Li et al. (2012)
miR-184	AKT2/mTOR	Induction of RPE differentiation	Jiang et al. (2016)
miR-410	RPE65, OTX2, LRAT, MITF	Inhibition of RPE development and differentiation	Choi et al. (2015, 2017)
miR-196a	PAX6, SIX3, LHX2, CHX10	Negative regulation of RPE development	Qiu et al. (2009)
miR-182	MITF, c-Met	Negative regulation of RPE proliferation and migration	Wang et al. (2016a)
miR-302d	CDKN1A, TGFβR2	Induction of RPE de-differentiation	Li et al. (2012); Jiang et al. (2018)
miR-183/-96/-182	OTX2, NRL, PDC, DCT	Triggering of neuronal cells differentiation	Davari et al. (2017)
miR-27b	NOX2	Negative regulation of RPE proliferation and migration	Li et al. (2018)
miR-34	c-Met, LGR4	Modulation of RPE proliferation and migration	Hou et al. (2013, 2016)
miR-451a	ATF2	Inhibition of RPE proliferation and migration	Shao et al. (2019)
miR-218	RUNX2	Inhibition of RPE cells proliferation	Yao et al. (2019)
miR-26b	Unknown	RPE cell proliferation promotion	Zhang et al. (2017b)
miR-203a	SOCS3	Regulation of RPE differentiation	Chen et al. (2020b)
miR-194	ZEB1	Inhibition of EMT of RPE cells	Cui et al. (2019)
miR-148	Unknown	RPE EMT promotion	Takayama et al. (2016)
miR-124	RhoG	Control of EMT	Jun and Joo (2016)
miR-125b/let-7a	Unknown	Promotion RPE fate during differentiation	Shahriari et al. (2020)
miR-29b	AKT2	Regulation of RPE EMT	Li M. et al. (2016)
miR-328	PAX6	Promotion of RPE proliferation	Chen et al. (2012)
miR-21	TGFβ	Cell proliferation and migration promotion	Usui-Ouchi et al. (2016)

and sheet-like microvilli into the interphotoreceptor matrix. The long microvilli engulf the tips of the rod and cone photoreceptor outer segments; this ensures that the segments are orientated in the appropriate plane for optics and maximizes the cellular surface for efficient epithelial transport. The short microvilli mainly function in the turnover of POS through phagocytosis. Moreover, RPE cells are constantly exposed to oxidative stress due to exposure to light and the generation of reactive oxygen species (ROS) following phagocytosis of POS (Plafker et al., 2012). A robust endogenous antioxidant defense mechanism is therefore critical. This is characterized by the production of endogenous antioxidants such as glutathione, whose production decreases with age and/or incidence of degenerative diseases (Beatty et al., 2000). Oxidative stress alters RPE integrity and promotes the activation of the inflammation system, activating complementary cascades and upregulating the production of cytokines and chemokines. Notably, the mature RPE itself maintains tissue homeostasis through long-term cell survival, with little evidence of cell turnover and a stem cell compartment for *de novo* cell production. This static homeostasis suggests that as RPE cells age, substantial tissue changes occur that compromise RPE function and morphology, triggering causes of AMD (Fuhrmann et al., 2014). Based on its structural characteristics, the RPE plays crucial roles in retinal homeostasis, of which a primary role is the formation of the outer BRB that prevents the entrance of toxic molecules and plasma components into the retina (Rizzolo, 2007). Breakdown of the BRB can lead to visual loss in a number of ocular disorders, including diabetic retinopathy (DR).

MicroRNAs Are the "Traffic Wanders" of the RPE Homeostatic Center

Maintenance of RPE layer integrity is controlled by junctional complexes, characterized by the presence of multiple proteins including occludin, claudins, and junctional adhesion molecules. This type of cell–cell adhesion establishes a barrier between the subretinal space and choriocapillaris (Harhaj and Antonetti, 2004). The first evidence for the role of miRNAs in RPE homeostasis was reported from studies on both Dicer and Dgcr8 cKO mice, where ultrastructural analysis of RPE revealed disorganized basal infoldings with large cytoplasmic vacuoles and debris accumulation at the interface of Bruch's membrane, resulting in atrophy and disruptions to the integrity of the RPE (Sundermeier et al., 2017; Wright et al., 2020). Furthermore, miRNA studies on miR-204 revealed its prominent functional role in the formation and maintenance of the epithelial barrier of the RPE. Wang et al. (2010) demonstrated that miR-204 significantly alters claudin 10 and 19, tight-junction proteins both highly expressed in human RPE, while miR-204/211 affects transthyretin (TTR) secretion, an important marker for epithelial barrier integrity and critical for vitamin A transport across the RPE (Cichon et al., 2014). In agreement with this, overexpression of the miR-204/211 family in primary hfRPE induced high expression levels of RPE-specific genes, triggering cell reprogramming to RPE epithelial cells characterized by hexagonal shape with junctional multiplexes (Adijanto et al., 2012). Additionally, it has also been observed that miR-148a induced reduction in *ZO-1* expression and disruptions to RPE morphology (Takayama et al., 2016). Moreover, the expression of miR-20b/106a and miR-222/221 families are likely to be essential for maintaining RPE barrier properties and function and have crucial functional roles in RPE homeostasis (Ohana et al., 2015). As part of the BRB, the RPE transports water, ions, and metabolic waste from the subretinal space to the choriocapillaris (Marmor, 1990; Hamann, 2002), where it takes up nutrients such as glucose, vitamin A, retinol, and fatty acids to the photoreceptors (Ban and Rizzolo, 2000; Marmorstein, 2001). To regulate transport across the RPE, various pumps, channels, and transporters are distributed specifically to either the apical or the basolateral membrane. In particular, it has been shown that Kir7.1 is the most abundant potassium channel localized at the apical membrane in the RPE (Yang et al., 2008).

Kir7.1 mediates the interactions between retinal photoreceptors and RPE following transitions between light and dark. Wang et al. (2010) also provided the earliest evidence that miR-204 indirectly suppresses Kir7.1 via *TGF-βR2* repression in human RPE and thus likely limits potassium efflux across the RPE apical membrane. Additionally, the Kir7.1 channel is functionally coupled to other apical membrane potassium transporters, and the recycling capacity of this channel is maintained by high levels of miR-204 expression. In this context, supporting these observations, miR-204$^{-/-}$ mice showed a reduced efflux of K^+ transport, which accumulates in the RPE and induces cell swelling and alteration of subretinal space (Zhang et al., 2019). Furthermore, in several studies, it has been shown that miR-204 controls the expression of its host gene, namely, *TRPM3*, which conducts Ca^{2+} and Zn^{2+} ions in renal cell carcinoma (Hall et al., 2014). Depletion of miR-204 may also increase *TRPM3* expression, resulting in general alterations to ion transportation in the RPE. On the other hand, TRPM3 also cooperates with ZO-1 to maintain junctional permeability and barrier function at the apical membrane and to sense light-induced Ca^{2+} levels in the photoreceptor matrix during the visual cycle (Zhao et al., 2015). The latter suggests that the loss of miR-204 could alter the homeostasis of RPE through multiple mechanisms. Other miRNAs have also been identified as crucial regulators of ion channels. Noteworthy among them, Naso et al. (2020) demonstrated that miR-211-mediated inhibition of *Ezrin* releases a Ca^{2+} microdomain flux into cells through potentiation of TRPML1 channel sensitivity, a lysosomal cation channel implicated in lysosomal biogenesis and function (Gomez et al., 2018). miR-211$^{-/-}$ mice show severely compromised vision characterized by an accumulation of phagolysosomes which contain poorly processed POS in the RPE and cone dystrophy (Naso et al., 2020). Confirming the central relevance of miRNAs in RPE homeostasis, miRNAs also take part in cellular responses to environmental stresses such as hypoxia, oxidative stress, and inflammation. Remarkably, oxidative stress stimulates the production of miRNAs that play a role in connecting the antioxidant defense system with imbalances in redox state. Among those, miR-626 represses *Keap1*, which results in the promotion and activation of Nrf2-dependent antioxidant signaling to protect RPE against oxidative stress (Xu et al., 2019). In line with this finding, Lin et al. (2011) showed antiapoptotic effects of miR-23 against oxidative injury through regulation of *Fas*, suggesting the relevance of miR-23 as a key regulator of ROS-mediated cell death/survival. Finally, miR-30b has been reported to increase the cellular antioxidant defense against oxidative stress by targeting the catalase gene. Conversely, miR-144 has recently been described to exert its apoptotic function by targeting *Nrf2*. In response to oxidative stimuli, miR-144 directly represses *Nrf2*, causing reductions to endogenous levels of glutathione and leaving RPE cells susceptible to oxidative stress (Jadeja et al., 2020). These changes in miRNA expression levels suggest dual roles for miRNAs, giving rise to protective or pathological apoptotic phenotypes. Remarkably, Nrf2 also protects retinal tissues from vascular inflammation. A number of additional studies hint at miRNAs playing a role in counteracting the chronic neovascularization in RPE by regulating the expression patterns of proangiogenic factors. For example, miR-9 has been demonstrated to target *Srpk1* gene, controlling the alternative splicing of angiogenic *VEGF165* in human RPE cells under oxidative stress (Yoon et al., 2014). Notably, overexpression of miR-9 effectively reduces the mRNA levels of proangiogenic *VEGF165a* in hypoxia, while the inhibition of miR-9 decreases antiangiogenic *VEGF165b* mRNA levels (**Figure 2**). These findings further imply that alterations in specific miRNAs (listed in the **Table 2**) may be used as pathophysiological biomarkers and, additionally, that the precise control of their expression patterns could offer therapeutic strategies to counteract the onset and progression of diverse retinal inherited disorders.

VISUAL CYCLE IN THE RPE

Vision relies on the functional interaction between RPE and photoreceptors. RPE takes an active part in the visual cycle, as it expresses the enzymes required for reisomerization of the 11-*cis* chromophore from all-*trans*-retinal, which is essential for initiating the photoreceptor response to light. All-*trans*-retinal is reduced to all-*trans*-retinol within the photoreceptors and then transported to the RPE, where it is esterified by LRAT (Saari and Bredberg, 1989; Ruiz et al., 1999). The all-*trans*-retinyl ester product is then isomerized by RPE65 and hydrolyzed to release 11-*cis*-retinol (Redmond et al., 2005). Importantly, phagocytosis and autophagy are crucial cellular mechanisms required for maintaining RPE/PRs homeostasis and supporting the visual system. Light exposure to photoreceptors is accompanied by photo-oxidation of proteins and phospholipids of the outer segments (Beatty et al., 2000). To maintain their function, the POS are shed on a circadian basis (LaVail, 1976, 1980) and phagocyted by the RPE, where they fuse with lysosomes for the degradation and recycling of the ingested POS cargo (Anderson et al., 1978). Alterations to any of the above activities of the RPE is thought to be the primary cause in retinal pathologies, including AMD, because the RPE is vital for the health, survival, and function of the adjacent retinal PRs and choroid (Nandrot et al., 2004, 2006). Remarkably, oscillations in genes related to these cellular processes were observed to respond rapidly to changes in the light environment supporting combinatorial light-dependent and circadian-mediated regulation of visual POS turnover. Phagocytosis and degradation of POS occurs by a non-canonical form of autophagy termed LC3-associated phagocytosis (LAP), which exploits the effectors of autophagy in the process of phagocytosis (Kim et al., 2013; Ferguson and Green, 2014). Importantly, during LAP process, the phagocytic machinery takes advantage of autophagy components Atg5, Atg7, Atg3, Atg12, and Atg16l1 for the lipidation of LC3, resulting in LC3 recruitment to the phagosome and uses a BECN1-PIK3C3/VPS34 complex lacking Atg14. LAP is also independent of the autophagy preinitiation complex consisting of ULK1/Atg13/FIP200 (Kim et al., 2013). Engulfed POS enters the phagosome on a daily basis, the Atg12-Atg5-Atg16L1 complex is recruited, as is the lipidated form of LC3 (LC3-II). Only then does the lysosome fuse with the phagosome forming

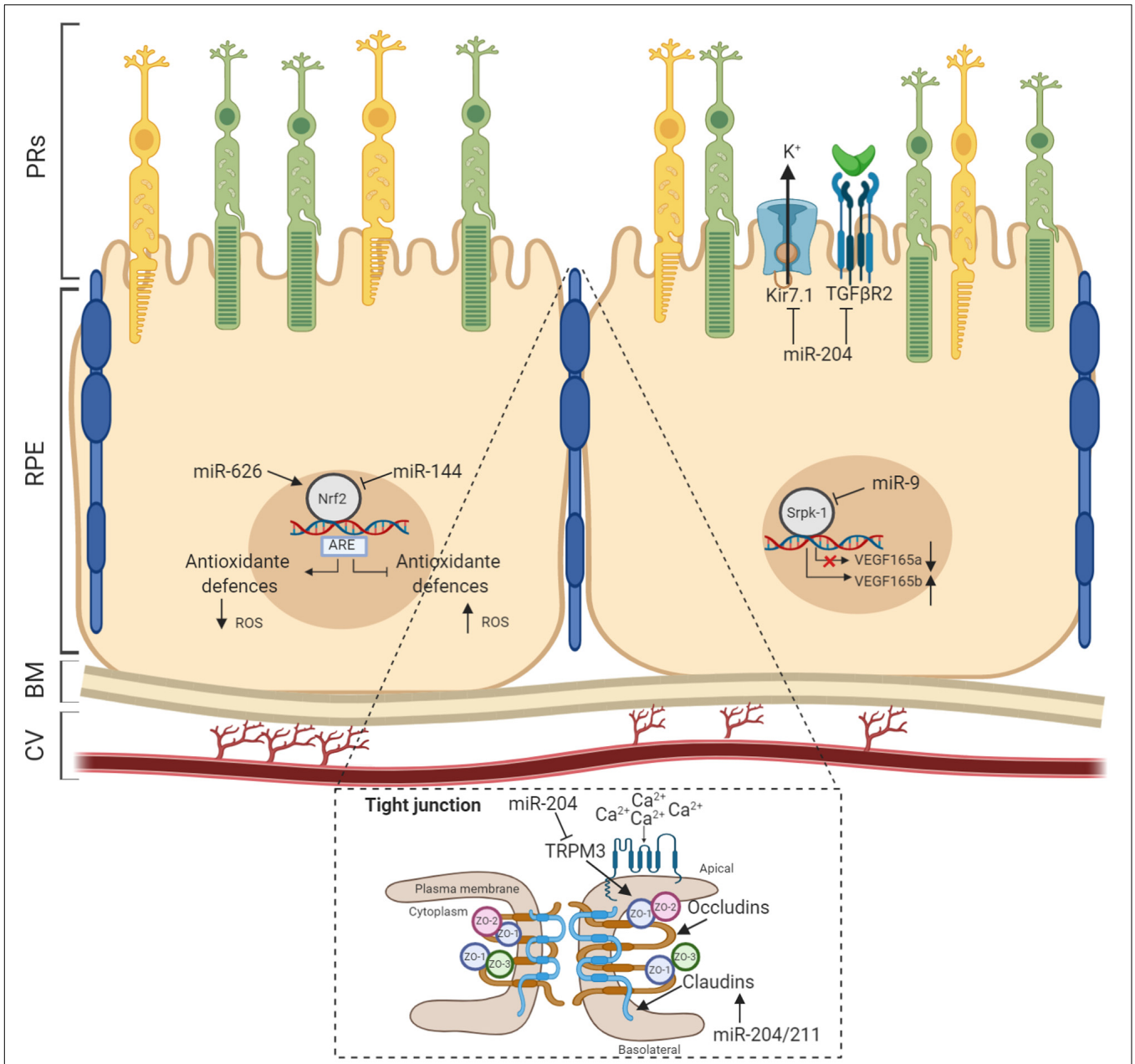

FIGURE 2 | MicroRNAs (miRNAs) act as modulators of RPE homeostasis. Several miRNAs have been demonstrated to influence the correct functioning of the RPE homeostatic machinery. Recognized miRNAs species associated to specific RPE functionality are shown with validated targets. BM, Bruch's membrane; CV, choroidal vasculature.

the phagolysosome leading to degradation of the POS cargo (Ferguson and Green, 2014).

The MicroRNAs as Key Players in the Phagocytosis and Cell Clearance

The relevance of miRNAs in RPE phagocytosis and cell clearance soon became evident from studies on the RPE from Dgcr8 cKO mice. Specifically, these mice displayed significant decreases in the phagocytotic process accompanied by the presence of lipid droplet-like structures and a lower recovery rate of the visual chromophore 11-*cis*-retinal in the RPE (Sundermeier et al., 2017). Importantly, the daily phagocytosis of POS requires flexibility and the correct orientation of the cytoskeleton in the RPE (Law et al., 2015); uptake and intracellular processing of extracellular material are mediated by both actin and myosin, as well as microtubules for the fusion of lysosomes and phagosomes (Stossel, 1977). Several studies identified Ezrin and ezrin–radixin–moesin (ERM)-binding phosphoprotein 50 (EBP50) as the complex responsible for the polarization dynamics

TABLE 2 | miRNAs involved in the regulation of RPE homeostasis.

miRNA	Target	Function	References
miR-204	TGFβR2, SNAIL2	Regulation of claudins and Kir 7.1 in RPE physiology	Wang et al. (2010)
miR-211	EZRIN	Potentiation of TRPML1 channel sensitivity	Naso et al. (2020)
miR-128/miR-148	ABCA1	Reduction of cellular cholesterol efflux	Hou et al. (2013)
miR-21	p21, Cdc25A, DAXX PPARα	Influence on p53 pathway in retinal microglia Inflammation amelioration	Chen et al. (2017); Morris et al. (2020)
miR-382	NR3C1	Acceleration of intracellular ROS generation	Chen et al. (2020a)
miR-144	NRF2	Worsening of oxidative stress	Jadeja et al. (2020)
miR-626	KEAP1	Protection of RPE cells from oxidative injury	Xu et al. (2019)
miR-195	BCL-2	Oxidative stress increase	Liu et al. (2019)
miR-302a/miR-122	Unknown	Induction of neovascularization after oxidative stress	Oltra et al. (2019)
miR-34a	SIRT1 HSP70	Reduction of oxidative stress tolerance Cryoprotective effect by refolding proteins misfolded in the presence of oxidative stress	Ryhanen et al. (2009); Tong et al. (2019)
miR-601	CUL3	Protection of RPE cells from hydrogen peroxide	Chen et al. (2019)
miR-103	NKILA	Protection of RPE cells from hypoxia	Zhou et al. (2018)
miR-125b	HK2, 15-LOX, SYN-2	Prevention of oxidative stress	Lukiw et al. (2012)
miR-1307/miR-3064/miR-4709/miR-3615/miR-637	KLHl7, RDH11, CERK1, AIPL1, USH1G	Regulation of RPE oxidative stress	Donato et al. (2018)
miR-374a	FAS	Protection of RPE cells against oxidative conditions	Donato et al. (2018)
miR-17	MnSOD, $TRXR_2$	Aggravation of oxidative RPE damage	Tian et al. (2016)
miR-23a	GLS1	RPE cells sensitization to H_2O_2	Li D.D. et al., 2016
miR-30	Catalase	Antioxidant defense reduction	Haque et al. (2012)
miR-23	FAS	Protection of RPE cells against oxidative damage	Lin et al. (2011)
miR-20/miR-126/miR-150/miR-155	VEGF-A, PDGFβ, NF-κβ, Endothelin, p53	Protection of RPE cells against oxidative stress	Howell et al. (2013)
miR-340	iASPP	Affection of UVB-mediated RPE cell damage	Yan et al. (2018)
miR-141	KEAP1	Protection of RPE from UV radiation	Cheng et al. (2017)
miR-217	SIRT1/HIF1A	Inflammation amelioration	Xiao and Liu (2019)
miR-15a	ASM, VEGF-A	Regulation of proinflammatory pathways	Wang et al. (2016c)
miR-27	TLR4	Inflammation strengthening	Tang et al. (2018)
miR-22	NLRP3	Protection of RPE inflammatory damage	Hu et al. (2019)
miR-155	MC5R, BACH1, SHIP1, CEBPB, IKK	Modulation of inflammatory response	Kutty et al. (2010a), Muhammad et al. (2019)
miR-146a	IL-6, IL-8, TRAF6, IRAK1	Expression regulation of inflammatory genes	Hao et al. (2016)
miR-223	NLRP3	Negative inflammation regulation	Sun et al. (2020)
miR-138	SIRT1	Negative effect on vascular function	Qian et al. (2019)
miR-9	SRPK-1, CEBPA, CEBPB	Biomarker for chronic neovascularization Importance in maintaining RPE cell function	Kutty et al. (2010b, 2013), Yoon et al. (2014)
miR-93	VEGF-1	Choroidal neovascularization involvement	Wang et al. (2016b)
miR-31/miR-150	VEGF, HIF, PDGFβ	Dynamic regulation of neovascularization	Shen et al. (2008)
miR-200	ZEB1, ZEB2, FLT1	CNV involvement	Chung et al. (2016)

of the RPE microvilli (Bonilha et al., 1999; Kivela et al., 2000; Nawrot et al., 2004). Notably, at the apical surface of RPE, Ezrin and EBP50 can associate with the cellular retinaldehyde-binding protein (CRALBP) in a complex necessary for the release of 11-*cis*-retinal or uptake of all-*trans*-retinol (Bonilha et al., 2004). Preliminary evidence for the role of miRNA in phagocytosis was reported by Murad et al. (2014) showing that inhibition of miR-184 results in the upregulation of *Ezrin* gene and causes downregulation of the phagocytosis in ARPE-19 cells, altering RPE homeostasis. Interestingly, RPE primary culture from AMD donors also showed downregulation of miR-184, consistent with an altered visual cycle (Murad et al., 2014). Moreover, recently, an interesting study showed that miR-302d-3p interrupts phagocytosis in the RPE cells. The latter was correlated to a role for miR-302d-3p in promoting RPE dedifferentiation by targeting $P21^{Waf1/Cip1}$, a cyclin-dependent kinase inhibitor (Jiang et al., 2018). A number of additional miRNAs have been implicated to phagocytosis process, including miR-410 (Choi et al., 2017), miR-194 (Cui et al., 2019), and miR-25 (Zhang et al., 2017a) (**Figure 3**). miR-25 was the first miRNA to be well characterized *in vivo*. Oxidative stress promotes miR-25 expression in the RPE cells by STAT3 signaling, this in turn decreases phagocytosis through direct miR-25-mediated targeting of *IGTAV* and *PEDF*. Interestingly, subretinal injection of antago-miR-25 in a rat model of retinal degeneration, induced by sodium iodate (SI)-mediated

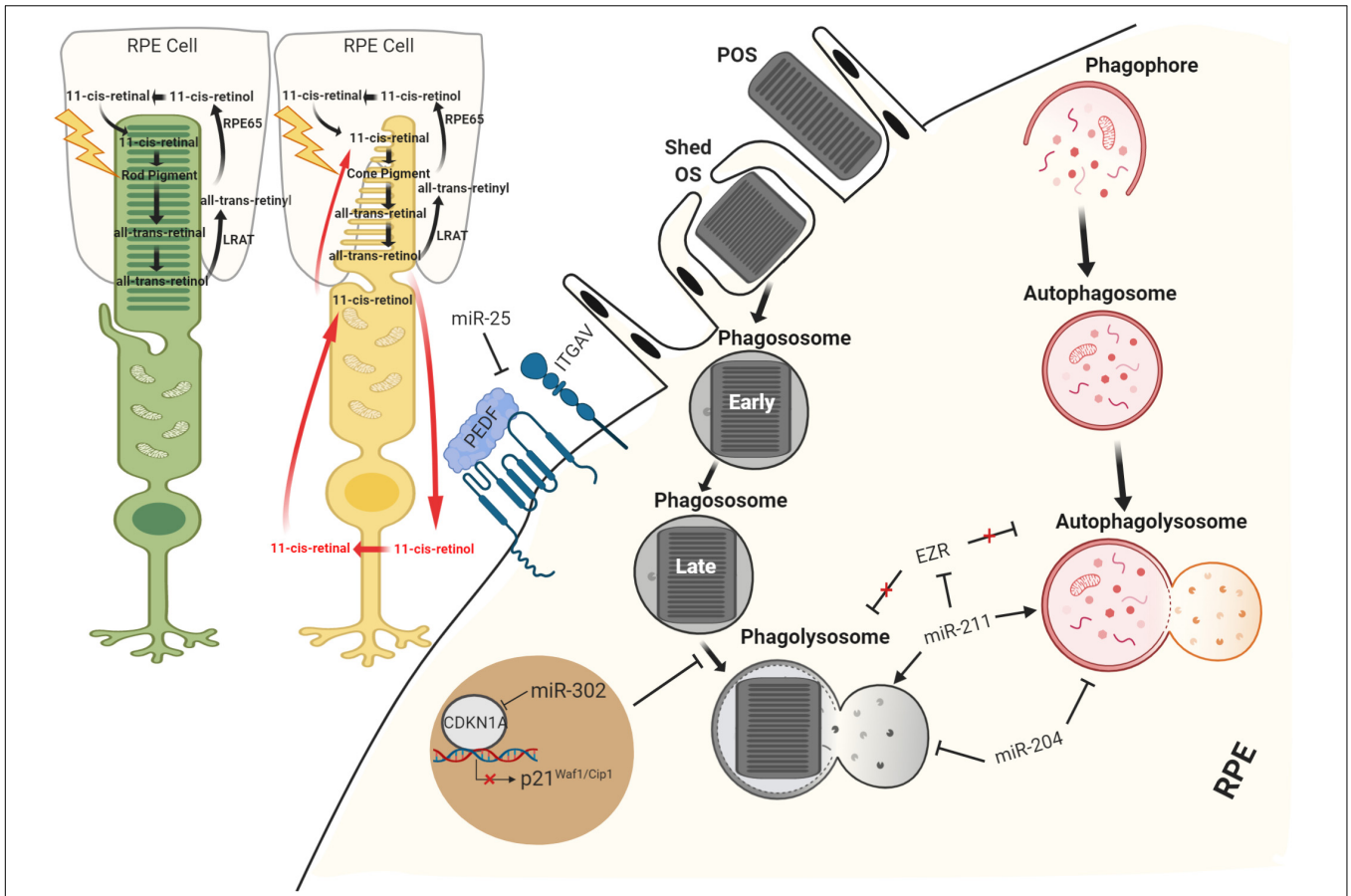

FIGURE 3 | Schematic view of miRNA regulation in RPE recycle. miRNAs have been demonstrated to function both as supporters of visual system and as important regulators of RPE homeostasis maintaining processes, phagocytosis, and autophagy.

oxidative stress, regressed the impairments of phagocytosis and also ameliorated the RPE degeneration (Zhang et al., 2017a). Similarly, *in vitro* studies suggest that inhibition of miR-410 is also able to induce phagocytic capabilities by increasing gene and protein expression of RPE-specific factors (Choi et al., 2017). A recent study has also reported that miR-204 is also involved in phagocytosis and in the processing of phagocyted POS by lysosomes. More specifically, miR-204$^{-/-}$ mice are characterized by retinal degeneration caused by the incomplete degradation of POS and lysosomal accumulation of rhodopsin (Zhang et al., 2019). The study revealed that the absence of miR-204 causes an increase in *Rab22a*, an inhibitor of endosomal maturation. Rab22a is essential to the maturation of early autophagosomal/endosomal intermediates that do not fuse with lysosomes. Thus, miR-204 reduction determines an accumulation of undigested POS, showing a blockage of phagolysosome activity (Zhang et al., 2019). Mice lacking miR-204 also showed inefficient transportation of 11-*cis*-retinal from RPE to the photoreceptors associated with significant reduction to specific gene expression levels (i.e., *RPE65*, *LRAT*, and *TTR*) required for visual pigment regeneration (Zhang et al., 2019). Importantly, severe retinal defects were observed in patients affected by a dominant mutation in miR-204, as the genetic cause of a retinal degeneration associated to other ocular manifestations (Conte et al., 2015). A number of additional miRNAs have been validated to modulate autophagy and recycle of visual components in the RPE (**Table 3**). miR-30 was also found to play an important role in autophagy by downregulating *Beclin 1* and *Atg5* expression (Yu et al., 2012). Moreover, Lian et al. (2019) demonstrated that miR-24 plays an important role in regulating autophagy in the RPE. This study identified *chitinase-3-like protein 1* (CHI3L1) as a main target. Importantly, *CHI3L1* was suggested to activate the AKT/mTOR and ERK pathways resulting in aberrant autophagy and RPE dysfunction (Lian et al., 2019). Notably, the circadian clock and light are the major regulators of POS phagocytosis and autophagy in the RPE. Several miRNAs were identified to play a role in modulating circadian timing and light-induced responses (Xu et al., 2007; Huang et al., 2008; Pegoraro and Tauber, 2008). Among these, miR-211 is of particular importance because it shows a light-dependent expression pattern (Krol et al., 2010) and it is relevant in homeostasis maintenance of retinal cells through diurnal lysosomal biogenesis in the RPE (Naso et al., 2020). miR-211$^{-/-}$ mice are characterized by defective lysosomal biogenesis and degradative capacities, resulting in the accumulation of POS within the RPE. While miR-211 activation induced autophagy and rescued the anomalies in lysosomal

TABLE 3 | miRNAs in recycling visual pigments.

miRNA	Target	Function	References
miR-137/miR-363/miR-92/miR-25/miR-32	RPE65	Negative regulation of visual cycle	Masuda et al. (2014), Zhang et al. (2017a)
miR-124	SOX9, LHX2	Visual cycle inhibition	Masuda et al. (2014)
miR-183/miR-96/miR-182	BDNF	Protection against retinal light injury	Li et al. (2015)
miR-204	RAB22A	RPE endolysosome function alteration	Zhang et al. (2019)
miR-211	EZRIN	Modulation of lysosomal biogenesis and retinal cell clearance	Naso et al. (2020)
miR-184	EZRIN	Efficiency of POS uptake increase	Murad et al. (2014)
miR-410	RPE65	Induction of phagocytic capabilities	Choi et al. (2017)
miR-302	CDKN1A	RPE phagocytosis inhibition	Jiang et al. (2018)
miR-25	IGTAV, PEDF	Phagocytosis decrease	Zhang et al. (2017a)
miR-29	LAMTOR/p18	Autophagy enhancement	Cai et al. (2019)
miR-1273	MMP2, MMP9, TNF-α	Autophagy/lysosome pathway modulation	Ye et al. (2017)
miR-617	MYO7A	Lysosome movement in RPE cells influence	Tang et al. (2015)

biogenesis. This study also showed that miR-211 targets and represses *Ezrin*; this in turn promotes a Ca^{2+}-mediated activation of calcineurin, which leads to TFEB nuclear translocation, thus inducing the expression of lysosomal and autophagic genes (Naso et al., 2020).

miRNA FUNCTIONS IN RPE DISEASES

Age-related macular degeneration is a multifactorial, degenerative disease and the most common cause of vision impairment and blindness in the elderly population, with approximately 30–50 million people affected worldwide. The susceptibility to develop "dry" and "wet" AMD forms is dependent on a combination of genetic components and environmental factors. AMD, as well as other forms of retinal degeneration, is often similar and associated with impaired function of the RPE. AMD is characterized by deposits of lipofuscin and extracellular protein aggregates called "drusen" determining progressive dysfunction of autophagy and both RPE and photoreceptor cell death. Interestingly, studies carried out on AMD highlighted that Dicer1 mRNA was reduced in the RPE, but not into neural retina, by 65 ± 3% compared to control eyes, while there was no differences in *Drosha* and *Dgcr8* mRNAs in AMD eyes (Kaneko et al., 2011). To define the relevance of Dicer1 reduction in the RPE, Kaneko et al. (2011) crossed *Dicer1*f/f mice with *BEST1* Cre mice, which express Cre recombinase under the control of the RPE-cell-specific BEST1 promoter. Results showed that these mice displayed RPE cell degeneration in comparison to controls. However, Dicer1 dysregulation in these mice was associated to Dicer1 capacity of silencing toxic *Alu* transcripts rather than miRNA biogenesis (Kaneko et al., 2011). To verify the hypothesis that miRNA dysregulation is implicated in the pathophysiology of AMD, global expression profiles of miRNAs have been investigated. Several studies examined + circulating miRNAs as well as tissue-specific miRNA expression patterns from age-matched control and affected individuals. The results show a number of discrepancies in the number and type of miRNA identified, probably due to the procedures applied to quantify miRNAs and selection of analyzed samples. In spite of these discrepancies, it is evident that most of miRNAs participated to oxidative stress, inflammation, lipid metabolism, and angiogenesis. Remarkably, miRNA expression profiles in both AMD mouse and rat models exhibited overlapping results, suggesting that the main role of miRNAs in RPE is to protect against oscillations in gene expression and counteract environmental stress, in an effort to preserve RPE cellular homeostasis (Berber et al., 2017). **Table 4** lists the most commonly reported miRNAs identified in

TABLE 4 | miRNAs in AMD.

miRNA	Target	Function	References
miR-184	LAMP1, EZRIN AKT2/mTOR	Affection of RPE phagocytosis Prevention of RPE dysfunction and AMD	Murad et al. (2014); Jiang et al. (2016)
miR-34a	TREM2	Drusen formation in AMD	Smit-McBride et al. (2014); Bhattacharjee et al. (2016)
miR-24	CHI3L1	RPE protection from degeneration	Lian et al. (2019)
miR-34	SIRT1, TREM2	Modulating age-related conditions, including AMD	Bhattacharjee et al. (2016); Tong et al. (2019)
miR-30	ITGB3, CRP, PON2, RB1, RPGR, EDNR	Modulating AMD pathogenesis	Haque et al. (2012)
miR-302	CDKN1A	Contribution to the pathogenesis of both atrophic and exudative AMD	Jiang et al. (2018)
miR-155	CFH	Activation of immune-related signaling	Lukiw et al. (2012)
miR-200	ZEB1, ZEB2, VEGFR1	Modulation of pathological ocular angiogenesis	Chung et al. (2016)
miR-20/miR-126/miR-150/miR-155	VEGF-A, PDGFβ, NF-B, endothelin, p53	Protection of RPE cells against oxidative stress	Howell et al. (2013)
miR-9	CEBPA, CEBPB	Maintenance of RPE function	Kutty et al. (2010b)
miR-204	RAB22A	Modulation of endolysosomal and/or autophagy pathways	Zhang et al. (2019)
miR-29	LAMTOR/p18	Contribution to AMD progression	Cai et al. (2019)
miR-223	CCL3, NLRP3, STAT3	Regulation of inflammation during retina degeneration	Fernando et al. (2020)
miR-302/miR-122	VEGF	Regulation of vasculogenesis	Oltra et al. (2019)

pathological RPE conditions. Interestingly, the most frequently altered expression identified was that of miR-146a, which occurred in both "dry" and "wet" AMD patients and was found in the RPE of different AMD animal models. Remarkably, *in vivo* and *in vitro* studies of specific miR-146a functions demonstrated key roles including repressing expression of *IL-6* and *VEGF-A* genes and inactivating the NF-κB signaling pathway in the RPE. The latter, together with the gain-of function studies for miR-146a, suggests a crucial role for this miRNA in controlling genetic pathways essential to innate immune responses, inflammation, and the microglial activation state, which are major features in the pathogenesis of AMD (Hao et al., 2016). This further suggests that miR-146a may represent a useful disease biomarker and, additionally, a valuable therapeutic target for AMD treatment. Moreover, recent findings reported the role of the epithelial–mesenchymal transition (EMT) in the RPE as a pathological feature of the early stages of AMD (Shu et al., 2020). Remarkably, transforming growth factor-beta (TGFβ) has been identified as a key cytokine orchestrating EMT, and its alteration has been largely associated with onset and progression of several ophthalmological diseases, including AMD (Wang et al., 2019). Interestingly, dynamic changes in expression levels of several miRNAs were associated with TGF-β signaling during EMT in the RPE (Li D.D. et al., 2016). Among these, miR-29b was the most significantly downregulated. The function of the miR-29b was examined in ARPE-19. Additional consequences of miR-29b downregulation included the downregulation of E-cadherin and ZO-1, upregulation of α-smooth muscle actin (α-SMA), and increased cell migration. *In vitro* analyses showed that overexpression of miR-29b was sufficient to reverse TGF-β-mediated EMT through targeting Akt2. In agreement with this, silencing of the Akt2 abolished miR-29b-mediated repression of EMT process (Li M. et al., 2016). Similarly, a detailed study by Jun and Joo (2016) demonstrated that miR-124 was also involved in EMT in the ARPE-19. miR-124 overexpression induced occludin (OCLN) and ZO-1 and repressed α-SMA by directly targeting Ras homology growth-related (RHOG) (Jun and Joo, 2016). Besides miR-29b and miR-124, other miRNAs have been shown to negatively control the EMT. The levels of the ZEB1 protein, a key transcription factor in EMT, in ARPE-19 cells were decreased in the presence of miR-194 duplexes and elevated on miR-194 inhibition. In agreement with these observations, the levels of expression of ZEB1 target genes were reduced in response to miR-194 overexpression as a consequence of alterations in ZEB1 repression. The miR-194 targeting of ZEB1 has also been confirmed *in vivo*. Exogenous administration of miR-194 ameliorated the pathogenesis of proliferative vitreoretinopathy in a rat model (Cui et al., 2019). Accordingly, a recent study also showed that both miR-302d and miR-93 exert their functions in regulating TGFB signaling in the RPE (Fuchs et al., 2020). Altogether, these data highlight a potential use for miRNA: as a promising therapeutic resource for the treatment of ocular disorders. An additional key player involved in AMD and pathophysiology of RPE is miR-211. Indeed, two independent studies based on genome-wide searches for novel AMD risk factors identified miR-211 and its host gene,

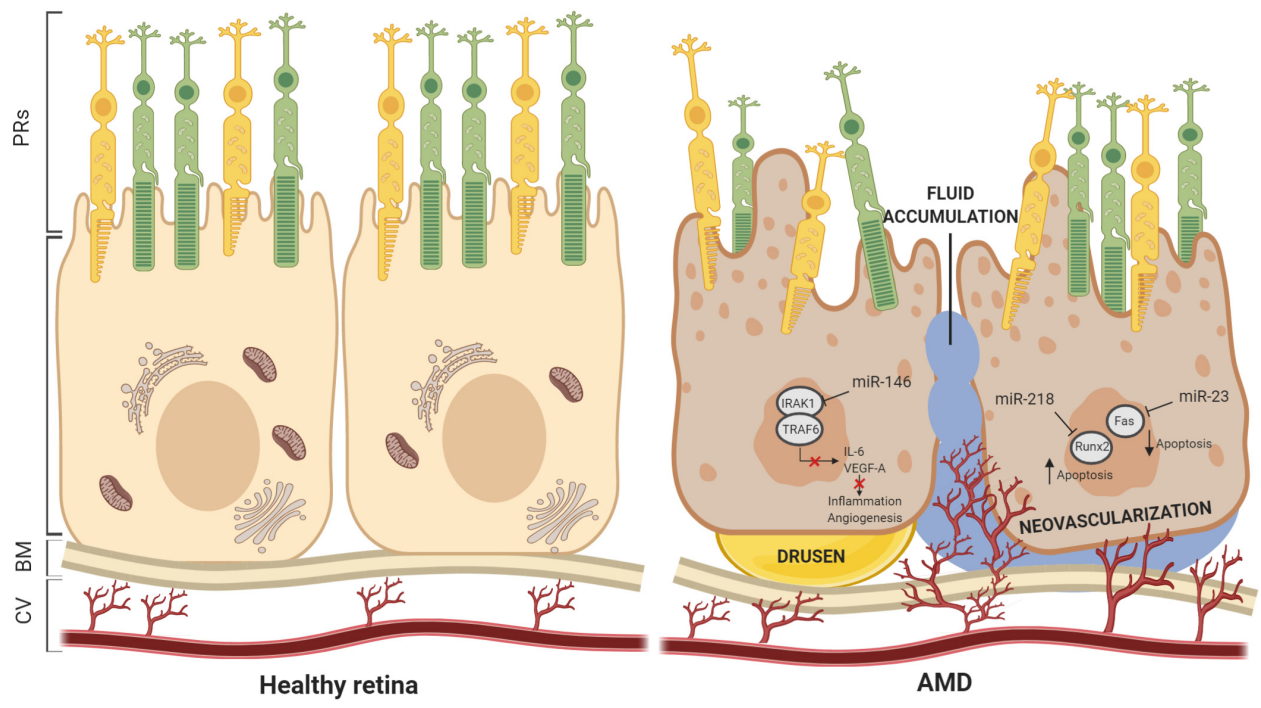

FIGURE 4 | Schematic view of miRNAs dysfunction in AMD. miRNAs have been demonstrated to play key roles in maintaining the structure and functionality of Bruch's membrane and RPE cells. Incidence of drusen and neovascularization, fluid accumulation, and vascular leakage are often consequences of miRNAs dysfunction. In this scheme, we report examples of miRNAs dysregulation associated with AMD pathogenesis. BM, Bruch's membrane; CV, choroidal vasculature.

namely *TRPM1*, as a locus-containing risk factors for AMD (Persad et al., 2017; Orozco et al., 2020). Remarkably, the RPE phenotype in the miR-211$^{-/-}$ mice was similar to that observed in animal models for AMD conditions. Moreover, miR-211 gene delivery via gene therapy was also explored to promote RPE and PR survival in AMD (Cunnusamy et al., 2012). These studies, together with *in vitro* studies demonstrating the function of miR-211 in the physiology of RPE, support that miR-211 is central to RPE homeostasis and, consequently, may serve as a precious molecular tool to counteract AMD disease. A number of additional examples of miRNAs involved in inflammation, oxidative stress, and angiogenesis have already been shown to be potential therapeutic targets for AMD. miR-23a is downregulated in RPE cells from AMD patients, and overexpression of this miRNA in ARPE-19 reduces cell apoptosis by regulating *Fas* (Lin et al., 2011). On the contrary, upregulation of miR-218 accelerates the apoptosis of ARPE-19 cells, negatively regulating *Runx2*. These finding reveal that miR-218/*Runx2* axis could be a therapeutic target for retinal diseases (Yao et al., 2019) (**Figure 4**).

CONCLUSION

We are in the process of increasing our knowledge on the role of miRNAs as key regulators of RPE homeostasis, function, and survival. miRNAs play an important role in contributing to the precise control of RPE homeostasis in both physiological and pathological states. These insights are opening up avenues to a wide range of novel research areas, including gene-independent medicine, and offers an exclusive class of biomarkers for diagnostic analysis. Recent preclinical studies indicate that miRNAs-based gene therapy is nearing the transition to clinical trial stages. As a single miRNA has the potential to modulate and orchestrate entire molecular programs, future studies are required to shed new light on how they may be safely used as key factors stabilizing and/or resetting the RPE state in pathological conditions. However, recent findings suggest that they are highly promising molecular tools to treat and counteract retinal degeneration including the AMD onset and progression.

AUTHOR CONTRIBUTIONS

DI, GG, and IC have contributed to wrote the manuscript and prepared the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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miRNAs and Müller Glia Reprogramming During Retina Regeneration

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The use of model systems that are capable of robust, spontaneous retina regeneration has allowed for the identification of genetic pathways and components that are required for retina regeneration. Complemented by mouse models in which retina regeneration can be induced after forced expression of key factors, altered chromatin accessibility, or inhibition of kinase/signaling cascades, a clearer picture of the key regulatory events that control retina regeneration is emerging. In all cases, Müller glia (MG) serve as an adult retinal stem cell that must be reprogrammed to allow for regeneration, with the end goal being to understand why regenerative pathways are blocked in mammals, but spontaneous in other vertebrates such as zebrafish. miRNAs have emerged as key gene regulatory molecules that control both development and regeneration in vertebrates. Here, we focus on a small subset of miRNAs that control MG reprogramming during retina regeneration and have the potential to serve as therapeutic targets for treatment of visual disorders and damage.

Keywords: miRNA, Müller glia, retina, regeneration, zebrafish

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INTRODUCTION

In mammals and humans, the extent of spontaneous repair after retina injury or disease is either non-existent or extremely limited (Karl and Reh, 2010). Rather than regenerate, damaged mammalian retinas commonly undergo reactive gliosis and scar formation (Bringmann et al., 2006). This lack of a complete regenerative response to damage directly limits the treatment options for retinal based diseases such as age-related macular degeneration or Stargardt's disease (Link and Collery, 2015; Zarbin, 2016). Numerous strategies are currently being tested to address this limitation, including gene therapy approaches and transplantation of stem cell-derived progenitor cells (MacLaren et al., 2006; Pearson et al., 2012; Cehajic-Kapetanovic et al., 2015; Roska and Sahel, 2018; Stern et al., 2018). An attractive alternative strategy for treatment is to induce endogenous MG-derived regeneration of the retina as is observed in fish and amphibians (Hamon et al., 2016; Lahne et al., 2020). Zebrafish have the ability to regenerate a large array of tissues and organs (Gemberling et al., 2013). One goal for these studies is to determine the factors and pathways that allow for persistent and spontaneous regeneration. Focusing on the retina, knowledge gained from zebrafish studies (Wan and Goldman, 2016; Yao et al., 2018; Hoang et al., 2020; VandenBosch et al., 2020; Zhou et al., 2020) can be applied to identify common mechanisms that induce mammalian retina regeneration. Here, we will focus on the explicit role of miRNAs during MG reprogramming.

RETINA REGENERATION IN ZEBRAFISH

The retina forms from the central nervous system (CNS) and develops into a three-layered structure consisting of seven main types of cells and numerous other cell types identified by single cell RNAseq (Macosko et al., 2015). The structure, function, cell types, and genes expressed in the retina are largely conserved among vertebrates, supporting the notion that information gained from models capable of spontaneous regeneration might apply to mammals whose regenerative capacity isn't clear (Hitchcock and Raymond, 2004; Stenkamp, 2007; Hamon et al., 2016).

The three main layers that constitute the retina include the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). The INL and ONL are separated by a thin synaptic layer called the outer plexiform layer (OPL), and the INL and GCL are separated by a thick synaptic layer called the inner plexiform layer (IPL). The ONL contains rod and cone photoreceptors. The INL contains three types of interneurons: bipolar cells (BCs), horizontal cells (HCs), and amacrine cells (ACs). Ganglion cells (GCs) populate the GCL, collect information from BCs, and send signals to the brain for higher order visual processing. In addition to these neuronal cell types, Müller glia (MG) constitute the main glial cell type spanning all three layers of the retina.

MÜLLER GLIA-DERIVED REGENERATION

The unique behavior and placement of MG following damage led to hypotheses that they play an integral role in retina regeneration. Multiple lines of evidence, largely from zebrafish, strongly support MG as the source of retina progenitors after damage. First, new retinal progenitor cells (RPCs) formed in the INL migrate along MG processes to the ONL (Raymond and Rivlin, 1987; Vihtelic and Hyde, 2000; Wu et al., 2001; Raymond et al., 2006). Second, MG become mitotic after damage (Braisted et al., 1994; Vihtelic and Hyde, 2000; Wu et al., 2001; Faillace et al., 2002; Yurco and Cameron, 2005; Raymond et al., 2006). Third, gene expression profiles of MG and RPCs are very similar (Hoang et al., 2020). Most directly, the Raymond lab showed that zebrafish MG produce rod precursors during development and also produce RPCs that can differentiate into any retina cell type following damage (Bernardos et al., 2007). The mechanism by which MG produce RPCs is by dedifferentiation of the MG, owing to the fact that shortly after damage, zebrafish MG begin to produce markers of neural progenitors such as Pax6, α-tubulin, and BLBP (Fausett and Goldman, 2006; Raymond et al., 2006; Thummel et al., 2010).

Following damage in zebrafish, signaling cascades induce MG to dedifferentiate to a stem cell-like state and reenter the cell cycle, followed by asymmetric division for self-renewal and for the generation of proliferating RPCs (Nagashima et al., 2013). These cells cluster along MG processes and then migrate to sites of damage where they exit the cell cycle and differentiate into new cells that can replace any damaged cell type (**Figure 1**; Fausett and Goldman, 2006; Bernardos et al., 2007; Thummel et al., 2008b, 2010; Montgomery et al., 2010; Ramachandran et al., 2010; Qin et al., 2011; Powell et al., 2012; Taylor et al., 2012). Though the current understanding of retina regeneration is ongoing, a number of factors have been identified that transition the retina through the various stages of MG-derived retina regeneration (Wan and Goldman, 2016; Lahne et al., 2020; **Figure 2**). Here, we will focus on the identification and role of miRNAs during MG reprogramming and retina regeneration.

miRNAS

miRNAs are highly conserved ∼22 nucleotide (nt) RNAs that post-transcriptionally regulate gene expression (Krol et al., 2010; Bartel, 2018; Gebert and MacRae, 2019). Primary miRNA transcripts are initially processed in the nucleus into ∼70 nt precursor structures by a multi-protein complex referred to as the Microprocessor, the main component of which is Drosha (Kim, 2005). After export from the nucleus, cytoplasmic processing is accomplished by another multi-protein complex that includes the enzyme Dicer, which yields ∼22 nt double stranded RNAs. One of the strands is subsequently assembled into an RNA Induced Silencing Complex (RISC) (Schwarz et al., 2003; Filipowicz, 2005) containing one or more members of the Argonaute protein family (Peters and Meister, 2007). miRNA-mediated gene silencing occurs by pairing between miRNAs and their target mRNAs, usually in the 3′ UTR. Once paired, miRNAs inhibit translation and induce deadenylation leading to mRNA degradation (Giraldez et al., 2006; Guo et al., 2010).

miRNAS AND RETINA REGENERATION

miRNAs were first discovered in *C. elegans* where they control development (Lee et al., 1993; Wightman et al., 1993), but they have now been shown to play important roles in a number of biological processes including metabolism, cancer, metastasis, and regeneration (Alvarez-Garcia and Miska, 2005). miRNAs have been implicated in regeneration in a number of biological models ranging from planaria to mice (Yin and Poss, 2008; Williams et al., 2009; Thatcher and Patton, 2010) and in zebrafish have been shown to regulate regeneration of the heart, fin, muscle, liver, lens, and inner ear hair cells (Tsonis et al., 2007; Liu et al., 2008, 2012; Thatcher et al., 2008; Yin et al., 2008; Song et al., 2010). Knockdown of Dicer in the adult zebrafish retina prior to constant intense light damage reduced the ability of MG to produce proliferating RPCs in response to damage (Rajaram et al., 2014a). This indicated a general requirement for miRNAs during retina regeneration. After damage, most miRNA expression levels remain unchanged or undergo only small changes during regeneration. However, specific subsets of zebrafish miRNAs show both up- and down-regulation throughout the regenerative process (**Figure 3**; Rajaram et al., 2014a). Similarly, a small subset of miRNAs have been implicated in controlling the reprogramming of mammalian Müller glia (Wohl and Reh, 2016b; Wohl et al., 2019).

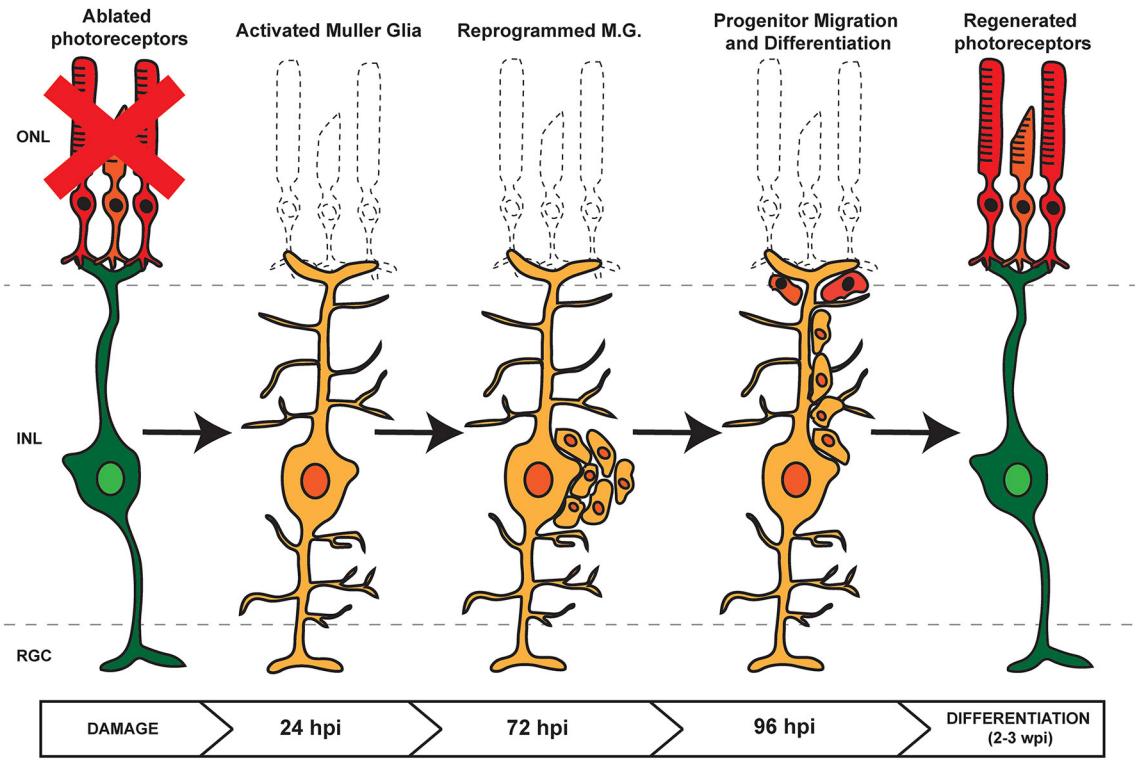

FIGURE 1 | Retina Regeneration in Zebrafish. In response to retinal damage or cell loss, zebrafish Müller Glia (MG) are activated and undergo dedifferentiation. Asymmetric division allows for self renewal and the generation of proliferating retinal progenitor cells (RPCs) which cluster along MG processes. As regeneration proceeds, the RPCs migrate to the site of damage before differentiating into any lost or damaged cell types.

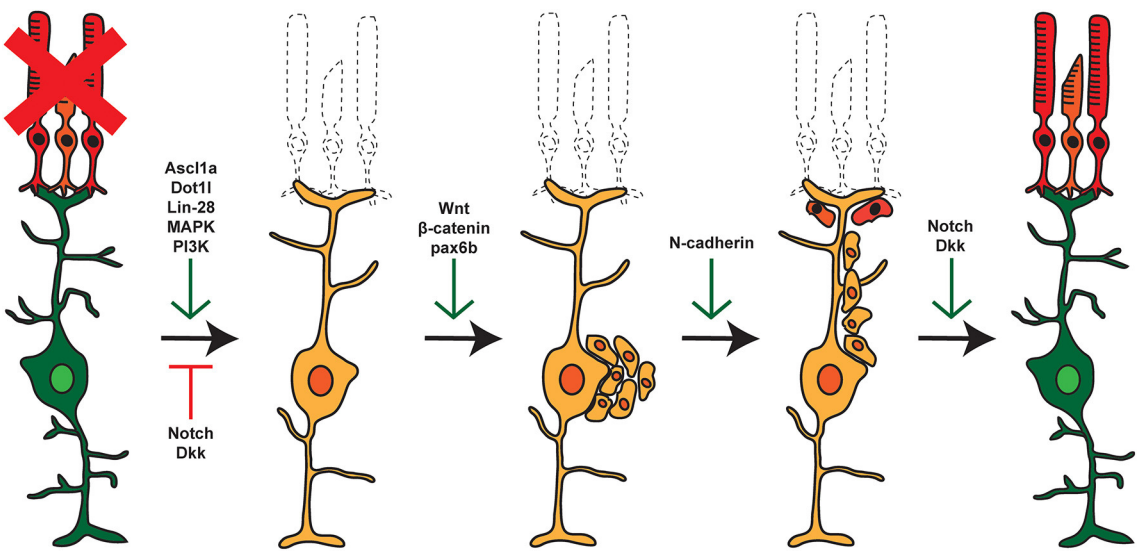

FIGURE 2 | Pathways and factors involved in retina regeneration. Following damage in the zebrafish retina, multiple pathways are activated controlling dedifferentiation of MG, re-entry into the cell cycle, asymmetric cell division, generation of proliferating RPCs, and eventual differentiation into replacement cell types. Green arrows represent factors that are active at the given step, whereas red arrows represent factors that are repressed or inactive at that time.

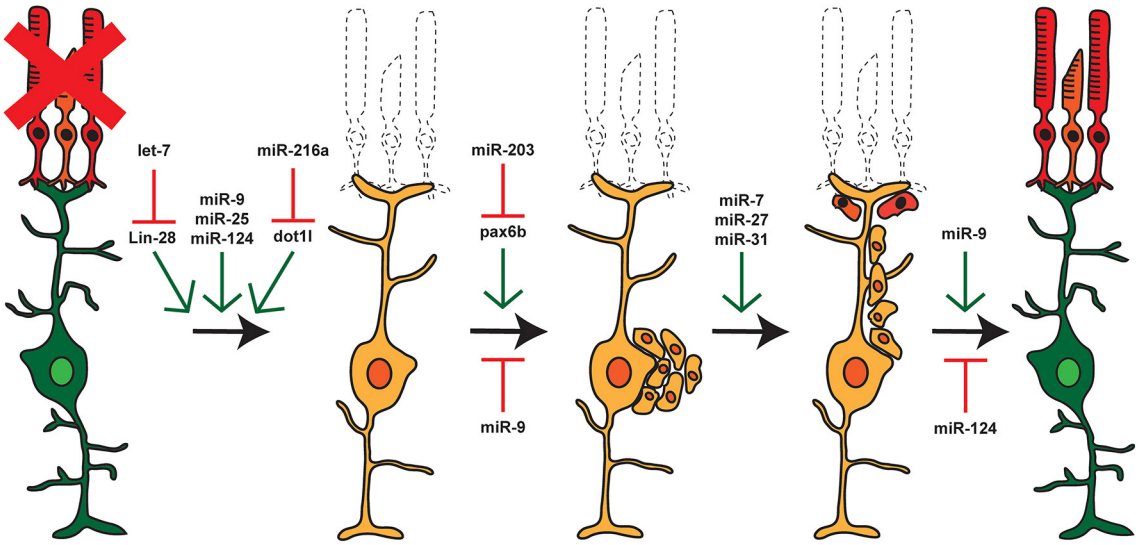

FIGURE 3 | miRNAs and Retina Regeneration. The adult zebrafish retina expresses ~200 miRNAs but only a small subset of these miRNAs are differentially expressed during regeneration. RNAseq and other analyses after damage or during distinct stages of regeneration have identified miRNAs and select target mRNAs, as indicated. Green arrows represent miRNAs that are expressed or active at that given step, and red arrows represent miRNAs that are not expressed or inactive.

let-7/LIN-28

One of the first demonstrations of a role for miRNA involvement in retina regeneration was by the Goldman lab focusing on *let-7* (Ramachandran et al., 2010). Using a puncture damage model in zebrafish, they used a candidate gene approach to identify pluripotency factors whose expression changes during retina regeneration. One of those factors was Lin-28, an RNA-binding protein that was first discovered to regulate development in *C. elegans* as part of a double negative feedback loop with *let-7* (Moss et al., 1997; Reinhart et al., 2000). After retina damage, Lin-28 is induced downstream of the transcription factor Ascl1 and knockdown of Lin-28 inhibits retina regeneration (Ramachandran et al., 2010; Zhao et al., 2017). Lin-28 activation leads to the repression of *let-7* expression which in turn derepresses the expression of multiple regeneration and pluripotency factors including Ascl1a (Ramachandran et al., 2010). *Let-7* has 12 family members; Lin-28 inhibits the production of most *let-7* family members by recruiting a uridylyl transferase to *pre-let-7* transcripts leading to uridylation, inhibition of processing, and subsequent decay (Hagan et al., 2009; Heo et al., 2009). For MG, it appears that expression of *let-7* maintains the differentiated state, but that induction of Lin-28 after injury allows dedifferentiation by reducing *let-7* levels. This is consistent with a role for regulation of Lin-28 by *let-7* during development as well (La Torre et al., 2013; Fairchild et al., 2019).

miRNA-203/PAX6B

In a screen to identify differentially expressed miRNAs during zebrafish MG-derived retina regeneration, *miR-203* was found to be downregulated and that artificially maintaining its expression blocked retina regeneration (Rajaram et al., 2014b). Previously, *miR-203* downregulation had been shown to be required for caudal fin regeneration and it is similarly downregulated during mouse skin regeneration (Lena et al., 2008; Thatcher et al., 2008; Yi et al., 2008). These data supported a role for *miR-203* in promoting differentiation and repressing stemness. Elevated levels of *miR-203* inhibit proliferation of RPCs and are essential for the formation of clusters of RPCs that are commonly observed along MG processes (**Figure 1**; Rajaram et al., 2014b). However, *miR-203* does not play a role in dedifferentiation of MG during earlier stages of regeneration, indicating a temporal expression pattern throughout the regenerative processes.

In a search for potential mRNA targets for *miR-203*, bioinformatic and reporter analyses identified the Paired-box gene 6b (Pax6b) (Rajaram et al., 2014b). Pax6 expression is essential for eye development across species (Shaham et al., 2012; Baker et al., 2018). There are two Pax6 paralogs in zebrafish (Pax6a and Pax6b) with distinct roles during retina regeneration (Thummel et al., 2010). Misregulation or loss of Pax6 expression can lead to multiple mammalian visual system defects, most commonly aniridia or nystagmus (Lima Cunha et al., 2019). Consistent with a role for *miR-203* controlling progenitor proliferation and cluster formation, the Hyde lab had previously shown that *pax6b* in zebrafish is expressed in NPCs and is required for the formation of clusters (Thummel et al., 2008a). *miR-203* must be repressed during regeneration to allow for *pax6b* expression and the formation of RPC clusters on MG.

miR-9/miR-124/PTB/nPTB

The Fu laboratory discovered that repression of the hnRNP protein Polypyrimidine tract-binding protein 1 (PTB1) can

convert fibroblasts into a neuronal cell type fate (Xue et al., 2013). PTB1 is a ubiquitously expressed regulator of alternative splicing that itself undergoes alternative splicing to autoregulate its levels (Wollerton et al., 2004). A neuronal paralog of PTB (PTB2 or nPTB) controls multiple neuronal alternative splicing events and its levels are regulated by an alternative splicing event controlled by PTB1 (Boutz et al., 2007; Makeyev et al., 2007). PTB1 is expressed in neuronal precursor cells and glia; nPTB is expressed during neuronal induction and maturation, with the expression of both paralogs decreasing during neuronal differentiation (Boutz et al., 2007; Hu et al., 2018). Two regulatory loops control PTB and nPTB expression through the action of *miR-9* and *miR-124,* respectively, along with the transcription factors REST and BRN2 (Makeyev et al., 2007; Hu et al., 2018). Intriguingly, it was recently shown that targeted destruction of mRNAs encoding PTB in the mouse retina can cause MG to dedifferentiate (Zhou et al., 2020). Using an NMDA damage model in mice, targeting of mRNAs encoding PTB by CRISPR/CasRx led to MG dedifferentiation and replacement of damaged ganglion cells. Also, targeted depletion of mRNAs encoding PTB by antisense oligonucleotides led to the conversion of astrocytes to dopaminergic neurons (Qian et al., 2020). Together, the data support the surprising finding that targeting a single, widely expressed regulator of alternative splicing can drive the conversion of glia to neurons.

Even though targeting of PTB provides an attractive single gene approach for retina regeneration, an alternative would be to deliver *miR-9* and *miR-124* to not only regulate PTB and nPTB expression, but also to upregulate the transcription factors REST and BRN2 (Hu et al., 2018). It will be crucial to determine whether the expression of PTB and nPTB are controlled in the retina by the *miR-9 and miR-124* regulatory loops. If so, regulation of REST by these regulatory loops could control expression of NeuroD which plays a role in neuronal cell fate, and Ascl1 which is required for retina regeneration (Ramachandran et al., 2010; Cherry et al., 2011). Similarly, regulation of BRN2 could in turn control expression of neuronal maturation genes including NEUN and NLGN2. Gene profiling experiments did not observe significant changes in PTB between control and neurogenic MG (Hoang et al., 2020) so it may be that increased expression or delivery of *miR-9* and *miR-124* mimics might drive broader overall gene expression changes to induce retina regeneration rather than just targeting PTB (Wohl and Reh, 2016b).

miR-9/miR-124/ASCL1

The Reh lab used dissociated mouse MG cultures to identify factors and miRNAs that can stimulate reprogramming of retinal cell fate (Pollak et al., 2013; Wohl and Reh, 2016b). Induced overexpression of the transcription factor Asc1 can reprogram mouse MG into neurogenic RPCs and the effects of Ascl1 overexpression can be augmented by parallel overexpression of both *miR-124* and *miR-9* (Wohl and Reh, 2016b). This agrees nicely with the regulatory loops controlling neuronal induction and maturation controlled by *miR-124* and *miR-9* but to date, whether these loops control gene expression in the retina is not clear (Hu et al., 2018).

miR-25/*let-7*/miR-124/miR-9

In a follow up study to the effects of *miR-9* and *miR-124* on reprogramming of mammalian MG, the Reh lab profiled miRNA expression patterns in sorted mouse MG and RPCs, and also utilized a conditional mouse model with a MG-specific deletion of Dicer (Wohl and Reh, 2016a; Wohl et al., 2017, 2019). After Dicer knockdown, the most significantly altered gene was Brevican (BCAN) which is targeted by *miR-9*. More broadly, loss of Dicer led to a dramatic loss of retinal architecture indicating an important role for miRNAs in the maintenance of homeostasis, and also supporting the overall importance of miRNAs in regeneration, a process which is blocked by the loss of Dicer (Rajaram et al., 2014a). When comparing sorted MG and RPCs, Ascl1 expression was found to be enhanced by either overexpression of *miR-25* and *miR-124* or by downregulation of *let-7* (Wohl et al., 2019). Targeting of Lin-28 by *let-7* can explain indirect regulation of Ascl1.

miR-25 is expressed as part of the highly conserved *miR-106b/25* cluster with roles in DNA damage response, cell cycle regulation, cell proliferation, migration, and differentiation (Sarkozy et al., 2018). Additional potential target mRNAs for *miR-25* include REST, Tpm1, Itgb1, Ctdsp1, Rcor1, and Ccnd2 (Sarkozy et al., 2018; Wohl et al., 2019). Besides targeting the protein components of the REST complex, another key predicted target of *miR-25* (and *let-7*) is the Wnt inhibitor Dickkopf 3 (Dkk3) (Huo et al., 2016; Wohl et al., 2019). This is consistent with the requirement for Wnt activation during retina regeneration in zebrafish and possibly mice (Osakada et al., 2007; Ramachandran et al., 2011; Kara et al., 2019).

miR-124 is one of the most abundant miRNAs in the adult brain and is thought to be a master regulator of neuronal differentiation, including its role in regulating PTB expression (Yeom et al., 2018) and targeting of REST (Wohl et al., 2019). *miR-124* is known to reduce the expression of a small phosphatase specific for phosphoserines in the C-terminus of RNA Polymerase II called SCP1, which is a repressor of neuron-specific transcription in nonneuronal cells and is also a component of REST (Cao et al., 2007; Makeyev et al., 2007; Visvanathan et al., 2007).

miR-216a/DOT1L

miR-216a is another well-known miRNA that plays a role in gliogenesis during retinal development by indirectly regulating Notch signaling (Olena et al., 2015). For MG-derived regeneration, *miR-216a* can be thought of as a gatekeeper miRNA in reprogramming events, as its expression holds MG in a quiescent state until the retina is damaged (Kara et al., 2019). One mechanism for how *miR-216a* can serve as a gatekeeper controlling the early steps of regeneration is by targeting mRNAs encoding the Disruptor of telomeric silencing 1-like (DOT1l) gene (Kara et al., 2019). Dot1l plays a role in many

chromatin-associated functions such as gene-transcription, heterochromatin formation, and DNA repair, as well as the response to DNA damage and chemotherapy responsiveness (McLean et al., 2014). After retinal damage in zebrafish, *miR-216a* is downregulated allowing increased expression of Dot1l which leads to activation of Wnt target genes, presumably by altering chromatin accessibility surrounding these genes (Kara et al., 2019). The idea that dedifferentiation of MG involves changes in chromatin accessibility is expected and has been experimentally supported (Jorstad et al., 2017; Mitra et al., 2018; VandenBosch et al., 2020). When combined with Ascl1 overexpression in an NMDA damage model in adult mice, the addition of the general histone deactylase inhibitor trichostatin A (TSA) stimulated neuronal regeneration which was otherwise only observed in developing mice <12 days old (Jorstad et al., 2017). Also, after puncture damage in zebrafish, inhibition of histone deacetylases by valproic acid suppressed the formation of MG-derived NPCs (Mitra et al., 2018). Further work is needed to identify specific genes whose chromatin accessibility changes during MG dedifferentiation and eventual re-differentiation, but miRNA control is an attractive regulatory mechanism that might allow for fine-tuned control of signaling cascades that are induced after cellular damage.

miR-7/miR-27/miR-31

In a screen to identify differentially expressed miRNAs during zebrafish retina regeneration, *miR-7, miR-27, and miR-31* were all found to be upregulated at 72 h post light damage and targeted knockdown of these miRNAs led to decreased numbers of proliferating cells (Rajaram et al., 2014a). The timing of overexpression and the effects of loss of function of these miRNAs during regeneration suggest that they function during continued RPC proliferation and migration, similar to the proposed role for Pax6a (Thummel et al., 2010; Rajaram et al., 2014b). Exact targets for these miRNAs remain to be identified but related experiments summarized below might provide hints to possible mRNA targets for these miRNAs.

miR-7 regulates multiple signaling pathways including epidermal growth factor receptor (EGFR), insulin-like growth factor (IGF), Hedgehog, Notch, and the mammalian target of rapamycin (mTOR) pathways, as well as being a key regulator of *pax6a* in mice (Needhamsen et al., 2014; Baba et al., 2015; Zhao et al., 2015). In the forebrain, *miR-7* regulates *pax6* to spatially control the origin of dopaminergic neurons (de Chevigny et al., 2012).

miR-27 promotes blood vessel development, particularly in the eye (Liu et al., 2020). *miR-27* also plays an important role in mitochondrial dynamics as it inhibits degradation of damaged mitochondria by regulating PINK1 and also by inhibiting mitochondrial fission factor (MFF) expression, which increases mitochondrial membrane potential (Tak et al., 2014; Kim et al., 2016). Loss of *miR-27c* in the retina decreases proliferation of MG-derived RPCs during regeneration, a similar phenotype to what occurs with loss of *miR-27a and miR-27b* in muscle progenitor cell proliferation (Crist et al., 2009; Lozano-Velasco et al., 2011; Rajaram et al., 2014a).

miR-31 is a well-studied miRNA with a major target being transcripts encoding the myogenic determining factor Myf5 (Crist et al., 2012). *miR-31* levels affect both satellite cell differentiation *ex vivo* and muscle regeneration *in vivo*, making *miR-31* a miRNA of great interest in regard to stem cell research. *miR-31* is a regulator of many signaling pathways relevant to developmental biology and cancer including the Prlr/Stat5, TGFβ, and Wnt/β-catenin pathways (Lv et al., 2017). Additionally, *miR-31* has been shown to coordinate signals from BMP, TGFβ, and Wnt pathways in intestinal stem cells to regulate their proliferation, regeneration, and homeostasis, further reinforcing its impact in progenitor proliferation during regeneration (Tian et al., 2017).

DISCUSSION

miRNAs regulate gene expression by binding to 3′ UTR elements leading to deadenylation and subsequent degradation of mRNA targets (Giraldez et al., 2006; Guo et al., 2010). Target recognition typically involves imperfect base pairing, often within the seed region (nucleotides 2-8) that is commonly used to predict miRNA targets (Li et al., 2008; Broughton et al., 2016; Bartel, 2018). Because the base pairing interaction is imperfect, miRNA target prediction algorithms can identify candidate mRNAs, but experimental validation is necessary to confirm direct silencing. Thus, for all of the miRNAs discussed above, there are likely additional mRNA targets that could affect the same processes, ranging from regeneration to signaling cascades. Further work is required to identify the complete set of miRNAs that regulate retina regeneration and the target genes they control.

Because miRNAs are largely conserved among vertebrates, the expection is that discoveries across species will illustrate general principles and uncover common mechanisms. Fortunately, it does not appear that there are hundreds of miRNAs that regulate MG reprogramming and, for the subset that has been identified, they comprise an attractive class of regulatory molecules, especially because retinal architecture and MG gene expression patterns are evolutionarily conserved suggesting that elucidating overall gene regulation will help to understand the inability of mammalian MG to initiate regeneration. It remains possible that species-specific networks or species-specific factors might control the passage of MG from quiescence to reactivity and further to the generation of proliferating RPCs, but the weight of evidence thus far seems to suggest that activating MG-derived regeneration cascades in mammals will be possible by derepression of existing pathways as opposed to delivery of species-specific genes (Ahmad et al., 2011; Lust and Wittbrodt, 2018; Hoang et al., 2020).

THERAPEUTIC miRNA

The accessibility of the eye and the small size of miRNAs raises the possibility of delivering miRNA mimics or antisense RNAs (antagomirs) that block miRNA function for therapeutic

purposes. The challenges for such experiments are at least three-fold: (1) how to target injected miRNAs to MG; (2) whether single injections will be sufficient to induce a regenerative response; and (3), avoidance of off-target effects if high concentrations are required. While direct injections of miRNAs or antagomirs are possible, the discovery that extracellular vesicles (EVs) can be used to deliver therapeutic cargo opens an exciting possibility for cell-specific delivery (Mead and Tomarev, 2020). Recently, it has become increasingly clear that miRNAs can engage in cell-cell signaling via EVs (Maas et al., 2017; O'Brien et al., 2020). Transfer of miRNAs or other cargo by EVs might play a role in patterning the retina during development and may also be a key part of degeneration and regeneration (Bian et al., 2020). Indeed, retina regeneration can be induced by delivery of EVs (Didiano et al., 2020). Although EVs were shown to induce the early stages of retina regeneration, the effects were quite modest. However, as a therapeutic tool, it may be possible to load EVs with specific miRNAs or other small molecules for delivery to MG after intravitreal or subretinal injection. The miRNAs described in this review may be candidate miRNAs for the development of designer EVs that could be targeted to MG to induce retina regeneration.

AUTHOR CONTRIBUTIONS

GK made the figures. All authors contributed to the writing.

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Sophisticated Gene Regulation for a Complex Physiological System: The Role of Non-coding RNAs in Photoreceptor Cells

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Photoreceptors (PRs) are specialized neuroepithelial cells of the retina responsible for sensory transduction of light stimuli. In the highly structured vertebrate retina, PRs have a highly polarized modular structure to accommodate the demanding processes of phototransduction and the visual cycle. Because of their function, PRs are exposed to continuous cellular stress. PRs are therefore under pressure to maintain their function in defiance of constant environmental perturbation, besides being part of a highly sophisticated developmental process. All this translates into the need for tightly regulated and responsive molecular mechanisms that can reinforce transcriptional programs. It is commonly accepted that regulatory non-coding RNAs (ncRNAs), and in particular microRNAs (miRNAs), are not only involved but indeed central in conferring robustness and accuracy to developmental and physiological processes. Here we integrate recent findings on the role of regulatory ncRNAs (e.g., miRNAs, lncRNAs, circular RNAs, and antisense RNAs), and of their contribution to PR pathophysiology. We also outline the therapeutic implications of translational studies that harness ncRNAs to prevent PR degeneration and promote their survival and function.

Keywords: photoreceptors, cones, rods, retina, non-coding RNAs, microRNAs, lncRNAs, circRNAs

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INTRODUCTION

Photoreceptors (PRs) are specialized neuronal cells adapted to the conversion of light stimuli into electrical signals. Rods are sensitive to dim light and essential for night vision, while cones enable high acuity daylight vision and color perception (**Figure 1A**). PRs are among the cell types of our organism that are exposed to high levels of cellular stressors (e.g., light exposure and sustained protein synthesis). First, PRs have an elevated metabolic demand due to the high rates of ion transport, opsin protein turnover, and trafficking from inner (IS) to outer segments (OS) (**Figure 1A**). This high energy requirement, which is almost double for a cone compared to a rod cell (Ingram et al., 2020), renders PRs vulnerable to fluctuations in energy flow. The high rate of protein synthesis poses a challenge to proteostasis (Athanasiou et al., 2013) and may expose PRs to endoplasmic reticulum stress. Second, the inevitable photo-oxidative stress requires continual clearance of Reactive Oxygen Species (Organisciak and Vaughan, 2010). In addition, PRs need to remove the toxic retinoid byproducts of the visual cycle (**Figure 1A**).

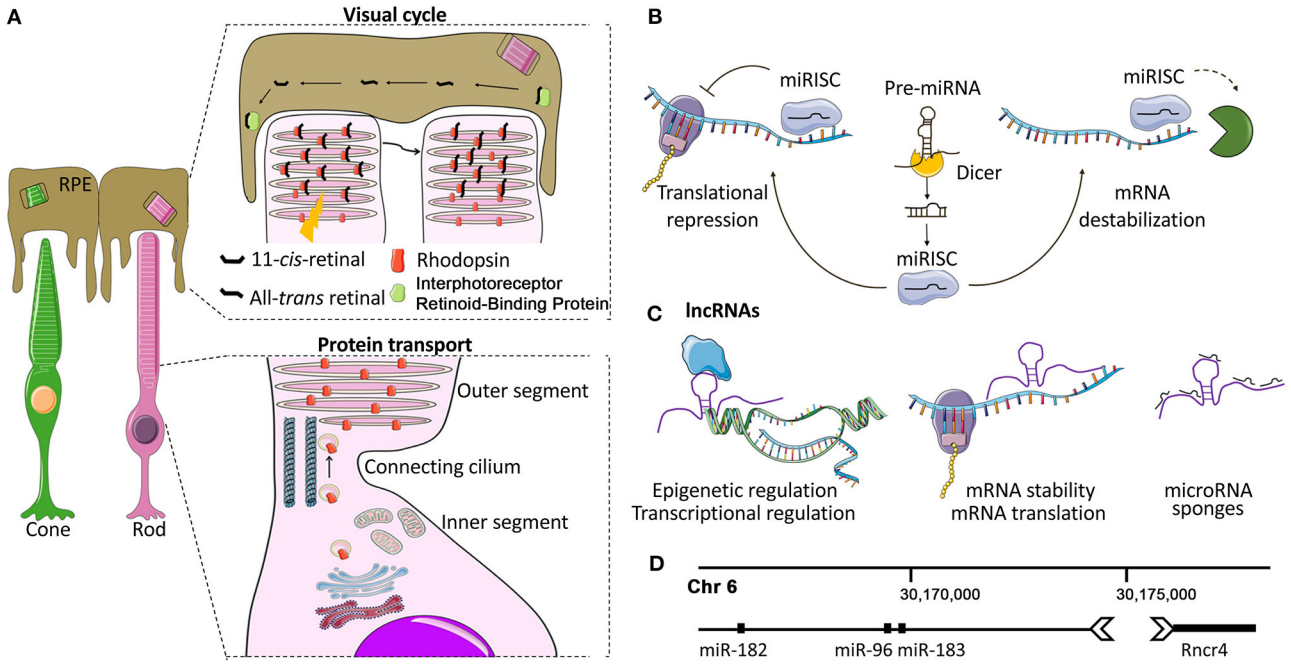

FIGURE 1 | Photoreceptors and non-coding RNA mechanisms of gene regulation. **(A)** Photoreceptor structure and function. Each photoreceptor (PR) comprises a synaptic terminal that partakes in interactions with second-order retinal cells, an inner segment (IS) that contains cell organelles, and a connecting cilium that joins the IS with the outer segment (OS). The OS is a finely articulated sensory cilium (Pearring et al., 2013). It envelopes a series of stacked lamellae (membranous discs) that contain the light-sensitive opsin proteins (e.g., Rhodopsin and cone opsins) which convert photons into electrochemical signals via phototransduction cascade (Pearring et al., 2013). The PR OS physically interacts with the adjacent retinal pigment epithelium (RPE). This dynamic interplay between PRs and RPE is essential for the phagocytosis of the shed OS (Kevany and Palczewski, 2010), the transport of the circulating glucose from the choroid (Ait-Ali et al., 2015), the maintenance of ionic and osmotic balance as well as for phototransduction and visual cycle. **(B)** Overview of miRNA biogenesis and mode of action. Mature miRNAs are 20-25-nt long ncRNAs. In the canonical biogenesis pathway, they are enzymatically released from longer precursors with cleavage steps that take place in the nucleus and in the cytoplasm and are controlled by the Drosha/DGCR8 and the Dicer-TRBP complex, respectively (Krol et al., 2010b; Reh and Hindges, 2018). The mature miRNA, loaded into the RNA-induced silencing complex (RISC), recognizes and binds to complementary sequences of mRNA targets, mostly localized to 3′ untranslated regions (3′ UTR), inducing gene silencing (Krol et al., 2010b; Reh and Hindges, 2018). **(C)** Overview of lncRNA biogenesis and mode of action. LncRNAs interact with a range of cellular molecules such as other RNAs (miRNAs, mRNAs), DNA, and proteins or peptides. These interactions represent the lncRNA interactome and play a variety of roles in cell development and the pathogenesis of various diseases (Kazimierczyk et al., 2020). LncRNAs can act by different mechanisms, still not completely elucidated: (a) nuclear LncRNAs can act as epigenetic regulators, recruiting activator/repressor chromatin modifying complexes on their target promoters; (b) they can regulate transcription by guiding or preventing the recruitment of TFs on the promoters of their targets or on other active chromatin sites; (c) cytoplasmic lncRNAs may be endowed with post-transcriptional activity by regulating mRNA stability and translation, modulating mRNA degradation, and by acting as miRNA sponges (Gourvest et al., 2019). **(D)** miR-183/96/182-Rncr4: an example of crosstalk between lncRNAs and miRNAs in PRs. Graphical representation of the region of mouse chromosome 6 where the lncRNA Rncr4 (retinal non-coding RNA 4) and the miR-183/96/182 cluster are localized. Rncr4 is expressed in PRs and is transcribed in the opposite direction of the pri-miR-183/96/182. Its expression stimulates pri-miR-183/96/182 processing. Their regulatory crosstalk is important for the correct formation of the PR and inner nuclear layers (Krol et al., 2015). Details on the molecular mechanism are provided in the text.

The structural and functional integrity of PRs is crucial for vision. Defects or mere fluctuations in any of the key molecular processes involved in PR homeostasis (e.g., energy metabolism including retinal blood flow, lipofuscin clearance, protein sorting, vesicle, and intra-flagellar transport, etc.) due to genetic changes or environmental insults can lead to PR dysfunction, cell death, and ultimately to blindness (Pearring et al., 2013). Because of their high metabolic activity, PR dysfunction is often the only phenotypic read-out of mutations in genes with ubiquitous expression, such as in the case of key ciliary genes (e.g., *CEP290* and *RPGR*) or splicing factors (e.g., *PRPF31*).

Non-coding RNAs (ncRNAs) are established as key regulators of several developmental and physiological processes. Here we discuss the role of regulatory ncRNAs in conferring robustness to PR development and function. Because of space limitations, ncRNAs that impact PR homeostasis through

Abbreviations: AAV, adeno-associated viral vector; asRNA, antisense RNA; CC, connecting cilium; ceRNA, competitive endogenous RNA; circRNA, circular RNA; cKO, conditional knockout; CNV, choroidal neovascularization; Dgcr8, DiGeorge Critical Region 8; ERG, electroretinography; hPSCs, human pluripotent stem cells; IS, inner segment; KO, knockout; lincRNAs, large intergenic non-coding RNAs; lncRNA, long non-coding RNA; MALAT1, Metastasis Associated Lung Adenocarcinoma Transcript 1; MEG3, Maternally Expressed 3; miRNA, microRNA; ncRNA, non-coding RNA; ONL, outer nuclear layer; OS, outer segment; P, postnatal day; PRs, photoreceptors; RGC, retinal ganglion cells; RPC, retinal progenitor cells; RP, Retinitis Pigmentosa; RPE, Retinal Pigment Epithelium; TRPM1/3, transient receptor potential cation channel subfamily M member 1/3; TUG1, Taurine Upregulated Gene 1.

non-cell-autonomous processes (e.g., expression in other retinal cell-types or extracellular vesicles; Xu et al., 2019; Morris et al., 2020; Wooff et al., 2020) are not discussed in this mini-review.

miRNA-MEDIATED REGULATION IS ESSENTIAL FOR PHOTORECEPTOR MATURATION, FUNCTION, AND SURVIVAL

MiRNAs have emerged as an intriguing class of regulatory ncRNAs because of their powerful and well-conserved mechanism of sequence-specific post-transcriptional gene regulation (Bartel, 2018; **Figure 1B**). In particular, each miRNA is predicted to recognize on average 200 mRNA targets (Bartel, 2018), allowing for a pleiotropic fine-tuning of correlated pathways that confers robustness to biological processes (Ebert and Sharp, 2012). This multiple targeting, together with sequence similarities (i.e., miRNA families) and functional redundancy complicate the study of miRNA function.

An important piece of information to start deciphering miRNA function in the retina came from studies which defined miRNA expression in this tissue (Karali and Banfi, 2019). PR-expressed miRNAs were identified either by RNA *in situ* hybridization approaches (Kapsimali et al., 2007; Karali et al., 2007, 2010; Xu et al., 2007; Zhuang et al., 2020) or by small RNA-Seq analysis of sorted cone cells (Busskamp et al., 2014). More detailed information on the miRNA complement of PRs may derive from single-cell based sequencing approaches adapted to small RNA analysis, as already reported for mRNAs (Macosko et al., 2015; Lukowski et al., 2019; Peng et al., 2019). Besides their PR-specific expression, altered miRNA expression profiles in retinal degeneration models, even at early pre-symptomatic stages, underscored miRNA significance in PR pathophysiology (Loscher et al., 2007, 2008; Genini et al., 2014; Saxena et al., 2015; Palfi et al., 2016; Anasagasti et al., 2018).

Photoreceptor-Specific Depletion of miRNA Biogenesis Impacts Photoreceptor Morphogenesis and Function

Functional studies based on global disruption of miRNA processing in post-mitotic PR cells, mostly through generation of *Drosha/Dgcr8* or *Dicer1* conditional knockout (cKO) mouse models, demonstrated the importance of miRNAs for PR function and survival. In one example, miRNA depletion in mature rod PRs of *Dicer1* conditional-knockout (cKO) mice led to an early-onset severe retinal degeneration (Sundermeier et al., 2014). The initial disorganization of rod OS in cKO mice was followed by an almost complete loss of PR nuclei in the ONL, depletion of the visual chromophore and severely reduced rod-mediated scotopic electroretinography (ERG). However, the cKO retinas did not display primary defects in phototransduction or in the visual cycle, suggesting that observed degeneration could be due to defects in rod maturation or homeostasis.

MiRNA depletion in adult differentiated cones following conditional *Dgcr8* ablation, led to loss of cone OS and impaired cone-mediated responses both in ERG as well as in *ex vivo* whole-cell patch clamp tests (Busskamp et al., 2014). However, cone numbers did not diminish significantly, suggesting there was no direct impact on cone cell death. Adeno-associated viral vector (AAV)-based reintroduction of the PR-enriched miRNAs miR-182 and miR-183 was sufficient to prevent cone OS loss *in vivo*, while expression of the miR-183/96/182 cluster in three-dimensional optic cup cultures from mouse embryonic stem cells induced the formation of additional PR structural components (i.e., IS, CC, and OS).

More recently, the conditional loss of *Dicer1* in developing cones was shown to cause early onset cone dystrophy (Aldunate et al., 2019). Already at 3 weeks of age, Dicer1-depleted cones had shortened and abnormal OS. This structural disorganization gradually led to loss of cone PRs and reduced photopic vision. Conversely, rod survival and function were not affected.

Considering the well-established mutual interdependence between the retina and the retinal pigment epithelium (RPE), it is indisputable that proper miRNA expression in the latter tissue is also essential for PR morphogenesis and function. This was demonstrated with the conditional deletion of *Dicer1* in the developing RPE which dramatically impacted on OS formation and PR survival in a non-cell-autonomous manner (Ohana et al., 2015).

Functional Significance of Specific miRNAs in PRs

Loss-of-function studies of specific miRNAs have provided further insights on miRNA function in PRs (**Table 1**). A well-studied example is the light-regulated polycistronic miRNA cluster composed of miR-183/96/182 (Krol et al., 2010a). Collectively, this miRNA cluster accounts for almost half of the total miRNA population in human retina samples. MiR-182 and miR-183 are the most highly expressed retinal miRNAs (Karali et al., 2016), and are highly enriched in PRs (Karali et al., 2007, 2010; Xu et al., 2007; Zhu et al., 2011; Lumayag et al., 2013; Busskamp et al., 2014; Sundermeier et al., 2014). Sponge-mediated inactivation of the miR-183/96/182 cluster in mouse mature rods did not induce apparent morphological or functional defects under normal light conditions. Instead, it sensitized retinas to bright light-induced retinal degeneration, suggesting this cluster has a protective role under stress conditions (Zhu et al., 2011). On the contrary, constitutive gene-trap-based inactivation of the miR-183/96/182 cluster in mice not only increased susceptibility to light damage but led to an early-onset progressive retinal degeneration associated with defects in the phototransduction cascade and the PRs' synaptic connectivity (Lumayag et al., 2013). In agreement, miR-183/96/182 *null* mice, obtained by targeted recombination, display profound defects in vision and other sensory functions, mainly due to impaired terminal differentiation of sensory neurons. PR ciliogenesis and OS formation was delayed leading to early-onset degeneration (Fan et al., 2017). Finally, the double miR-183 and miR-96 KO mouse had defects in cone polarization and OS morphogenesis that gradually led to PR degeneration, underscoring their importance for PR maturation and maintenance (Xiang et al., 2017). The authors proposed that the effect of miR-183 and miR-96 on PR development is mediated through the regulation

TABLE 1 | Summary of representative non-coding RNAs with a role in photoreceptor function and maintenance.

ncRNAs	Function and main features	Therapeutic perspectives	References
miRNAs			
miR-182, miR-183, miR-96	Their expression is light-regulated; inactivation is linked to PR and synaptic transmission dysfunction, impairment of cone maturation and maintenance, with progressive degeneration; increased expression preserves cone OS in *Dicer1*-ablated PRs		Krol et al., 2010a; Zhu et al., 2011; Lumayag et al., 2013; Busskamp et al., 2014; Fan et al., 2017; Xiang et al., 2017; Wu et al., 2019; Peskova et al., 2020; Zhang et al., 2020a
miR-124	Predominantly localized to PR IS; promotes maturation and survival of cone photoreceptors by targeting *Lhx2*	Intravitreal miR-124 administration decreases microglia infiltration and PR cell death in mice after light-induced damage	Karali et al., 2007; Sanuki et al., 2011; Chu-Tan et al., 2018
miR-204, miR-211	Silencing in medaka-fish has a negative impact on PR maintenance and function, leading to increased apoptosis Genetic inactivation of miR-211 in mice leads to progressive cone dystrophy, cone loss, and alterations in visual function. A point mutation in the miR-204 mature sequence associated with retinal dystrophy and coloboma in humans	AAV-mediated subretinal delivery in mouse models of RP promotes PR survival and preserves retinal function through a synergistic effect on innate immunity, inflammatory response and cell death	Conte et al., 2015; Barbato et al., 2017; Karali et al., 2020
miR-6937-5p	Upregulated at early stages of retinal degeneration in *rd10* mice	AAV-mediated inhibition in *rd10* mice delays PR demise and vision loss	Anasagasti et al., 2018, 2020
LncRNAs			
TUG1	Involved in PR development and survival; loss-of-function is associated with defects in PR differentiation, structural defects of the OS and increased apoptosis		Young et al., 2005
Vax2os1	Controls cell cycle progression and proliferation of PR progenitors; increased expression delays PR differentiation and enhances apoptosis		Alfano et al., 2005; Meola et al., 2012
MEG3	Significantly upregulated in light-induced retinal degeneration	*MEG3* silencing protects against light-induced retinal degeneration; regulates PR apoptosis by preventing p53 degradation	Wang et al., 2012; Zhou et al., 2012; Zhu et al., 2018
MALAT1	*MALAT1* expression is lost in rod PRs with putative degeneration, suggesting its involvement in rod PR survival and retinal preservation	Intravitreal injection of *MALAT1*-siRNA in mice with diabetes-induced and in oxygen-induced retinopathy reduced secondary PR loss	Lukowski et al., 2019; Wang et al., 2020; Zhang et al., 2020b
RNCR4 (BB283400)	Regulates proper thickness of PR and inner nuclear layers by regulating pri-miR-183/96/182 processing		Krol et al., 2015
circ-Tulp4	Acts as a miR-26a/671/204 sponge; AAV-circ-Tulp4-shRNA-treated retinas show severe PR degeneration		Chen et al., 2020

PRs, Photoreceptors; OS, Outer Segment; IS, Inner Segment; AAV, adeno-associated viral vector; RP, Retinitis Pigmentosa.

of the taurine transporter *Slc6a6*. Recently, miR-183 KO mice presented mild yet progressive defects in ERG responses (Zhang et al., 2020a). Additionally, miR-182-depleted mice showed a thinner IS/OS layer, a progressive reduction of scotopic and photopic ERG responses and increased sensitivity to light-damage compared to control littermates (Wu et al., 2019). In contrast, a study assessing the potential redundancy of the cluster members reported the absence of retinal defects in single, double, and triple mir-183/96/182 mutant zebrafish (Fogerty et al., 2019).

Discrepancies in the phenotypic severity among the above-mentioned models may stem from differences in the methodology, developmental timing, and cell-type specificity of the cluster ablation. Nevertheless, the phenotypes are consistent with the role of the miR-183/96/182 cluster in PR differentiation and synaptic connectivity, signal transduction, transmembrane transport, cell-adhesion, regulation of circadian rhythm and apoptosis (Xu et al., 2007; Dambal et al., 2015; Palfi et al., 2016). Moreover, several mRNA targets that partake in these processes were shown to be regulated by the 183/96/182 cluster members such as *Crb1* (Krol et al., 2015), *Mitf, Adcy6* (Xu et al., 2007), *Rnf217* (Xiang et al., 2017; Zhang et al., 2020a), *Rac1, Slc6a9* (Palfi et al., 2016), *Slc1a1*

(Krol et al., 2010a), *Slc6a6* (Xiang et al., 2017), and *Casp2* (Zhu et al., 2011).

Another miRNA that contributes to PR survival is miR-124, an abundant neuronal-specific miRNA expressed in all neural cells of the retina (Kapsimali et al., 2007; Karali et al., 2007, 2011, 2016; Liu et al., 2011; Sanuki et al., 2011). In adult PRs, miR-124 is predominantly localized in the IS (Karali et al., 2007; Sanuki et al., 2011). Upon degeneration induced by photo-oxidative damage, miR-124 aberrantly redistributed from PRs to the inner retina (Chu-Tan et al., 2018). This mislocalization of miR-124 was likely mediated by extracellular vesicles, as it was impaired following exosome-depletion (Wooff et al., 2020). MiR-124 depletion in a mouse KO for *Rncr3*, its main host gene, resulted in cone mislocalization and demise. Rod PRs were not significantly affected indicating that miR-124 is important primarily for cone survival by targeting *Lhx2* (Sanuki et al., 2011).

The miR-204/211 family is expressed in diverse ocular tissues consistent with its pleiotropic role in eye development (Shiels, 2020). MiR-204 and miR-211 are intragenic miRNAs transcribed from introns of the *Trpm3* and *Trpm1* genes, respectively (Barbato et al., 2017; Shiels, 2020). In the posterior eye, miR-204 is strongly expressed in the RPE and INL (Karali et al., 2007, 2011, 2016) but also detectable in PRs (Conte et al., 2015). Although PRs are not the primary site of miR-204 expression, morpholino-mediated knockdown of miR-204 adversely impacted PR maintenance and function in medaka-fish (Conte et al., 2015). Similarly, the targeted inactivation of miR-211 caused progressive cone dystrophy characterized by reduced cone-elicited ERG responses and cell density in mouse. Transcriptome analysis suggested that miR-211 can impact retinal metabolism by controlling genes involved in glucose and lipid metabolism (Barbato et al., 2017). The higher metabolic demand of cones (Cheng et al., 2020) that renders them susceptible to energy fluctuations, could explain why cones (rather than rods) were predominantly affected by miR-211 loss (Barbato et al., 2017). Likewise, the rapid light-regulated turnover of miR-204/211 in the murine retina, similar to that of the miR-183/96/182 cluster, has been postulated to facilitate activity-dependent expression changes in neuronal cells (Krol et al., 2010a). Remarkably, a heterozygous point mutation within the seed region of miR-204 segregated with a dominant retinal dystrophy characterized by severe PR loss and diminished ERG responses, presumably through a gain-of-function mechanism (Conte et al., 2015).

ROLE OF SPECIFIC lncRNAs IN PHOTORECEPTORS

Long non-coding RNAs (lncRNAs) are a class of transcripts longer than 200 nucleotides with limited protein-coding potential. They exceed mRNAs in quantity and participate in various biological processes and functions across different cell types (Blackshaw et al., 2004; Briggs et al., 2015; Quek et al., 2015; Clark and Blackshaw, 2017; Kopp and Mendell, 2018). LncRNAs can act as decoys of transcription factors (TF) and miRNAs (Hansen et al., 2013), interact with chromatin modifiers to regulate epigenetic states (Rinn and Chang, 2012), or participate in nuclear topological organization (Ip and Nakagawa, 2012) and in protein-complex scaffolding (Ribeiro et al., 2018; **Figure 1C**). LncRNAs have recently been found to contribute to PR function (**Table 1**).

The availability of high-throughput sequencing approaches has facilitated the comprehensive and unbiased identification of lncRNAs in the retina. Palczewski and colleagues identified a group of 18 highly conserved large intergenic non-coding RNAs (lincRNAs) in the retina, some of which localized to specific retinal layers, especially to the PR layer (Mustafi et al., 2013).

One of the first lncRNAs studied in the retina was the *Taurine Upregulated Gene 1 (TUG1)*. Loss-of-function of *TUG1* in the developing retina showed its implication in normal PR development, by acting on transcriptional regulation of PR-specific genes through a yet unknown mechanism. *TUG1* absence in the newborn retina led to structural defects in PR OS accompanied by increased apoptosis and expression changes of key TFs and marker genes of differentiated PRs (Young et al., 2005). A recent study reported the dynamics of the non-coding transcriptome in developing and mature PRs, focusing on rod-enriched lncRNAs controlled by the rod differentiation factor Nrl. The dynamic expression of the non-coding transcriptome during rod maturation is consistent with its functional relevance in the morphogenesis of OS membrane discs and synapse formation (Zelinger et al., 2017). The authors also identified a functional role of antisense RNAs (asRNAs), a subclass of lncRNAs that originate from the opposite strand of 20–40% of protein-coding genes, in PR formation. AsRNAs were identified as putative regulators of eye development because of their overlap with the mRNAs of TFs known to play key roles in vertebrate eye development (Alfano et al., 2005). Among them *Vax2os1*, where "os" stands for "opposite strand," shows an expression confined to the ventral portion of the eye and is predominantly detected in the layers where PR progenitors and differentiated cells reside. Consistent with this expression pattern, *Vax2os1* overexpression in the retina impaired cell cycle progression of PR progenitors and delayed PR differentiation. At later developmental stages, this perturbation led to increased apoptosis in the PR layer (Meola et al., 2012).

Recently, the *Maternally Expressed 3 (MEG3)* was shown to be involved in the pathogenesis of light-induced retinal degeneration. *MEG3* expression is significantly upregulated upon light insult whereas its silencing protects against light-induced retinal degeneration *in vivo* and *in vitro* by decreasing caspase 3/7 activity and proapoptotic protein (Bax) levels while upregulating antiapoptotic protein (Bcl-2) expression. Mechanistically, *MEG3* regulates PR apoptosis by acting as a p53 decoy, preventing MDM2-mediated p53 degradation (Wang et al., 2012; Zhou et al., 2012; Zhu et al., 2018).

Another lncRNA strongly upregulated in stress conditions is *MALAT1,* expressed in all retinal layers (Yao et al., 2016). *MALAT1* expression in rod PRs was reduced upon longer post-mortem times, prompting the authors to suggest that such downregulation may be linked to an early stage of rod PR degeneration (Lukowski et al., 2019).

An interesting example of crosstalk between lncRNAs and miRNAs in PR cells is represented by the lncRNA *Rncr4* (retinal non-coding RNA 4) and the miR-183/96/182 cluster (Krol et al., 2015; **Figure 1D**). *Rncr4* is expressed in maturing PRs in the opposite direction to the pri-miR-183/96/182. Its expression stimulates pri-miR-183/96/182 processing by acting on the activity of the DEAD-box RNA helicase/ATPase Ddx3x, an inhibitor of pri-miR-183/96/182 maturation in early postnatal PRs. Alteration of the timing of miR-183/96/182 formation led to early accumulation of mature miR-183/96/182 and caused profound irregularities in the thickness of the PR and inner nuclear layers (Krol et al., 2015).

A novel class of ncRNAs with tissue- and stage-specific expression patterns are the circular RNAs (circRNAs) whose circular characteristic confers increased stability compared to linear transcripts (Rybak-Wolf et al., 2015; Han et al., 2017). CircRNAs display high expression, evolutionary conservation, specificity of action and stability. They are involved in numerous biological processes and are differentially expressed in various diseases (Ghosal et al., 2013; Lukiw, 2013; Li et al., 2015; Peng et al., 2015; Zhao and Shen, 2017), including diabetic retinopathy, highlighting their potential as biomarkers and as predictors of response to treatments (Li et al., 2015; Chen et al., 2016; Shan et al., 2017; Zhang et al., 2017, 2018). Transcriptome profiling of circRNAs at five different developmental stages in wild-type and degenerating retinas (*rd8* mouse model; Mehalow et al., 2003) identified circRNAs with a dynamic and strong expression pattern. These circRNAs were found to target miRNAs in the retina, acting as competitive endogenous RNAs (ceRNAs). Specifically, *Cdr1as* acted as miR-204/677/378 sponge, *circHipk2* as a miR-124 sponge, *circTulp4* as a miR-26a/671/204 sponge, and *circAnkib1* as a miR-195a sponge. Functional analysis of *circ-Tulp4* revealed that its reduction leads to downregulation of miR-204-5p and miR-26a-5p targets (i.e., *Meis2, Cdh2, Mitf*, and *Pde4b*) which are major players in retinal development and function. Accordingly, AAV-*circTulp4*-shRNA-treated retinas showed severe PR degeneration with attenuated scotopic and photopic ERG responses confirming that *circTulp4* is indispensable for proper retinal function. The authors observed that the levels of some circRNAs, but not those of their corresponding linear transcripts, increased before disease onset in the retina of rd8 mice (Chen et al., 2020). More comprehensive transcriptome studies extended to additional models are needed to support the hypothesis that circRNA expression alterations may be early markers of PR degeneration.

PERSPECTIVES

Advancements in understanding the role of ncRNAs in PR development and function prompted the design of new strategies to promote PR survival (**Table 1**). Specifically, miRNA modulation can impact simultaneously on several biological processes that exacerbate PR degeneration as shown in the following examples of miRNA-based therapeutics. First, intravitreal delivery of miR-124 mimics reduced microglia infiltration and PR cell death following light-induced damage in mice, possibly by modulating *ccl2* levels (Chu-Tan et al., 2018). In a similar approach, the subretinal delivery of AAV-miR-204 in a mouse model of dominant RP (i.e., the transgenic *RHO*-P347S) promoted PR survival and preserved retinal function through synergistic effects on innate immunity, inflammatory responses, and cell death (Karali et al., 2020). miR-204 exerted this protective effect, at least partially, by downregulating the expression of *Siglec1* and *Xaf1* (Karali et al., 2020). Finally, AAV-mediated inhibition of miR-6937-5p in *rd10* mice delayed PR and vision loss (Anasagasti et al., 2020). This miRNA was upregulated at early stages of retinal degeneration in *rd10* mice (Anasagasti et al., 2018). Recently, intravitreal injection of *MALAT1*-siRNA in diabetes-induced mice reduced the functional and morphological damage of rods and cones, indicating its protective, likely indirect, effect against secondary PR loss (Zhang et al., 2020b). Similarly, *MALAT1* increase in oxygen-induced retinopathy was counteracted by intravitreal injection of MALAT1-siRNA, which significantly improved the retinal phenotype (Wang et al., 2020). Although these results highlight *MALAT1* silencing as a possible therapeutic strategy for different forms of retinopathy, the direct functional role of *MALAT1* in PRs remains unclear, especially in light of the results linking its reduction to PR degeneration (Lukowski et al., 2019). The discrepancy between the above observations warrant further investigation aimed at a better clarification of the role of *MALAT1* in the different retinal cell types.

These studies underscore the therapeutic potential of ncRNA-based approaches in mutation- and gene-independent approaches. Such strategies are particularly relevant for inherited retinal diseases due to their high genetic heterogeneity (Carrella et al., 2020). Moreover, they are appealing for gain-of-function and dominant negative mutations where gene supplementation is not appropriate to overturn the consequences of the genetic lesion. Similar attempts applied to non-retinal conditions yielded encouraging outcomes confirming the translational potential of ncRNA-based therapies in human disease (Hanna et al., 2019; Dammes and Peer, 2020).

AUTHOR CONTRIBUTIONS

SC and SB conceived the manuscript. SC and MK wrote the first draft of the manuscript. SB edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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MiRNA Regulatory Functions in Photoreceptors

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MicroRNAs (miRNAs) are important regulators of gene expression. These small, non-coding RNAs post-transcriptionally silence messenger RNAs (mRNAs) in a sequence-specific manner. In this way, miRNAs control important regulatory functions, also in the retina. If dysregulated, these molecules are involved in several retinal pathologies. For example, several miRNAs have been linked to essential photoreceptor functions, including light sensitivity, synaptic transmission, and modulation of inflammatory responses. Mechanistic miRNA knockout and knockdown studies further linked their functions to degenerative retinal diseases. Of note, the type and timing of genetic manipulation before, during, or after retinal development, is important when studying specific miRNA knockout effects. Within this review, we focus on miR-124 and the miR-183/96/182 cluster, which have assigned functions in photoreceptors in health and disease. As a single miRNA can regulate hundreds of mRNAs, we will also discuss the experimental validation and manipulation approaches to study complex miRNA/mRNA regulatory networks. Revealing these networks is essential to understand retinal pathologies and to harness miRNAs as precise therapeutic and diagnostic tools to stabilize the photoreceptors' transcriptomes and, thereby, function.

Keywords: miR-182, miR-183, miR-124, retina, retinal degeneration, photoreceptors, rods, cones

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INTRODUCTION

MicroRNAs (miRNAs) are small, non-coding RNAs, acting as quantitative regulators of gene expression, which are characterized by an average length of 22 nucleotides (nt) (Ghildiyal and Zamore, 2009; Ha and Kim, 2014). MiRNAs were discovered in 1993 in the nematode *Caenorhabditis elegans* (Lee et al., 1993). The biogenesis of miRNAs is divided into several steps (**Figure 1**). DNA sequences encoding for miRNAs are transcribed into primary miRNAs (pri-miRNAs) by RNA polymerases II/III (Lee et al., 2004; Borchert et al., 2006). Pri-miRNAs build hairpin-like structures or stem-loops by self-annealing. These structures are cleaved 11 base pairs from the hairpin stem by the miRNA-processing complex, consisting of Drosha ribonuclease and the double-stranded RNA binding domain partner protein DiGeorge critical region 8 (DGCR8): this forms the precursor miRNA (pre-miRNA), consisting of a 70-nt-long sequence and a 5′ phosphate and 2-nt overhang at the 3′ end (Lee et al., 2002; Ha and Kim, 2014). For the last step of miRNA maturation, the pre-miRNA is exported into the cytoplasm by the exportin-5 (XPO5)/RanGTP complex (Yi et al., 2003; Lund et al., 2004). At this point, Dicer endoribonuclease and its *trans*-activation response RNA-binding protein (TRBP) cleave the pre-miRNA and add a 5′

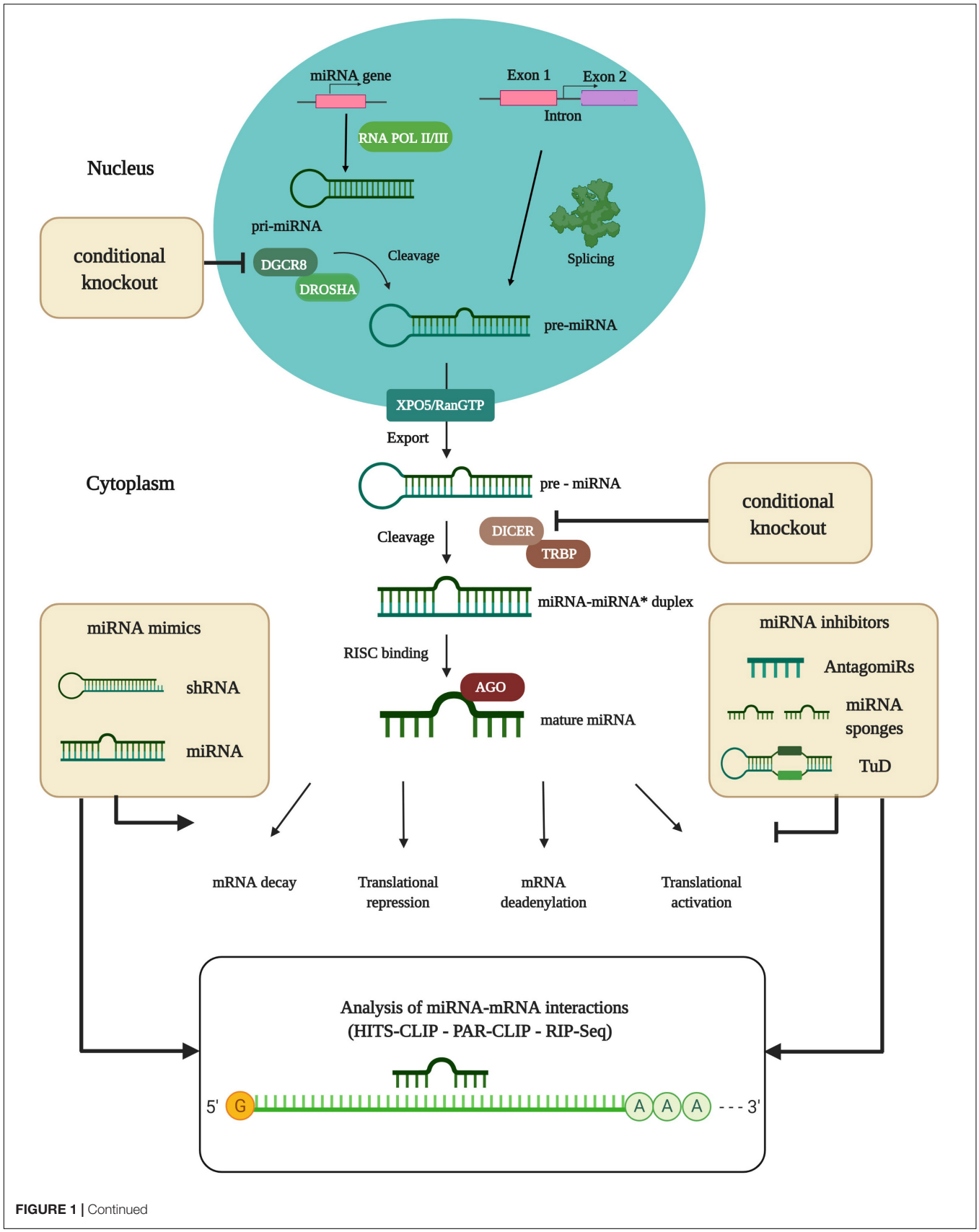

FIGURE 1 | Continued

FIGURE 1 | MiRNA biogenesis and experimental analysis of miRNA-mRNA interactions. DNA sequences encoding for miRNAs are transcribed into primary-miRNAs (pri-miRNAs) by partially complementary RNA polymerase II/III. Pri-miRNAs are subsequently cleaved by the miRNA-processing complex consisting of Drosha ribonuclease and the double-stranded RNA binding domain partner protein DGCR8 that form precursor-miRNAs (pre-miRNAs). The Drosha/DGCR8 complex and Dicer endoribonuclease are used for conditional knockout studies (cKO) to identify the actions of particular miRNAs. Full knockout of miRNA-processing machinery has proved lethal. The pre-miRNA is then transported into the cytoplasm by the exportin (XPO5)/RanGTP complex. In the cytoplasm, the Dicer/*trans*-activation response RNA binding protein (TRBP) nuclease complex cleaves the pre-miRNA, thereby producing the miRNA duplex. Finally, the duplex is loaded onto the Argonaute (AGO) protein as a part of the RNA-induced silencing complex (RISC) where one of the strands is removed. The remaining strand remains bound to the AGO protein, which is now ready to target mRNA. Translational efficiency is thereby reduced, mainly as a consequence of mRNA cleavage or deadenylation. Alternatively, miRNA biogenesis can proceed *via* splicing events where Drosha cleavage is replaced. MiRNA pathways can be modulated by miRNA mimics or inhibitors. Analysis of miRNA-mRNA interactions is done by RNAse digestion of AGO proteins, combined with next-generation sequencing (NGS) techniques like HITS-CLIP or PAR-CLIP, or novel techniques like RNA immunoprecipitation combined with NGS (RIP-Seq). shRNA, small hairpin RNA; TuD, Tough decoy. Figure was created with BioRender.com.

phosphate and a new 2-nt 3′ overhang by cutting the pre-miRNA ∼22-nt from the cleaving site of the miRNA processing complex. This step gives rise to the miRNA duplex (Lee et al., 2003). Finally, the Argonaute (AGO) protein, as a part of the RNA-induced silencing complex (RISC), binds to the miRNA and removes one of the strands. The remaining strand is bound to the AGO protein and ready to bind partially-complementary mRNA transcripts. MiRNA biogenesis can also occur through alternative pathways, where Drosha cleavage is replaced and the miRNAs are processed *via* splicing events when miRNAs reside in the introns of protein-coding genes (Westholm and Lai, 2011). The AGO proteins, as core components of the RISC complex, have a coordinating function for localizing mRNA transcripts, and are therefore essential for correct miRNA function (Bartel, 2009; Ha and Kim, 2014). A single miRNA can have thousands of *in silico* annotated target mRNAs, regulating multiple genes which often participate in the same biochemical pathway. This is due to similarities in the 3′ untranslated region (3′ UTR) of specific mRNAs which are bound by miRNAs (Lewis et al., 2005). However, experimental *in vivo* validations only result in very few mRNA targets upon miRNA manipulation (Rojo Arias and Busskamp, 2019). The RISC components are the most obvious targets for regulation. Nevertheless, other proteins that take over positive or negative control of miRNA effects have also been identified. Most miRNAs are translational repressors, or promote deadenylation and decay of mRNAs. However, miRNAs can also act as activators of translation by switching AGO2 from a repressor to an activator (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). In general, miRNAs have strong regulatory properties in many biological systems, including the eye, whilst regulating the transcription of genes in cells to maintain their homeostasis and function (Sundermeier and Palczewski, 2016). Hence, their dysregulation, especially during development, can lead to diverse pathological conditions like genetically-inherited disorders, neurodegenerative diseases, and cancer, as well as autoimmune and cardiovascular diseases (Ha, 2011; Dong et al., 2015; Chu-Tan et al., 2018). It is thought that miRNA regulatory networks provide robustness to biological systems whose faultless functioning is constantly being endangered by external and internal interferences (Ebert and Sharp, 2012). Approximately 25% of the human miRNA genes are structured in clusters. Their expression is normally tightly regulated, but altered in pathologies. Of note, epigenetic modifications have been reported that change the expression levels of proteins which are important for miRNA biogenesis (Kabekkodu et al., 2018). In addition, it has been reported that accelerated turnover of miRNAs depends on the activity of photoreceptors, i.e., their exposure to light (Krol et al., 2010a). Clusters are expressed as a polycistronic transcript, with a high sequence homology between the members. In general, clusters of miRNAs are formed by several miRNA genes that are located next to each other on the chromosome. The genes are transcribed as one long pri-miRNA, which is then processed into several individual pre-miRNAs (Altuvia et al., 2005). Each of the miRNAs has small differences in their seed sequence, leading to different mRNA targets (Dambal et al., 2015). However, if the seed sequences have diverged and their mRNA targets have diversified, they cooperate by targeting different genes in common pathways, which amplifies the downstream effects. The cooperative work of multiple miRNAs to target multiple functionally-related genes enables coordinated control of gene networks (Na and Kim, 2013). However, there are similarities in their binding properties to target mRNAs and they can compensate for each other's function (Jin et al., 2009).

Photoreceptors are the cells in the body with the highest metabolic activity, and are subject to high levels of external stress (Sung and Chuang, 2010). In this context, miRNAs play an important role in the functioning and survival of photoreceptors (Sundermeier and Palczewski, 2016). The fact that not all retinal diseases are linked to specific genes supports the idea that the dysregulation of certain miRNAs can cause the progression of retinal disorders. Experimental disruption of the miRNA processing machinery can lead to the loss of cone outer segments in humans, triggering their dysfunction, resulting in blindness (Busskamp et al., 2014). Furthermore, introducing gene-trap constructs downstream of a gene, expressing the miRNA cluster miR-182/96/183 that is highly expressed in the retina, leads to its inactivation and to progressive synaptic defects (Lumayag et al., 2013). Moreover, it has been shown that miR-124, as the most abundant miRNA in the brain, plays an important role in the progression of neovascular and atrophic form of age-related macular disease (AMD) (Chu-Tan et al., 2018). In this context, mimics of miR-124 in the eye decreased the inflammatory response in both forms of AMD, improving overall retinal function. Therefore, revealing miRNA regulatory pathways in retinal cell types is crucial to obtain a better understanding of the molecular processes that lead to ocular diseases for which there is currently no treatment. In addition, the investigation

of retinal miRNAs provides a basis for developing therapies to significantly improve the quality of life of affected patients. Here, we will emphasize the role of the highly-abundant miR-182/96/183 cluster and miR-124 within the retina. We will discuss how sophisticated experimental studies have revealed miRNA functions within different retinal cell types.

MiRNA REGULATION IN THE RETINA

Photoreceptor cells and retinal pigmented epithelium (RPE) cells are characterized by high rates of metabolism and protein synthesis. In addition, they are constantly exposed to toxic by-products of phototransduction. Moreover, they have to stay viable and functional under highly oxidizing conditions. All these processes cause high levels of stress to the cells, making them much more vulnerable to precocious death (Sundermeier and Palczewski, 2016). Acting as fine tuners of gene expression, miRNAs take over important functions with respect to photoreceptor survival and function (Sundermeier and Palczewski, 2016). On the other hand, *in vivo* and *in vitro* studies have shown that there are several miRNAs that are potentially associated with the cellular processes that lead to AMD (Wang et al., 2014; Chu-Tan et al., 2018). Investigating the miRNA transcriptome (miRNome) of retinal cells is the first step toward revealing miRNA regulatory pathways in health and disease. Studies have been conducted on the human and mouse miRNomes to reveal differences and similarities in miRNA regulatory pathways between human and mouse eye (Karali et al., 2010, 2016). In particular, research has focused on the most conserved miRNAs. This comparison is indispensable, because these two organisms show essential differences in the structure and function of the eye: however, mouse models are used more in vision research. These studies have revealed that one third of the retinal miRNAs expressed in human samples are also expressed in the mouse retina (Karali et al., 2016). Hence, mouse models are well-suited model systems for retinal miRNome studies for miRNAs that have been found to be expressed in both species. Almost a fifth of all known miRNAs are expressed in the retina, and a limited set of these miRNAs has been identified as playing an important role in the development and function of the retina (Krol et al., 2010b; Lumayag et al., 2013). This set consists of miR-182-5p, miR-183-5p, and miR-124-3p, as well as miR-96-5p and miR-9-5p. Moreover, miRNA expression is tissue specific and its regulation changes, depending on the developmental stage. This demonstrates that miRNAs are involved in important retinal maturation processes, and that their expression pattern is tightly controlled (Lagos-Quintana et al., 2002). The misregulation of miRNA expression is therefore a proximate cause of retinal degeneration and disease (Damiani et al., 2008; Arora et al., 2010; Georgi and Reh, 2010; Busskamp et al., 2014; Sundermeier et al., 2014; Ohana et al., 2015).

The length and sequence of mature miRNAs is highly heterogeneous: this is different from the canonical miRNA sequence (Morin et al., 2008). As a result, one miRNA can have several variants, called isomiRs, that are characterized by addition or deletion of nucleotides at the 3′ and/or 5′ end of the miRNA and/or substitutions within the sequence (Landgraf et al., 2007; Morin et al., 2008; Martí et al., 2010). Most miRNA-mRNA interactions are based on the binding of the miRNA seed sequence to its target mRNA (Helwak et al., 2013). Nucleotide substitutions at the 5′ end of the miRNA result in a modified seed sequence, resulting in a changed target specificity and far-reaching effects on miRNA functionality (Cammaerts et al., 2015). In the isomiR variant of miR-124-3p, a single nucleotide substitution in the seed region resulted in a change in its target specificity, when comparing with the canonical miRNA specificity (Karali et al., 2016). This resulted in an altered gene regulatory property and showed that gene regulation within the retina is complex but also necessary to ensure proper tissue function. The miR-124-3p and miR-183-5p isomiRs accounted for a large part of the retinal miRNome analysis (Karali et al., 2016). During miRNA biogenesis, either the 5′ or 3′ arm of the miRNA duplex is favorably cleaved by Drosha and Dicer: this becomes the mature miRNA (Khvorova et al., 2003; Schwarz et al., 2003). Still, next-generation sequencing (NGS) data have revealed that both arms are cleaved and detectable (Yang et al., 2011; Li et al., 2012; Neilsen et al., 2012; Zhou et al., 2012; Kang et al., 2013). The mature miR-183, miR-182, and miR-96 have almost identical seed sequences (Dambal et al., 2015). A single base difference in the seed sequence of miR-182 and miR-96 changes the binding property to the mRNA target sequence (Jalvy-Delvaille et al., 2012; Li et al., 2014). Nevertheless, their targets often lie in the same pathways, facilitating that these miRNAs control several parts of a cellular process (Dambal et al., 2015).

Disrupting miRNA regulatory pathways during development can have severe effects, such as aberrant photoreceptor layer architecture and progressive photoreceptor degeneration (Georgi and Reh, 2010). MiRNAs have been found to control transcription factors like *Pax6*, which is expressed in a spatiotemporal pattern in different tissues, including the developing retina, lens, cornea, and mature ocular cell types, during development (van Heyningen and Williamson, 2002; Kaspi et al., 2013). Analysis of the *Pax6* 3′ UTR has revealed that cooperative miRNA regulation of *Pax6* mediates developmental control and fine tuning of *Pax6* levels during development (Ryan et al., 2018). Changes in miRNA expression have also been investigated in a retinal degeneration model in which retinal damage was induced by light (Saxena et al., 2015). Transcriptomic analysis revealed that a large set of miRNAs regulates the immune response connected to the light-damage changes. This supported the theory that miRNAs play an important role in retinal degenerative diseases that are characterized by acute retinal damage (Veleri et al., 2015). In AMDs, miRNAs are often associated with the regulation of inflammatory processes which highlights the need for a better understanding of miRNA regulatory networks (Rutar et al., 2010; Chu-Tan et al., 2018).

Müller glia (MG) are the predominant glia in the retina: they nurture and protect retinal neurons, maintain the homeostasis of the retina, and support structural integrity (Bringmann et al., 2006). Consequently, the loss of mature MG can lead to impairment of the retinal structure (Byrne et al., 2013).

Neuronal loss leads to retinal remodeling, a process in which MG expand and fill the neuronal gaps. They form a glial scar, which is a major limiting factor regarding transplantation approaches to restore retinal function (Jones and Marc, 2005; Reh, 2016). To study the role of miRNAs in MG function, Dicer1 was specifically deleted in MG (Wohl et al., 2017). Here, it is of great importance that knockouts of the miRNA processing machinery are conditional (cKO) because full deletion during embryonic development in mouse models is lethal (Bernstein et al., 2003; Fukuda et al., 2007; Morita et al., 2007; Wang et al., 2007). The deletion of Dicer1 led to a significant decline in those miRNAs, called mGliomiRs, that are highly expressed in MG (Wohl and Reh, 2016b). The decline in MG miRNAs led in early phases to an increased number of MG, and in MG migration toward the outer nuclear layer. At later stages, glia accumulations and the deformation of the retinal architecture were found. A key player in this process was the miRNA miR-9 that targets the extracellular matrix molecule Brevican (encoded by *Bcan*). All these results led to the conclusion that miRNAs play an important role in MG function, which is required for the maintenance of retinal structure and function (Wohl et al., 2017). Moreover, overexpression of neuronal (Wohl and Reh, 2016a) or retinal progenitor miRNAs, in combination with inhibition of MG miRNAs (Wohl et al., 2019), can reprogram MG into late retinal progenitor cells that differentiate into bipolar-like neuronal cells. This suggests that miRNAs are involved in MG-reprogramming tool for retinal regeneration. Another Dicer conditional knockout mouse model was used to identify which miRNAs are important for retinal development (La Torre et al., 2013). Three different miRNAs, let-7, miR-125, and miR-9 were found to act as regulators, by changing the competence of retinal progenitor cells. In addition, the overexpression of these miRNAs accelerated retinal development. Other studies have investigated miRNA functions in cone photoreceptors, which are indispensable for daylight and high-acuity vision. Cone photoreceptor-specific miRNA-deficient mice showed a gradual depletion of DGCR8 protein over time, leading to a progressive loss of cone outer segments and low sensitivity to high light levels (Busskamp et al., 2014). Besides neurodegenerative retinal diseases, developmental genetic disorders such as microphthalmia, anophthalmia, and coloboma (MAC) cause structural eye malformations: a heterozygous mutation in the seed region of miR-204 has been described in MAC patients (Conte et al., 2015). Seed sequence modifications impact on the mRNA targets, resulting in photoreceptor alterations, reduced numbers of rod and cone photoreceptors, and increased levels of apoptosis. These findings highlight the important function of miR-204 during retinal development.

THE MiRNA CLUSTER 182/96/183 IN PHOTORECEPTORS

MiR-182/96/183 is a sensory-neuron enriched miRNA cluster. It is highly and prevalently expressed in mature photoreceptors and in the inner nuclear layer (INL) of the retina (Xu et al., 2007). In particular, miR-182 and miR-183, and also miR-96, play an important role in the maintenance and function of cone outer segments (Busskamp et al., 2014). MiR-96 also plays a major role in the cells of inner ear hairs (Lewis et al., 2009; Mencía et al., 2009). MiR-182, miR-96, and miR-183 are co-expressed on a single primary transcript and share high sequence homology, suggesting overlapping, but unique functions (Xu et al., 2007; **Figure 2**). As mentioned before, clusters can compensate for each other's function, which has been shown by targeted deletion of the miR-182 (Jin et al., 2009). Here, no changes in phenotype were observed, indicating that miR-183 or miR-96 had very likely a compensatory effect. In general, the cluster is responsible for global regulation of many downstream genes that are involved in several pathways such as synaptogenesis, synaptic transmission, and photoreceptor functions (Lumayag et al., 2013; Busskamp et al., 2014) and it has a protective effect on neurons by targeting Caspase-2 (Casp-2) (Zhu et al., 2011).

The expression of mature cluster miRNAs is low early in development, but increases after birth and is most abundant in the adult retina (Xu et al., 2007). The pri-miR-183/96/182 is highly expressed early in development but, due to reduced enzymatic processing, the expression of the mature miRNA cluster is delayed and dependent on the developmental stage. An interplay between a long, non-coding RNA [lncRNA Rncr4 (retinal non-coding RNA 4)] and the miRNA cluster has been described: this is crucial for postnatal retinal development (Krol et al., 2015). An enforced expression of mature miR-182/96/183 early in development can have negative effects on the morphology of the retinal layers. The regulation of miRNA cluster expression is indispensable for the correct development and function of the retina. Moreover, the miRNA cluster plays an important role in the formation of tight conjunctions between MG cells and photoreceptors (Krol et al., 2015). The cluster was found to have a dynamic diurnal expression pattern suggesting that its regulation is coupled to the circadian rhythm (Xu et al., 2007). Later, it was shown, that the miRNA cluster is reversibly up- and downregulated in the retina *in vivo* during light-dark adaption, independent of the circadian rhythm (Krol et al., 2010a). Additionally, re-expression of miR-182 and miR-183 prevented cone photoreceptor function loss *in vivo*, even after the miRNA processing machinery was disrupted. *In vitro*, the administration of the two miRNAs led to the formation of inner segments, connecting cilia, and short outer segments in stem-cell-derived 3D retinal organoids. In this way, the photoreceptors became light sensitive (Busskamp et al., 2014). Another study demonstrated that the cluster is an important regulator of *PAX6* and that it is important for retinal tissue morphogenesis (Peskova et al., 2020). To inhibit the cluster in organoid forming human pluripotent stem cells (hPSCs), a tough decoy approach was used. Also, abnormalities were observed in retinal organoid morphology, together with an upregulation of neuron- and retina-specific genes. A single knockout of miR-182 in mouse models did not lead to any significant changes in retinal architecture. However, deletion of both miR-183 and miR-96 caused defects in cone maturation (Xiang et al., 2017) linked to their target Slc6a6, a taurine transporter, which is needed for the maturation and maintenance

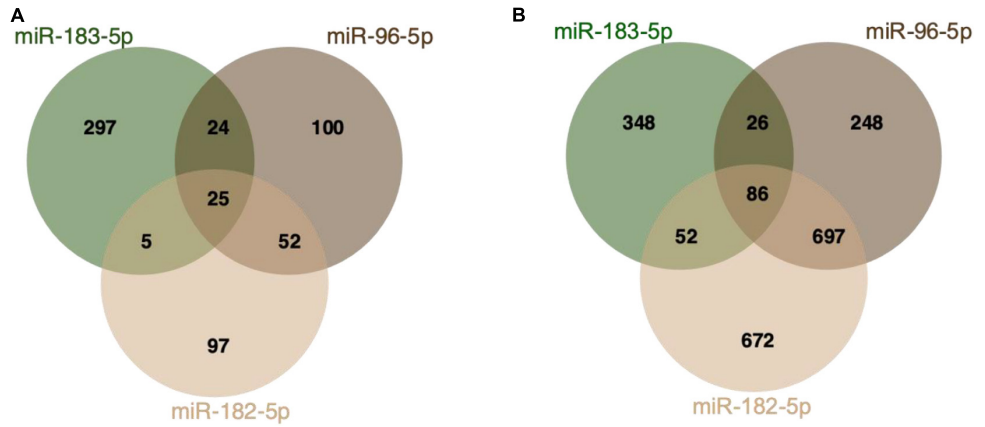

FIGURE 2 | MiR-182/96/183 cluster target interactions. **(A)** Venn Diagram displaying miRTarBase entries for miR-182/96/183 cluster targets (Chou et al., 2018). All three miRNAs share 25 validated common targets. **(B)** Predicted miR-182/96/183 targets based on TargetScan (release 7.2) (Agarwal et al., 2015). The analysis is based on analyzing the presence of target sites that match the seed region of each miRNA. All three miRNAs share 86 predicted biological targets.

of photoreceptors. The formation of correct synaptic connections between photoreceptors and postsynaptic retinal cells was also shown to be miRNA-dependent. A knockout mouse model generated from an embryonic stem cell clone (ESC), where a gene trap was inserted downstream of the first exon of the miR-182/96/183 gene, resulted in progressive synaptic defects in photoreceptors, and progressive retinal degeneration (Lumayag et al., 2013). MiR-182 has also been reported to impact axonal growth of retinal ganglion cells in *Xenopus laevis* (Bellon et al., 2017).

So far, only a handful of the thousands of annotated cluster miRNA targets have been experimentally validated in retinal cell types. As important biological processes fall under their regulation, further research in the coming years will provide deeper insights revealing their functions in health and disease. In summary, the miR-182/96/183 cluster is indispensable for proper retinal development and function, such as maintaining photoreceptor outer segments, synaptogenesis, and axonal growth. Therefore, it might be possible to use these miRNAs as therapies to cure neurodegenerative retinal diseases.

miR-124 AND ITS ROLE IN NEURODEGENERATION AND NEURONAL DIFFERENTIATION

miR-124 is one of the most abundant miRNAs in the brain, accounting for 25% of all brain miRNAs (Lagos-Quintana et al., 2002). It is also highly expressed in the retina, where it supports the maturation of photoreceptors. In addition, the partial loss of miR-124 during development leads to reduced opsin expression and cone photoreceptor death (Sanuki et al., 2011; Karali et al., 2016). This miRNA has three paralogs with six genomic copies. A complete miR-124 knockout has been generated in human induced pluripotent stem cells (hiPSC) that were subsequently differentiated to neurons and analyzed: loss of miR-124 led to morphological and functional alterations, as well as different neurotransmitter profiles and decreased long-term viability. However, the initiation of neuronal differentiation was independent of miR-124 (Kutsche et al., 2018). This finding was a bit surprising, as the overexpression of miR-124 in cell lines and embryonic stem cells mediates neuronal differentiation (Krichevsky et al., 2006; Makeyev et al., 2007); overexpression of miR-9 and miR-124 in human fibroblasts causes them to differentiate into neurons (Yoo et al., 2011). Additionally, in HeLa cells, delivery of miR-124 duplexes caused acquisition of neuronal gene profile (Lim et al., 2005). Because of its important role in central nervous system including retinal neurons, miR-124 dysregulation is connected to certain diseases, including Alzheimer's (AD), Parkinson's, and AMD (Smith et al., 2011; Wang et al., 2014; Sun et al., 2015). In the degenerating retina, miR-124 expression and its cellular location are altered in human and rodent tissues (Chu-Tan et al., 2018). It has been shown that miR-124 targets mRNAs which code for chemokines that are upregulated in neovascular and atrophic forms of AMD when physiological miR-124 levels are decreased. Intravitreal delivery of miR-124 reduced the chemokine expression levels, highlighting its anti-inflammatory properties. Photoreceptor death could be reduced, and overall retinal function was improved (Newman et al., 2012; Chu-Tan et al., 2018). The activation of the innate immune system is connected to the pathogenesis of certain retinal degenerative diseases, for example AMD or diabetic retinopathy (DR) (Edwards et al., 2005; Hageman et al., 2005; Hou et al., 2015). In the case of DR, elevated levels of monocyte chemotactic protein-1 (MCP-1) can be detected in tear fluid (Liu et al., 2010) and vitreous fluid (Wakabayashi et al., 2011; Chernykh et al., 2015). The same work showed that miR-124 takes over an anti-inflammatory role by targeting the 3′ UTR of the MCP-1 gene, therefore decreasing MCP-1 expression and inflammation (Dong et al., 2015). Altogether, anti-inflammatory properties of miR-124 have an impact as a therapeutic for treating retinal degenerative diseases (Chu-Tan et al., 2018).

OTHER KEY PHOTORECEPTOR MiRNAs

Other miRNAs, like the miR-181a and miR-181b have also been shown to control the expression of genes that are involved in mitochondrial biogenesis and function in the retina (Indrieri et al., 2019). In this regard, downregulation of these miRNAs increased mitochondrial turnover, thereby protecting photoreceptors from degeneration. Additionally, miR-181a and miR-181b are highly expressed in the retina, notably in retinal ganglion cells (RGCs), inner cell layers and in brain areas that are related to visual function (Ryan et al., 2006; Kapsimali et al., 2007; Karali et al., 2007). Furthermore, these two miRNA species represent about 17% of the cone photoreceptor miRNome (Busskamp et al., 2014). Gain- and loss-of-function approaches on these two miRNAs revealed that they impact on axonal growth and specification of retinal cells by fine tuning of the MAPK/ERK pathways (Carrella et al., 2015). Their expression is crucial for the formation of neural connections in the retina (Carrella et al., 2015). Also, miR-204 has been found to be expressed in photoreceptors and plays an important role in retinal development (Conte et al., 2015). In this connection, a single heterozygous point mutation was analyzed in miR-204, which is the only known miRNA mutation that causes inherited retinal dystrophy in humans (Conte et al., 2015). This mutation is within the miR-204 seed region and leads to an autosomal dominant phenotype. This mutation may impact as a loss of function, resulting in non-recognizable wild type target genes or as a gain of function *via* new unconventional targets of miR-204. The therapeutic potential of miR-204 has been investigated by subretinal delivery of AAVs carrying the miR-204 pre-miRNA (Karali et al., 2020). The administration led to a decrease in apoptosis of photoreceptors and microglia activation in mouse models displaying inherited retinal diseases. Due to this neuroprotective function, the use of miR-204 as a therapeutic agent represents a promising mutation-independent approach for curing forms of blindness.

SOPHISTICATED APPROACHES TO STUDY MiRNA REGULATORY PATHWAYS

Studying the miRNome of tissues that consist of different cell types, like the retina, can be challenging. For technical reasons regarding the detection of these small RNA molecules, only highly abundant miRNAs have been studied so far. *In vivo*, a high heterogeneity of neuronal cell types and progenitors may falsify the results of studying specific miRNA expression in defined cell types, due to the differences in coding and non-coding transcriptomes (Yaworsky and Kappen, 1999). This can lead to an insufficient view, and misinformation about the miRNome of specific cell types. Pooling neuronal samples to obtain sufficient material for transcriptomic studies masks cell-type-specific miRNomes and their target mRNAs. Moreover, miRNA regulatory networks can be complex, as they have a large number of targets including non-canonical binding events (Chi et al., 2012; Moore et al., 2015). The ongoing technological development and refining of assays facilitate more precise studies, providing consistent and reliable results. Labeling cells with cell-type-specific markers allows the isolation and a more narrowed miRNome analysis on a homogenous cell population. This has been achieved by using transgenic approaches, where green fluorescent protein (GFP) was exclusively expressed in mouse cone photoreceptors (Fei, 2003). Moreover, it is important to consider the developmental stage, especially when using animal models, because of changes in the expression of specific miRNAs as mentioned before (Xu et al., 2007; Krol et al., 2015). To investigate the effects of missing post-transcriptional gene regulation by miRNAs, *in vivo* and *in vitro* knockdown studies of the miRNA machinery and of certain miRNAs has been performed (Sanuki et al., 2011; Busskamp et al., 2014). In this context, the time point of manipulation is important in order to interpret the obtained results. It was shown that DGCR8 deletion had a time-delayed effect (Busskamp et al., 2014) due to the high stability of the DGCR8 protein, likely because of its phosphorylation and interactions with other proteins (Han et al., 2009; Herbert et al., 2013; Cheng et al., 2014). Hence, the effects of missing miRNAs could only be seen after postnatal day 30, with a fully developed retina, leading to an incomplete view of the impact of the missing miRNA processing machinery during development (Busskamp et al., 2014). Recent developments in genomic engineering have also facilitated the generation of complete miRNA knockouts, such as deleting all six miR-124 alleles by clustered regularly interspaced short palindromic repeats (CRISPR/Cas9). Thereby, it became obvious that also other miRNAs took over the regulatory space in absence of miR-124. The knockout of a highly abundant miRNA species is not leading to a vacuum of miRNA regulation and a sophisticated interpretation of the phenotype requires also to study effects of *de novo* upregulated miRNAs in the cells of interest. Hence, in comparison to genes, studying miRNA knockout effects is more complex and requires sophisticated system level analysis (Kutsche et al., 2018). Chemically-engineered oligonucleotides termed "AntagomiRs" are used for silencing endogenous miRNAs (Krützfeldt et al., 2005). This approach has been used for down-regulation studies of endogenous miR-124 (Cao et al., 2007; Visvanathan et al., 2007; Cheng et al., 2009; Åkerblom et al., 2012). Here, the results turned out to be controversial, suggesting that antisense nucleotides trigger only transient inhibition, and that the knockdown is not sufficient. This was seen especially in progenitor cells, as their high proliferation rate affected their efficacy. Moreover, so-called miRNA sponges were used to analyze effects of miRNA silencing on cellular processes. These miRNA sponges, holding multiple tandem binding sites to a miRNA of interest, are expressed from strong promoters and bind specifically to miRNA seed families. Nevertheless, their silencing efficacy is comparable to approaches using antisense nucleotides (Ebert et al., 2007). Interestingly, sponge cassettes have been delivered to specific retinal cell types by adeno-associated viruses (AAVs) to analyze miRNA actions in neuronal cells (Krol et al., 2010a) as well as in transgenic mouse models (Zhu et al., 2011).

Another approach to study miRNA functions focused on the robust and simultaneous suppression of different pairs or groups of miRNAs that are not related to each other (Hollensen et al., 2013). So-called "Tough Decoy" (TuD) inhibitors were designed that are characterized by hairpin structures carrying two or more miRNA recognition sites. TuD allows to suppress several miRNAs *via* one DNA-encoded RNA inhibitor, making them a valid approach for suppression studies of miRNA clusters or families. Yet, TuD design by predicting target mRNAs is challenging because miRNAs bind to their messenger RNAs by base pairing with 6–8 nucleotides only (Chi et al., 2009). In this respect, the biochemical isolation of AGO proteins with RNAse digestion, combined with next-generation sequencing (NGS) techniques such as HITS-CLIP and PAR-CLIP, have been developed to analyze miRNA-mRNA pairs (Chi et al., 2009; Tan et al., 2013). Novel techniques that do not rely on crosslinking for isolating miRNAs and mRNAs, such as RNA immunoprecipitation combined with NGS (RIP-Seq), have also helped to increase our understanding of the miRNA targetome in neurons (Malmevik et al., 2015). Thereby, the RIP-seq technique brings the advantage that the AGO protein is in direct contact with miRNAs and mRNAs within the RISC complex, providing the opportunity to take a snapshot of the ongoing gene-regulatory processes in a cell to analyze biologically active miRNAs and their targets. However, when analyzing miRNA/mRNA targets with low- or high throughput molecular assays, it should be noted that although the binding of the miRNA to the mRNA actually takes place, it does not result in a change of the macroscopic phenotype, thus having no biological effect (Pinzón et al., 2017). In this context, genome editing tools have helped to probe and validate miRNA/mRNA interactions in the last years, that evoke a change in the phenotype (Bassett et al., 2014). Further experimental validations such as luciferase reporter assays are indispensable and vitally important to analyze *in silico* predicted miRNA/mRNA interactions (Ko et al., 2009; Jin et al., 2013). Luciferase reporter assays are used in order to analyze, if miRNAs bind to the 3′ UTR of their target genes (Ko et al., 2009). Ultimately, the impact of miRNA regulation must also be studied at the protein level using highly sensitive quantitative techniques. Commonly used miRNA target prediction programs rely on the molecular rules of RISC/target binding (Mockly and Seitz, 2019). Computational algorithms have shown to be the driving force of predicting miRNA targets (Bentwich, 2005; Rajewsky, 2006; Doran and Strauss, 2007; Mazière and Enright, 2007). It is based on the programming alignment to identify the 3′ UTR and the complementary miRNA seed sequence to predict miRNA-mRNA interaction. Still, evidence suggests that these predicted interactions do not necessarily have a functional role (Didiano and Hobert, 2006). For instance, there is a clear discrepancy between predicted and validated miR-182/96/182 targets (**Figure 2**). Although the predictions of the targetome and genetic networks regulated by individual miRNAs are becoming more and more reliable, the interaction of different miRNAs must be taken into account to draw meaningful conclusions about biological effects of miRNAs on mRNA and protein levels (Rojo Arias and Busskamp, 2019). Therefore, it is indispensable to also validate physiologically relevant targets of miRNAs experimentally (Kuhn et al., 2008).

CONCLUSION

miRNAs impact on retinal development and function, especially on the survival and maintenance of photoreceptors. Therefore, it is not surprising that their misregulation is linked to various retinal degenerative diseases, as well as developmental genetic disorders. Increasing our knowledge of miRNAs is of great importance: to date, however, studies on miRNA regulatory networks are rare due to the complexity of the experimental procedures for small RNAs. Furthermore, these experiments require large amount of tissue samples, are expensive, and are limited in their application. The manipulations of miRNA regulatory networks are not trivial: the timing of the manipulation plays a crucial role as well. In addition, the knockout of highly abundant miRNAs results in other miRNAs taking over the regulatory space, which impedes a proper interpretation of the results for the manipulated miRNAs. This is especially important given the high annotated number of mRNA targets for any given miRNA species, because most studies to date have only experimentally validated a handful of targets. Aligning phenotypic characterizations with system level analysis will further provide deep mechanistic insights in order to understand complex miRNA regulatory pathways. Overall, although technological advances over the coming years will facilitate new discoveries of how non-coding RNAs impact on cellular functions, studying miRNA functions remains challenging, especially in the context of retinal degenerative diseases. Still, non-coding RNAs are key to understand comprehensively retinal functions in health and disease.

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JP: writing and conceptualizing of the original draft. JP, MZ, GP, AS, and VB: writing, review, and editing. VB: funding. All authors contributed to the article and approved the submitted version.

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A Comparative Analysis of Reactive Müller Glia Gene Expression After Light Damage and microRNA-Depleted Müller Glia—Focus on microRNAs

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Müller glia (MG) are the predominant glia in the neural retina and become reactive after injury or in disease. microRNAs (miRNAs) are translational repressors that regulate a variety of processes during development and are required for MG function. However, no data is available about the MG miRNAs in reactive gliosis. Therefore, in this study, we aimed to profile miRNAs and mRNAs in reactive MG 7 days after light damage. Light damage was performed for 8 h at 10,000 lux; this leads to rapid neuronal loss and strong MG reactivity. miRNAs were profiled using the Nanostring platform, gene expression analysis was conducted via microarray. We compared the light damage dataset with the dataset of Dicer deleted MG in order to find similarities and differences. We found: (1) The vast majority of MG miRNAs declined in reactive MG 7 days after light damage. (2) Only four miRNAs increased after light damage, which included miR-124. (3) The top 10 genes found upregulated in reactive MG after light damage include *Gfap*, *Serpina3n*, *Ednrb* and *Cxcl10*. (4) The miRNA decrease in reactive MG 7 days after injury resembles the profile of Dicer-depleted MG after one month. (5) The comparison of both mRNA expression datasets (light damage and Dicer-cKO) showed 1,502 genes were expressed under both conditions, with *Maff*, *Egr2*, *Gadd45b*, and *Atf3* as top upregulated candidates. (6) The DIANA-TarBase v.8 miRNA:RNA interaction tool showed that three miRNAs were found to be present in all networks, i.e., after light damage, and in the combined data set; these were miR-125b-5p, let-7b and let-7c. Taken together, results show there is an overlap of gene regulatory events that occur in reactive MG after light damage (direct damage of neurons) and miRNA-depleted MG (Dicer-cKO), two very different paradigms. This suggests that MG miRNAs play an important role in a ubiquitous MG stress response and manipulating these miRNAs could be a first step to attenuate gliosis.

Keywords: retinal degeneration, gliosis, injury, miR-125, let-7, stress response, Maff, Gadd45b

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INTRODUCTION

Müller glia (MG) are the predominant glia in the neural retina and fulfill, similar to astrocytes in the brain, a variety of crucial functions to ensure retinal homeostasis and proper neuronal function. These functions include neurotransmitter uptake and recycling via specific enzymes such as glutamine synthetase (GS), nourishment of neurons by providing lactate, tissue stabilization and structural support by forming the inner and outer limiting membrane, maintenance of the blood retinal barrier (BRB), and tissue protection (Reichenbach et al., 1993; Newman and Reichenbach, 1996; Bringmann et al., 2000, 2006; Bringmann and Reichenbach, 2001; Kuhrt et al., 2008; Reichenbach and Bringmann, 2013). The MG response to retinal injury is known as gliosis. There are two kinds of MG gliosis, an unspecific and a specific response. The unspecific response is characterized by hypertrophy (swelling) and the upregulation of the intermediate filament GFAP, which is considered a marker of reactivity. The specific response to neuronal damage includes the downregulation of the enzyme glutamine synthetase in models of massive and rapid photoreceptor loss such as light damage, or retinitis pigmentosa (Bringmann et al., 2006). The gliotic response requires substantial changes in gene expression and it is currently not known whether miRNAs play a role in this process.

miRNAs are short (18–24 nucleotide long) non-coding RNAs and act as transcriptional repressors. They are transcribed as a primary molecule that is processed by the microprocessor complex to a precursor miRNA, which is cleaved by the endoribonuclease Dicer1 into the mature and functional form. Mature miRNAs then bind predominantly (but not exclusively) on the 3′ untranslated region (UTR) of a messenger RNA (mRNA) and (1) inhibit translation into protein (imperfect complementary binding) or (2) induce mRNA decay (perfect complementary binding). This occurs in the RNA-induced silencing complex (RISC) in which the miRNA is bound to a protein called Argonaute 2 (Ago2) (Gurtan and Sharp, 2013; Roberts, 2015). miRNAs play important roles during retinal development by regulating cell division, cell maturation and cell fate specification. Because of this regulatory impact, miRNAs play a detrimental role in cancer and other diseases including retinal diseases (Sundermeier and Palczewski, 2012; Zuzic et al., 2019), but we are just beginning to understand the critical miRNAs and their mRNA targets in the retina.

We previously profiled the microRNAs (miRNAs) expressed in MG and studied their impact on MG function by deleting the endoribonuclease Dicer1 (Dicer-cKO^{MG}), the enzyme that generates mature miRNAs. In the Dicer-cKO^{MG} retinas, we observed a progressive retinal disorganization and a loss in rod photoreceptors and retinal function (Wohl et al., 2017). Interestingly, the deletion of Dicer1 in MG did not cause an upregulation of GFAP, the hallmark of gliosis. Moreover, the loss of rod photoreceptors and retinal disorganization had some similarities to the phenotype observed in end-stage retinitis pigmentosa. Since this phenotype displayed similarities to diseases characterized by massive photoreceptor loss, although no neuron was primarily affected, we aimed to profile the MG miRNAs and mRNA expression after light damage. Light damage is a commonly used model to study retinal degeneration that resembles age-related macular degeneration (AMD) or retinitis pigmentosa (Winkler et al., 1999; Beatty et al., 2000; Wenzel et al., 2001, 2005; Chen et al., 2004; Samardzija et al., 2006; Grimm and Reme, 2013; Luu et al., 2020). The light damage model is a very flexible model and can be altered with regard to light intensity (2,000–100,000 lux) and duration (minutes–days). Nevertheless, it is a very robust and well-studied model (Burns and Robles, 1990; de Raad et al., 1996; Chen et al., 2004; Rattner and Nathans, 2005; Gosbell et al., 2006; Ueki et al., 2008; Natoli et al., 2010; Ueki and Reh, 2012).

To our knowledge, there are no reports available about the miRNA profile of reactive MG. Therefore, in this study, we aimed to identify the miRNAs that might regulate genes involved in gliosis by performing global comparisons of reactive MG 7 days after light damage and MG after Dicer deletion (loss of miRNAs). We light damaged adult mice and analyzed neuronal loss and glial response after 1 week, when the vast majority of photoreceptors were lost, and MG were reactive (gliosis). miRNAs from light damaged MG were profiled using Nanostring technologies. We found that the vast majority of miRNAs highly expressed in MG declined after light damage, similar to what we found in the Dicer-cKO^{MG}. We used microarray for gene expression analysis in order to identify the genes that are upregulated the most 1 week after light damage and could represent potential miRNA targets. Most genes were associated with cell death and inflammatory response. Since the miRNA was similar, we compared that dataset with that from Dicer-cKO^{MG}. Thousand five hundred and two genes were expressed in both datasets, with *Atf3*, *Egr2*, *Maff,* and *Gadd45b* as top candidates, genes involved in stress response. The identified miRNAs, potentially targeting these genes, are miR-125b-5p, let-7c, and let-7d. This data suggests that independent from extrinsic influences, a common intrinsic glial stress program appears to be activated that is directed by MG miRNAs. This is particularly of importance for understanding and attenuating gliosis. MG miRNAs are therefore potential promising tools for counteracting glial alterations and this study is the first attempt to narrow down the list of candidates for subsequent downstream experiments.

MATERIALS AND METHODS

Animals and Cre Induction

All mice were housed at the State University of New York, College of Optometry and used in accordance with the Institutional Animal Care and Use Committee approved protocols (IACUC). *Rlbp1-creERT2* mice (obtained from Dr. Edward Levine, S129 background) were crossed to *R26-stop-flox-CAG-tdTomato* mice (Jackson Labs, also known as Ai14, #007908) and will be henceforth referred to as *RlbpCreER: stop$^{f/f}$-tdTomato* or wild type (wt). For light damage mice, Rlbp1-creERT:tdTomato mice were crossed to the albino Swiss Webster mouse (CSW 024, Charles River Laboratories) that carries the RPE65^{450Leu} gene (confirmed by genotyping). In addition, the Hes5-GFP mouse (Basak and Taylor, 2007) was used as another MG-specific reporter mouse established in the lab (Nelson et al., 2011). The

Hes5 mouse (S129 background) was also crossed to the Swiss Webser mouse. Genotyping was done using the primers listed in **Supplementary Table 1**. For the detection of RPE65 variants, a subsequent digest of the PCR product with the restriction enzyme MwoI was performed for 2 h at 37°C. Tamoxifen (Sigma, St. Louis, MO) was administered intraperitoneally at 75 mg/kg in corn oil for four consecutive days in adult mice (2–3 months of age) to initiate the recombination of the floxed alleles.

Light Damage

Mice were exposed to diffuse, cool, white light (bulbs are located on top of the cage). Food and water were placed in the cage to avoid blocking light exposure. Luminance (∼10,000 lux) was measured on the cage floor using a light meter. Mice were exposed to the light for 8 h and returned under normal lighting (12 h on/12 h off cyclic light) for recovery. Analysis was performed 7 days after light damage (LD).

Fluorescence Activated Cell Sorting (FACS)

All retinas were checked for successful recombination under the fluorescence microscope before every sort. For each sort, about 6–10 retinas were pooled and dissociated in DNase/Papain (75 μl/ 750 μl, respectively, Worthington) for 20 min at 37°C on the shaker, triturated, mixed with Ovomucoid (750 μl), centrifuged for 10 min at 300 × g and resuspended in 800 μl DNase/ Ovomucoid/ Neurobasal solution (1: 1: 10, respectively, Gibco) per retina. Cells were filtered through a 35 μm filter, sorted using an 85 micron nozzle, and collected into two chilled tubes. Cell sorts were performed using BD Aria III cell sorter (BD Bioscience). Debris was excluded from the sort and only all events in gate P1 were sorted (**Supplementary Figure 1A**). Cells with the brightest fluorescence were found in gate P3 ("positives," MG fraction), cells with no fluorescence in gate P2 ("negatives," neuronal fraction, **Supplementary Figure 1A′**), everything in between was excluded. Gating settings were kept throughout all sorts for undamaged and damaged retinas. The fraction of MG comprised about 1.7% **Supplementary Figures 1A″, *A‴***). Samples were collected in FBS-coated tubes containing Neurobasal medium. After collection, the tdTomato$^+$ MG fraction (P3) and the tdTomato$^-$ fraction (P2) was post-sorted to validate purity (**Supplementary Figures 1B–B″,C–C″**). Cells were spun for 10 min at 300 × g at 4°C, the pellet was homogenized in Qiazol (Qiagen) and stored at −80°C.

RNA Purification, miRNA, and mRNA Profiling

For miRNA profiling, the sorts of 44 light damaged retinas were pooled for RNA purification. RNA was extracted and purified with a miRNeasy Micro Kit in accordance with manufacturer's instructions (Qiagen). NanoString nCounter was used for miRNA expression analysis. Two hundred ng total RNA per sample (33 ng/μl) was submitted for NanoString analysis. NanoString data was analyzed using nSolver 4.0 software. The data represents counts of molecules normalized against 4 housekeeping genes (*β-actin*, *GAPDH*, *Rpl19*, and *B2m*), 8 negative controls, and 6 positive controls that were run with the samples. miRNA data after Dicer-cKO and for wild type MG was published before (Wohl et al., 2017), is available at GEO (GSE 103098). Raw data was *de-novo* normalized and analyzed together with the light damaged data. For Microarray, 12 retinas were used for controls and 10 retinas for light damage, FACS-purified, the RNA isolated and run on the Mouse Gene 1.0 ST microarray (Affymetrix) according to manufacturer's guidelines. The RIN numbers for the samples ranged from 8 to 10 with a mean of 8.98. The microarray data was normalized and analyzed with Affymetrix Power Tools software and TM4 Multi-Experiment Viewer software. RNA-Seq data of pigmented adult wild type MG was published before (Wohl et al., 2017), is available at SRA (NBCI, SRP115835) and was used for gene expression comparisons. Also, the datasets of control FACS purified MG and MG 36h after light damage, as well as 48h after NMDA damage from Hoang et al. (2020) were used for gene expression comparisons.

Fixation, Sectioning, and Immunofluorescent Labeling

Mouse eyes were fixed in 4% PFA for 30–60 min, treated with 30% sucrose in PBS overnight, embedded in O.C.T. embedding medium, and cross sectioned in 12 μm thick sections. For immunofluorescent staining, cells were incubated in blocking solution (5% milk block: 2.5 g non-fat milk powder in 50 mL PBS; with 0.5% Triton-X100) for 1 h at RT. Sections were incubated with primary antibodies (**Supplementary Table 2**) in 5% milk block overnight, secondary antibodies (Invitrogen/Molecular Probes, and Jackson ImmunoResearch, 1:500–1,000) for 1 h at RT and counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, 1:1,000).

Microscopy, Cell Counts, and Statistical Analysis

Live imaging was performed using Zeiss Observer D1 with AxioCam. Fixed cells were analyzed by Olympus FV1000 or Zeiss LSM 880 confocal microscope as well as Keyence BZX 800 for overview images. For retinal cross sections, two areas/section with 625 μm × 625 μm dimension at 200× magnification, four optical sections of 2 μm thickness, for two sections per mouse, of at least six light damaged and undamaged controls were counted. For Otx2 cell assessment in the ONL, vertical rows of cells were counted for six different areas per image. Values are expressed as mean ± standard deviation (S.D.). Statistical analyses were performed by Mann-Whitney (U) test for independent samples and the Wilcoxon test for dependent samples. Holm-Bonferroni method was used to correct for multiple comparisons.

miRNA-Target Interaction Analysis, Ago HITS-CLIP, Gene Ontology

For miRNA-mRNA interaction analysis, DIANA-TarBase v.8 (https://carolina.imis.athena-innovation.gr/diana_tools) was used, a database of experimentally supported miRNA:mRNA interactions (Karagkouni et al., 2018). It integrates information on cell-type specific miRNA–gene regulation, while thousands of miRNA-binding locations are reported. It is the first database

indexing more than 1 million entries, corresponding to ∼670 000 unique miRNA-target pairs. The interactions are supported by >33 experimental methodologies, applied to ∼600 cell types/tissues under ∼451 experimental conditions (Karagkouni et al., 2018). All gene names were inserted and miRNA candidates that were found in MG, selected and depicted. For miRNApathway analysis DIANA-mirPath v.3 was used. DIANA-mirPath can utilize predicted miRNA targets (in CDS or 3′-UTR regions) provided by the DIANA-microT-CDS algorithm or even experimentally validated miRNA interactions derived from DIANA-TarBase (Vlachos et al., 2015). STarMirDB (http://sfold.wadsworth.org/starmirDB.php) was used to find and visualize the individual miRNA binding sites in mRNA target 3′ UTRs (Rennie et al., 2016). The database allows a fast search of pre-computed results that were enriched for miRNA binding sites identified from CLIP data. The transcriptome-scale predictions results are categorized into seed and seedless sites in 3′ UTR, CDS and 5′UTR, and provide a list of sequences, thermodynamic and target structural features. A logistic probability model was used as a measure of confidence of the site being a miRNA binding site (threshold 0.5–0.7).

Ago HITS-CLIP [Argonoute2 **h**igh-**t**hroughput **s**equencing of RNAs isolated by **c**ross**l**inking **i**mmuno**p**recipitation developed in the Darnell lab (Chi et al., 2009)] data for whole undamaged and light damaged retinas; generated in the Natoli lab (available at BioRxiv), was used to evaluate whether MG miRNAs and mRNAs are Ago2 bound (Chu-Tan et al., 2020).

For mRNA gene ontology, ShinyGO v0.61 (http://bioinformatics.sdstate.edu/go/) was used. Genes were inserted, GO Biological Process selected, *P*-value cutoff (FDR, false discovery rate) of 0.05 chosen and the 20 most significant terms selected to be depicted.

RESULTS

Light Damage Leads to Neuronal Loss and Müller Glia Activation

In order to isolate reactive Müller glia (MG) from light damaged retinas, we crossed the MG reporter mouse with a mouse that has an amino acid variation (leucine instead of methionine) at position 450 in the retinal pigment epithelial protein RPE65, referred as RPE65^{450Leu} mouse (**Figure 1A**). This variant found in all mouse strains except C57BL/6 (which are commonly used as background mice) has been discovered as a genetic modifier of susceptibility to light-induced damage mice (Danciger et al., 2000; Wenzel et al., 2001, 2005). We aimed to profile reactive adult MG in the acute phase after damage and therefore chose 7 days after damage for analysis. Photoreceptor death begins as early as 24 h (37% of photoreceptors are lost, **Supplementary Figures 2A–E**). After 7 days, about 90% of photoreceptors (almost entire outer nuclear layer, ONL) were lost in the central retina (**Figures 1B–E**), while cell death was attenuated in the peripheral retina (**Figures 1F–H**).

This gradient of neuronal loss from center to periphery was reflected in the MG response. While GFAP protein was strongly upregulated in MG in the central retina (**Figures 1I,J/J′-K/K′**), MG in the periphery remained GFAP$^-$ (**Figures 1I,L/L′-M/M′**). As we were interested in profiling reactive MG in acute phase of injury, we chose 7 days post injury to have at least 50% reactive MG in the tissue (**Figure 1I**). GFAP upregulation in the central retina can be detected as early as 1 day after injury and lasts for several weeks (**Figures 1N/N′-S/S′**).

Since there was this substantial neuronal loss resulting in significant retinal thinning in the center retina, we next evaluated aspects of unspecific and specific glial responses due to this neuronal loss. We determined the number of MG cells and their location in the retinal tissue to assess potential proliferation and migration processes. In the center of undamaged control retinas, we found about 120 tdTomato+ MG per field and all were located in the center of the INL, expressing the nuclear marker Sox9 and cytoplasmic enzyme glutamine synthetase (GS, **Figures 2A–C/C′,E,F**, **Supplementary Figures 3A/A′ -E/E′**). Seven days after LD we found ∼130 tdTomato+ MG expressing Sox9 and GS in the lower INL and in the remaining OPL (1–2 cell layers, **Figures 2D/D′–E,F**, **Supplementary Figures 3F/F′-J/J′**) indicating that MG migrated toward the injury side. GS expression in the migrating MG was rather diffuse and could indicate a reduction of GS levels, which has been reported to be a specific MG response in models with massive photoreceptor loss (Bringmann et al., 2009). However, most glial genes were not found to be differently expressed in FACS-purified MG 7 days after light damage, except the intermediate filament GFAP (**Figure 2G**, **Supplementary Table 3**). MG comprise only 2–3% of the total retinal cell population (Jeon et al., 1998). Consequently, multiple retinas (biological replicates) need to be pooled for sufficient amounts of RNA from FACS-purified MG. This results in low technical replicate number. To test the robustness of the microarray dataset (6 biological replicates, one technical replicate), we compared the gene expression levels of known glial genes from our microarray dataset (log_2 of relative expression, fold change 7d light damage vs. control) with RNA-Seq data (fold change 36 h light damage vs. control) by performing a linear regression analysis. This 36 h light damage data set is from a recent global gene expression study [4 technical replicates of FACS-purified MG from pigmented Glast-Cre-GFP reporter mice (Hoang et al., 2020), **Supplementary Figure 4A**]. This analysis resulted in a high coefficient of determination (R^2) indicating a strong positive correlation of the gene expression patterns of these different methods and different mouse strains (**Supplementary Figure 4B**). A high correlation of differentially expressed genes of microarrays and RNA-Seq has been reported before (Rao et al., 2018) and was also demonstrated in a white paper from Illumina (Illumina_Inc., 2011). We next analyzed the rigor and robustness of our pooled MG samples and compared our RNA-Seq dataset of wild type RlbpCreERT:tdTomato mice (log_2 counts per million of adult pigmented wild type mice, 20 biological replicates, one technical replicate, **Supplementary Figure 4C**) with the Hoang et al. dataset. Hoang et al., generated two replicates of LD controls and for NMDA controls, respectively, which, since not significantly

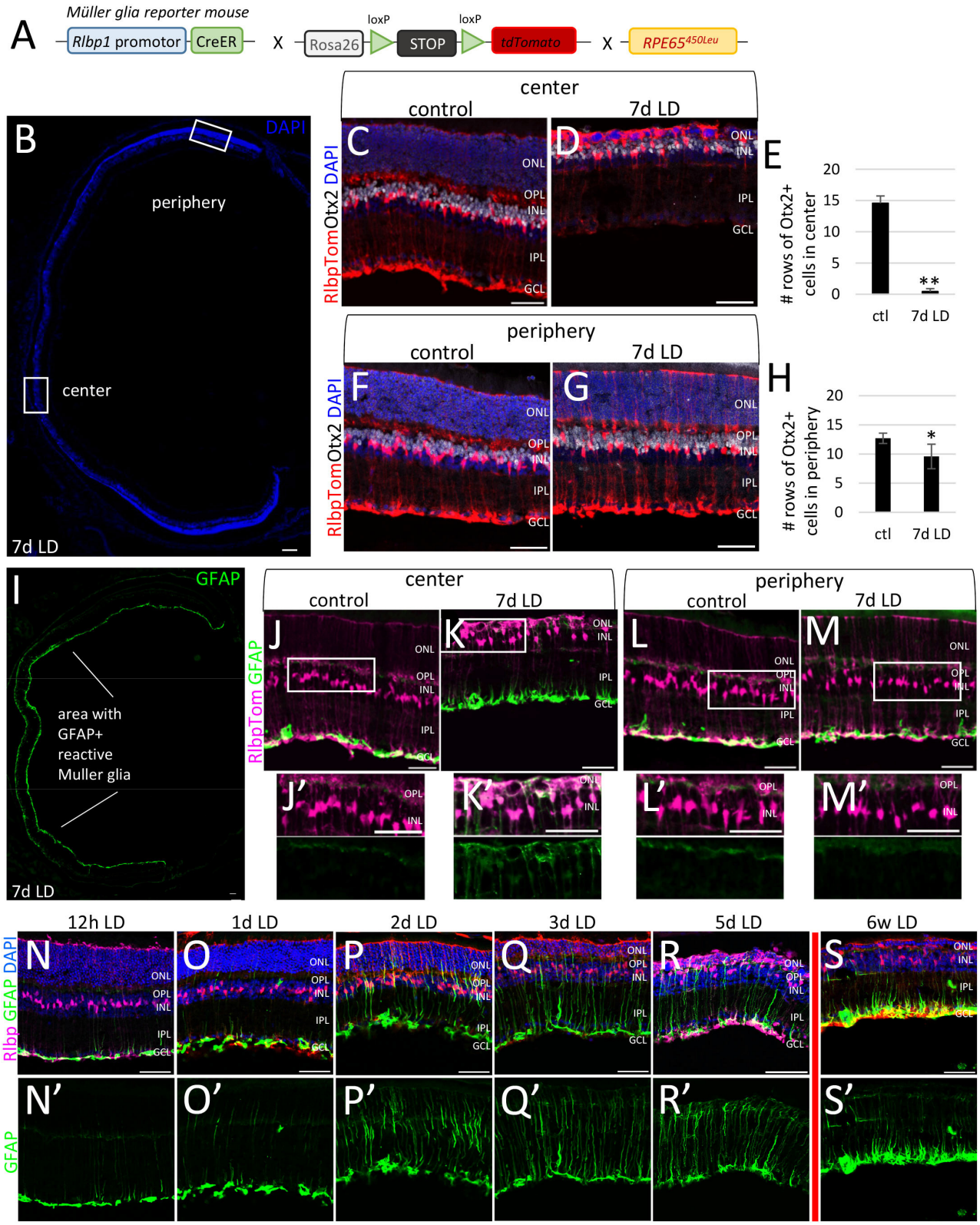

FIGURE 1 | Neuronal loss and gliosis in the central and peripheral retina after light damage. **(A)** Schematic of the *RlbpCreER: stop*$^{f/f}$*-tdTomato: RPE65*^{450Leu} mouse. **(B)** Cross section of DAPI-labeled retina 7 days after light damage with regions of analysis. **(C–G)** Immunofluorescence labeling for tdTomato (MG), Otx2, and DAPI nuclear staining of center **(C,D)** and peripheral areas **(F,G)** of undamaged and light damaged (7d LD) retinal sections. **(E)** Number of vertical cell rows of Otx2+

(Continued)

FIGURE 1 | photoreceptors in the ONL of the central retina in controls and 7 days after light damage (7d LD). **(H)** Number of vertical cell rows of Otx2+ photoreceptors in the ONL of the peripheral retina in controls and 7 days after light damage (7d LD). **(I–M/M′)** Immunofluorescence labeling for tdTomato (MG) and GFAP of an entire retinal cross section **(I)**, in the central **(J/J′-K/K′)** and peripheral areas **(L/L′-M/M′)** of undamaged and light damaged (7d LD) retinal sections. **(N/N′-S/S′)** Immunofluorescence labeling for tdTomato (MG), GFAP, and DAPI nuclear staining of the center retina 12 h, 1, 2, 3, and 5 days as well as 6 weeks after light damage (LD). Scale bar in B, I 100 μm, in C-S 50 μm. Significant differences are indicated: $*p < 0.05$, $**p < 0.01$, *U*-test, n control = 5, n LD = 7. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; LD, light damage.

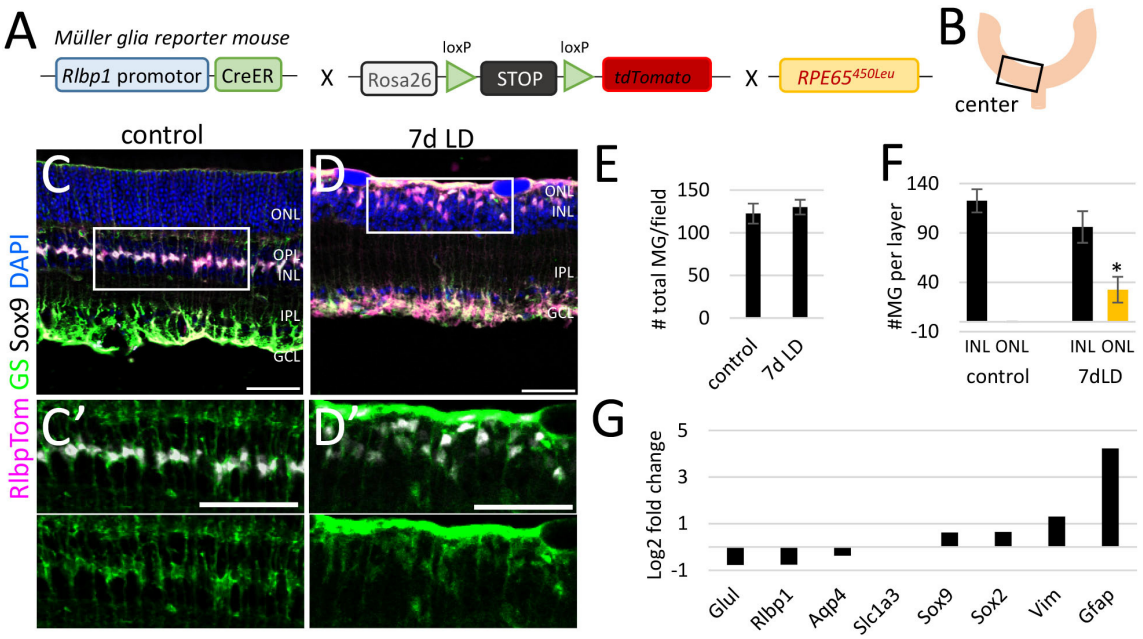

FIGURE 2 | Müller glia number and location in the central retina after light damage. **(A)** Schematic of the *RlbpCreER: stop*$^{f/f}$*-tdTomato: RPE65*^{450Leu} mouse. **(B)** Region of analysis. **(C/C′,D/D′)** Immunofluorescence labeling for tdTomato (MG), glutamine synthetase (GS), Sox9, and DAPI nuclear staining of retinal sections from undamaged controls and 7 days after light damage. **(E)** Number of MG per field in undamaged mice and 7 days after light damage (LD). **(F)** Number of MG in the INL and ONL in undamaged controls and 7 days after LD. **(G)** Expression levels (log_2 fold change light damage vs. control) of glial genes in undamaged (6 biological replicates, one technical replicate) and damaged retinas (five biological replicates, one technical replicate). Scale bars 50 μm. Significant differences are indicated: $*p < 0.05$, Wilcoxon-test, n control = 5, n LD = 5. ONL: outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; LD, light damage.

different, were averaged resulting in 4 technical replicates. Linear regression analysis showed a strong correlation ($R^2 = 0.99$) of both datasets (**Supplementary Figure 4D**).

Most Müller Glia miRNAs Decline After Light Damage

miRNA have been profiled for whole normal retina and after light damage (Karali et al., 2007, 2011; Hackler et al., 2010; Chu-Tan et al., 2020), but not in MG after light damage. In order to profile the miRNAs of reactive MG, we used the MG reporter mice, FACS-purified the MG from undamaged and damaged retinas (7 days after light damage) and performed Nanostring miRNA profiling. Six hundred miRNAs were quantified by solution hybridization using a NanoString nCounter assay. An advantage of this approach is that the NanoString assay does not require amplification, which might introduce bias and requires relatively small amounts of RNA (200 ng), which allows the analysis of small cell populations (Geiss et al., 2008). We focused on the miRNAs that previously were identified to be highly expressed in MG mGliomiRs (<20% expression in neurons) and shared miRs [similar expression levels in neurons (Wohl and Reh, 2016), **Figure 3A**]. We found that the vast majority of MG miRNAs declined after light damage (**Figure 3B**, **Supplementary Table 4**). This result resembled the miRNA changes we previously observed 1 month after Dicer deletion (Wohl et al., 2017). We compared the expression levels to those we obtained after Dicer deletion and found 11 miRNAs similarly reduced, including miR-204, miR-125-5p, and three let-7 family members. Interestingly, miR-16 and miR-1944 expression levels in reactive MG after light damage were lower than in the Dicer-CKO MG (**Figure 3C**). We also plotted the miRNAs that were less reduced after light damage. These miRNAs included miR-9, miR-181a, and three other let-7 family members (**Figure 3D**). let-7g, miR-135a, and miR-22 displayed only a reduction of about 10% compared to undamaged controls.

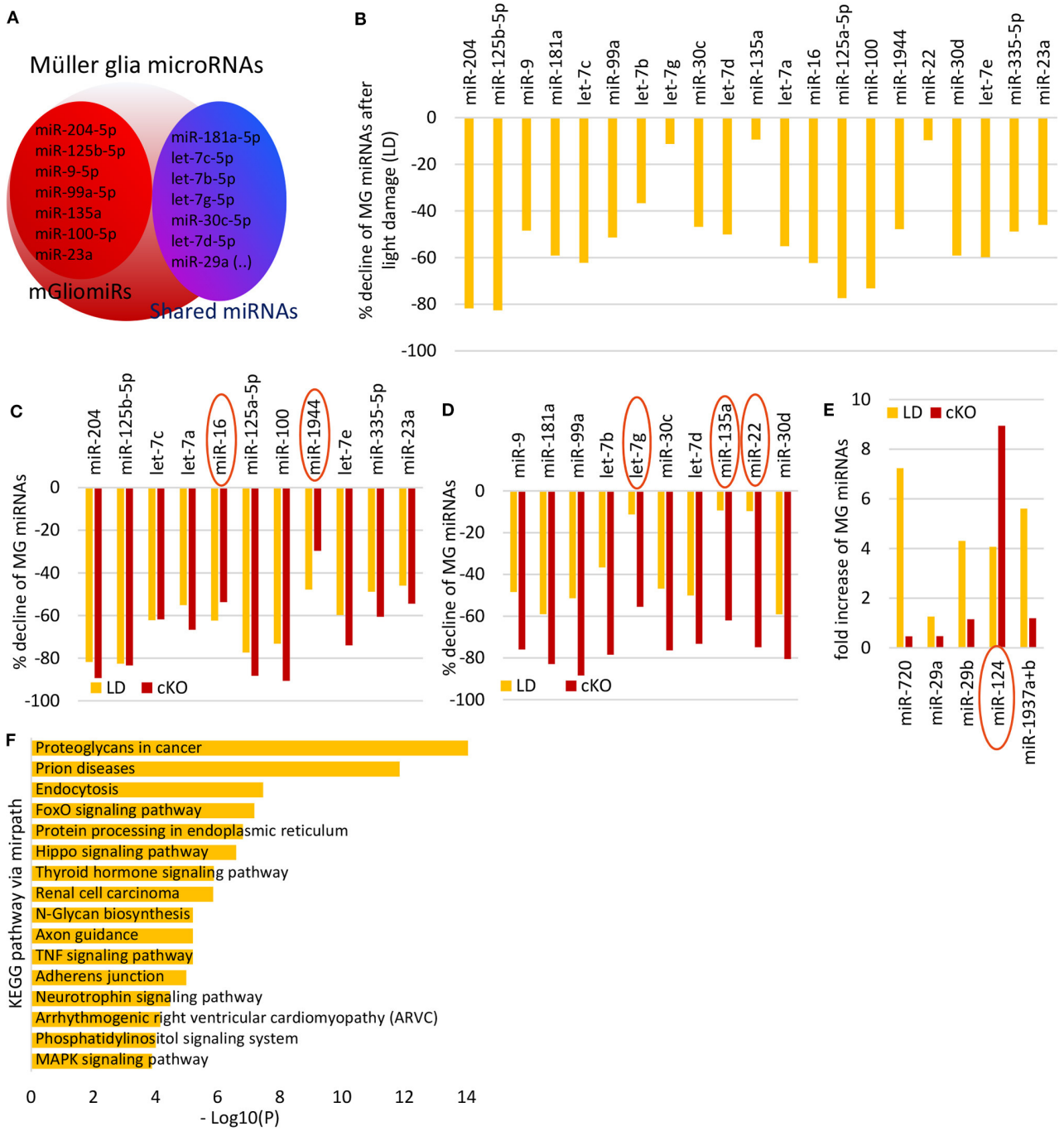

FIGURE 3 | Müller glia miRNA expression after light damage. **(A)** Schematic of miRNA highly expressed in MG i.e., mGliomiRs and shared miRs (modified after Wohl and Reh, 2016). **(B)** Percent decline of the top 21 highly expressed MG miRNAs after 7 days light damage compared to normal MG (cutoff 3,000 counts). **(C)** miRNAs with similar declined levels after light damage as after Dicer deletion (in percent). **(D)** miRNAs with less declined levels after light damage as after Dicer deletion (in percent). **(E)** Fold increase of 5 miRNAs that increased after 7 days light damage (cutoff 5,000 counts) compared to controls. **(F)** DIANA-mirPath v.3 analysis of the 21 MG miRNAs that declined after light damage.

Interestingly, from all miRNAs analyzed, only five increased after light damage, i.e., miR-720, miR-29a and b, miR-124-3p, and miR-1937a+b (**Figure 3E**, **Supplementary Table 5**). We next used DIANA-mirPath v.3 to see which pathways involve these MG miRNAs. Using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, we found that most of the MG miRNAs regulate proteoglycans in cancer, prion diseases, FoxO signaling pathway etc. (**Figure 3F**). Proteoglycans are found in

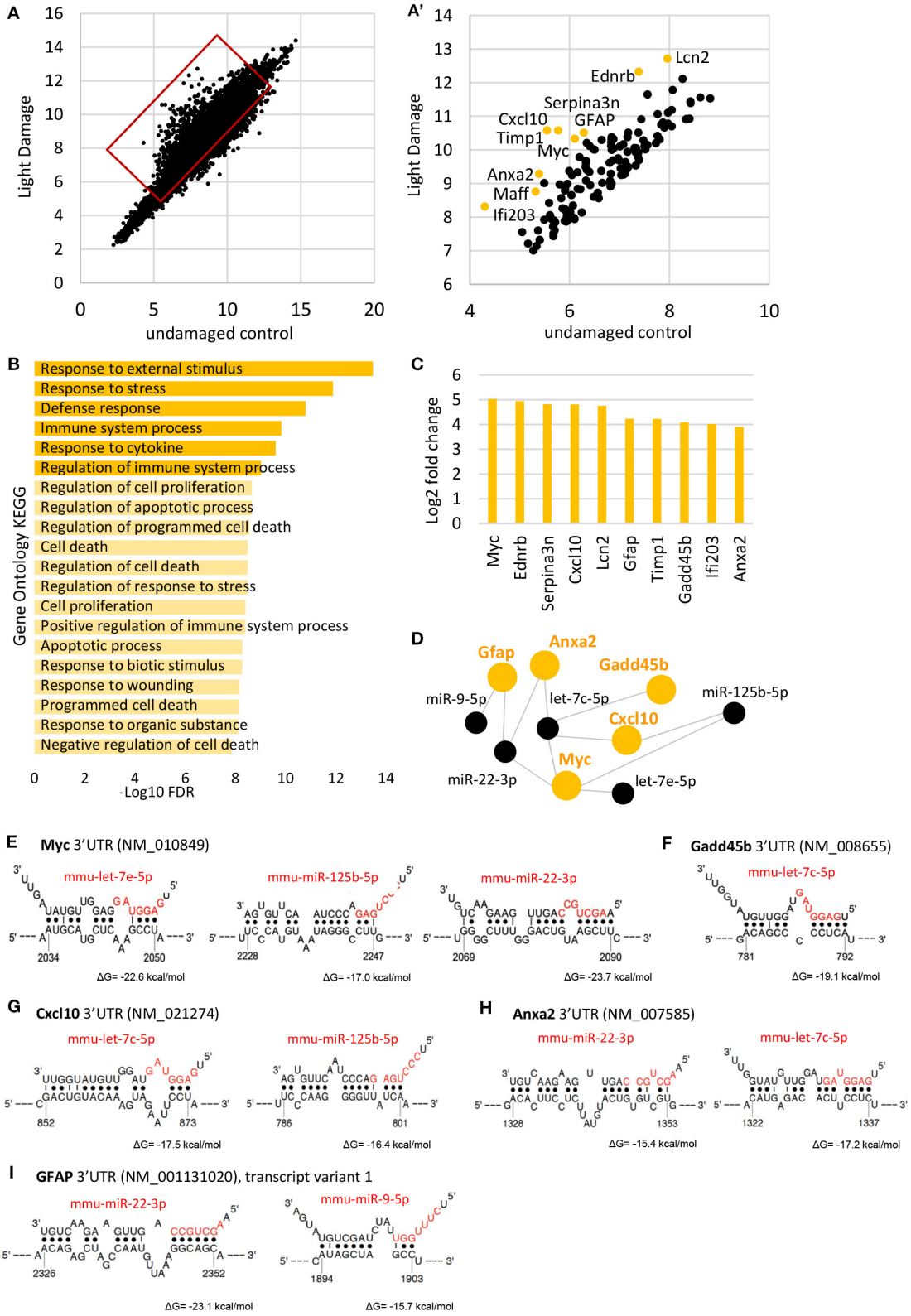

FIGURE 4 | Müller glia gene expression after light damage. **(A)** Scatterplot of genes expressed in undamaged controls and 7 days after light damage (LD) from microarray (log_2 of relative expression). **(A')** Scatter plot of the top 100 highly expressed genes after light damage. **(B)** Gene ontology of the top 100 genes upregulated after light damage using ShinyGO v0.61. **(C)** Expression levels of the top 10 genes upregulated after light damage compared to undamaged controls (five

(Continued)

FIGURE 4 | and six biological replicates, respectively, one technical replicate per condition). **(D)** DIANA-TarBase v.8 analysis of the top genes upregulated after light damage and MG miRNAs that declined and are likely to target these genes based on prediction and experimental confirmation. **(E–G)** Binding sites of identified miRNAs from D (DIANA-TarBase) in the 3′UTRs of *Myc* **(E)**, *Gadd45b* **(F)**, *Cxcl10* **(G)**, *Anxa2* **(H)**, and *Gfap* **(I)** using STarMirDB. The seed sequences of the particular miRNA are shown in red. Dots represent complimentary base pairing. ΔG indicates the structural accessibility at the target site, low values (e.g., −17 kcal/mol) indicate high accessibility.

the extracellular matrix (ECM) and plasma membrane of cells and are probably relevant for cell migration. Prions are proteins that trigger abnormal protein folding in the brain leading to neurological disorders. FoxO are a subgroup of forkhead family transcription factors and are involved in cell processes including cell death, DNA repair, cell cycle arrest (Carter and Brunet, 2007).

miRNAs are predominantly found in the cytoplasm of a cell where they act as repressors, being bound to Ago2. However, they can also be found in multivesicular bodies or cell organelles including the nucleus (Leung, 2015). This means a miRNA identified in a cell does not necessarily regulate gene expression. We therefore used Ago HITS-CLIP data of total retina from a recently published study from the Natoli lab (Chu-Tan et al., 2020), to assess if any of the MG miRNAs are bound to Ago2 (**Supplementary Figure 5A**). Although this dataset is from whole retina and not cell type specific, our previous results comparing MG miRNA expression with retinal neuronal miRNA expression allows us to tentatively assign the HITS-CLIP results for some of the MG-specific miRNAs (mGliomiRs). All MG miRNAs including the mGliomiRs (e.g., miR-204-5p, miR-125b-5p, and miR-9 etc.) and shared miRs (e.g., miR-181a, let-7 family etc.) were bound to Ago2, except miR-1944 (**Supplementary Figure 5B**). For the upregulated candidates after light damage, we found miR-29a-5p, miR-29b-3p, and miR-124-3p bound to Ago2. Since the data is whole retina, we also plotted the photoreceptor specific miRNAs (miR183/182/96 cluster) as reference, since the vast majority of cells in the whole retina are photoreceptors (**Supplementary Figure 5B**, **Supplementary Table 6**).

Taken together, we found that almost all highly expressed MG miRNAs declined 7 days after light damage, a profile that resembled the Dicer-cKO MG 1 month after deletion. We next analyzed the gene expression patterns of reactive MG after light damage and compared them to that found in Dicer-depleted MG.

Gene Expression Analysis of Reactive Müller Glia After Light Damage and Potential miRNA Regulators

For gene expression analysis, we used microarray analysis and plotted the data ($\log_2$ of relative expression) from light damaged retinas against undamaged controls (**Figure 4A**). We focused on the top 100 genes upregulated after light damage and carried out gene ontology analysis using ShinyGO (http://bioinformatics.sdstate.edu/go/, **Figure 4A′**). We found that these genes are involved in processes of stress and defense, cell death/apoptosis, and cell proliferation (**Figure 4B**). We identified the top 10 highly upregulated genes in the MG 7 days after light damage (at least 3.9-fold increase compared to normal MG expression) which were: *Myc*, (Myelocytomatosis oncogene, alternative symbol c-myc), *Ednrb* (Endothelin receptor type B) *Serpina3n* (Serine (or cysteine) peptidase inhibitor, clade A, member 3N), *Cxcl10* (Chemokine ligand 10), *Lcn2* (Lipocalin 2), *Gfap*, *Timp1* (*Tissue inhibitor of metalloproteinases), Gadd45b* (Growth arrest and DNA damage inducible beta), *Ifi203* (interferon activated gene 203), and *Anxa2* (Annexin 2) (**Figure 4C**, **Supplementary Table 7**). From these genes, *Gfap* has been reported in a variety of studies and is known as a gene for reactive glia. However, less is known about the other genes. So far, *Cxcl10*, *Myc*, *Timp1*, *Serpina3n*, and *Ednrb* have been reported to be upregulated in retinas after light damage (Rattner and Nathans, 2005; Rutar et al., 2015; Mansouri et al., 2020) or other injury/diseases models such as retinal detachment (Rattner and Nathans, 2005), glaucoma (Naskar and Thanos, 2006), or retinal ischemia-reperfusion (Abcouwer et al., 2013). *Lcn2* encoding for lipocalin 2 [also known as NGAL or oncogene 24-3 (Abcouwer et al., 2013)], which was also found to be upregulated in reactive astrocytes (Zamanian et al., 2012) and was reported for whole retina after light damage (Chen et al., 2004); Most of these studies analyzed whole retina, and a MG-specific expression was only confirmed for *Cxcl10* (Rutar et al., 2015), *Ednrb* and *Serpina3n* (Rattner and Nathans, 2005) after light damage. Annexin 2 was shown to be expressed in normal MG (Grosche et al., 2016), but not for reactive glia. We used again the dataset provided by Hoang et al., in order to see if these genes were differentially expressed 36 h after light damage (Hoang et al., 2020). We found all genes increased 36 h post light damage (fold change compared to controls, **Supplementary Figure 4E**). Please note that the time point of analysis was very early after injury (36 h vs. 7days) and the injury paradigms were different from ours (3,000 lux, 4 h duration vs. 10,000 lux, 8 h duration).

Next, we used DIANA-TarBase to identify the MG miRNAs that might regulate these genes (Karagkouni et al., 2018). DIANA-TarBase is a reference database devoted to the indexing of experimentally supported microRNA (miRNA) targets. It integrates information on cell-type specific miRNA–gene regulation, while thousands of miRNA-binding locations are reported. We analyzed the 21 MG miRNAs that decreased, and the top 10 genes upregulated after light damage. Using this tool, we found 5 of the 10 genes (*Gfap, Anxa2, Cxcl10, Myc,* and *Gadd45b*) are regulated by the miRNAs miR-9-5p, miR-125b-5p, let-7c-5p, and let-7e-5p **Figure 4D**). In order to identify the binding sites of the miRNAs in the mRNA molecule STarMirDB was used, a database that provides information (sequence, thermodynamic and target structural features) of seed and seedless sites in 3′UTR, CDS and 5′UTR, identified from CLIP (cross-linking immunoprecipitation) data.

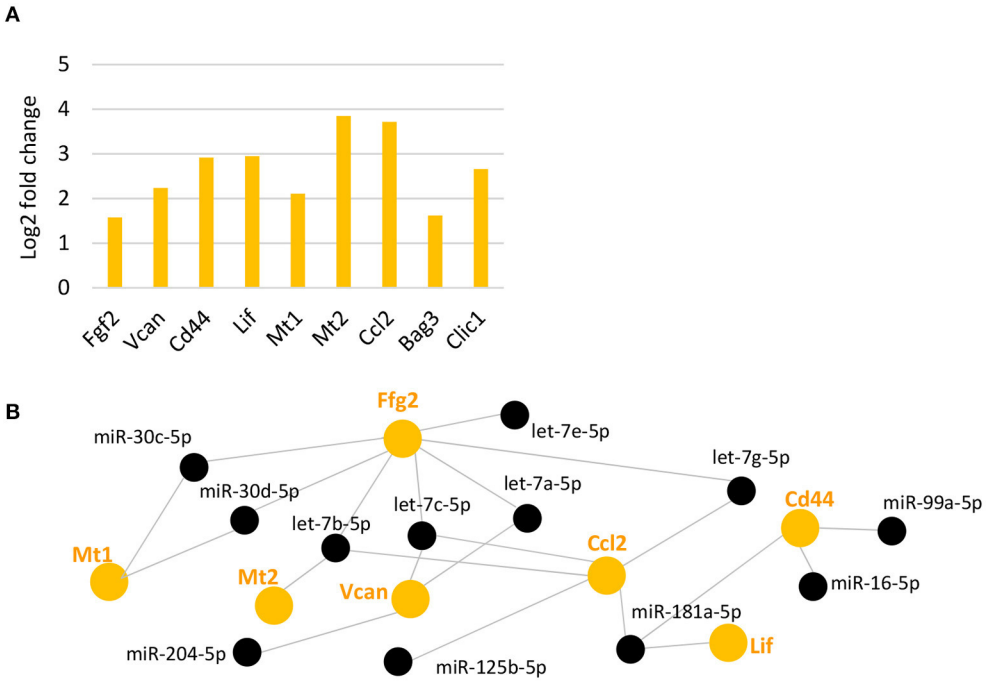

FIGURE 5 | Upregulated reported genes after light damage and their potential miRNA regulators. **(A)** Expression levels (fold change of $\log_2$ relative expression) of reported genes upregulated after light damage compared to undamaged controls (five and six biological replicates, respectively, one technical replicate per condition). **(B)**: DIANA-TarBase analysis of genes in A and MG miRNAs that declined and are likely to target these genes based on prediction and experimental confirmation. FDR, false discovery rate.

We found binding sites in the 3′UTR of the particular mRNA molecule for all DIANA-TarBase predicted miRNAs, with high structural accessibility at the target site ($\Delta G \sim -17$ kcal/mol, **Figures 4E–I**).

The myc-let-7 interactions has been reported for different tissue and species and indicates a conserved mechanism (Chang et al., 2009; Kim et al., 2009; Ramachandran et al., 2010; Buechner et al., 2011; Leppert et al., 2011; Wong et al., 2011; Gunzburg et al., 2015; Maldotti et al., 2016; Balzeau et al., 2017). Interestingly, we did not find any of these mRNAs bound in the whole retina Ago HITS-CLIP data set, except *Gfap*. This could imply an indirect regulation via other genes. It is also possible that these genes were not detectable due to low MG input (since MG comprise only 2% of the retina). It was shown before that strong signals in MG-enriched samples might not be detected in whole retina samples (Diaz Quiroz et al., 2014). However, *Gfap* together with other glial markers such as *Glul* (glutamine synthetase), *Scl1a3* (glutamate aspartate transporter GLAST), *Sox2*, *Sox9*, *Vim* (Vimentin), and *Aqp4* (Aquaporin4) were bound to Ago2 in RISC, suggesting that the transcripts of these genes are regulated by miRNAs in retinal cells (**Supplementary Figure 5C**).

We next analyzed genes that have been reported in previous studies to be upregulated after light damage in MG (Grosche et al., 1995; Hartig et al., 1995; Ueki et al., 2008; Bringmann et al., 2009) or whole retina (Chen et al., 2004) (see complete list in **Supplementary Table 8**). From this list of genes, we found 10 genes with an at least 3.9-fold increase in gene expression 7 days after light damage (**Figure 5A**). These genes included: *Fgf2,* encoding for the fibroblast growth factor 2, which is known to be expressed in MG after damage (Guillonneau et al., 1998); *Vcan* and *Cd44,* encoding for the extracellular matrix protein versican and the cell surface receptor CD44 (CD44 antigen) respectively [see review Bringmann et al., 2009)]; *Lif*, encoding for leukemia inhibitory factor that actives the STAT (signal transducer and activator of transcription) pathway (Ueki et al., 2008); *Mt1* and *Mt2* encoding for the proteins metallothionein 1 and 2, found in whole retina after light damage (Chen et al., 2004); *Ccl2* encoding the chemokine (C-C motif) ligand 2 (Abcouwer et al., 2013; Chu-Tan et al., 2018; Mansouri et al., 2020); *Bag3*, encoding for BCL2-associated athanogene 3, and *Clic1,* encoding for the chloride channel protein chloride intracellular channel 1. Both, *Bag3* and *Clic1* were reported for light damaged retinas (Chen et al., 2004). Although the light damage paradigms and/or time points of analysis from the other studies were different to ours, we found the same genes upregulated. We next used DIANA-TarBase to see if MG miRNAs regulate these genes. We found that *Fgf2, Vcan, Cd44, Lif*, *Mt1,* and *Mt2*, as well as *Ccl2* are regulated by MG miRNAs and predominantly by members of the let-7 family (**Figure 5B**). Interestingly, *Mt2, Lcn2, Ccl2, Bag3*, and *Clic1* were also found to be bound to Ago2 in RISC (**Supplementary Figure 5D**).

Taken together, light damage causes a decline in the vast majority of MG miRNAs, which might in turn be responsible for some of the increases in specific genes that occur in MG

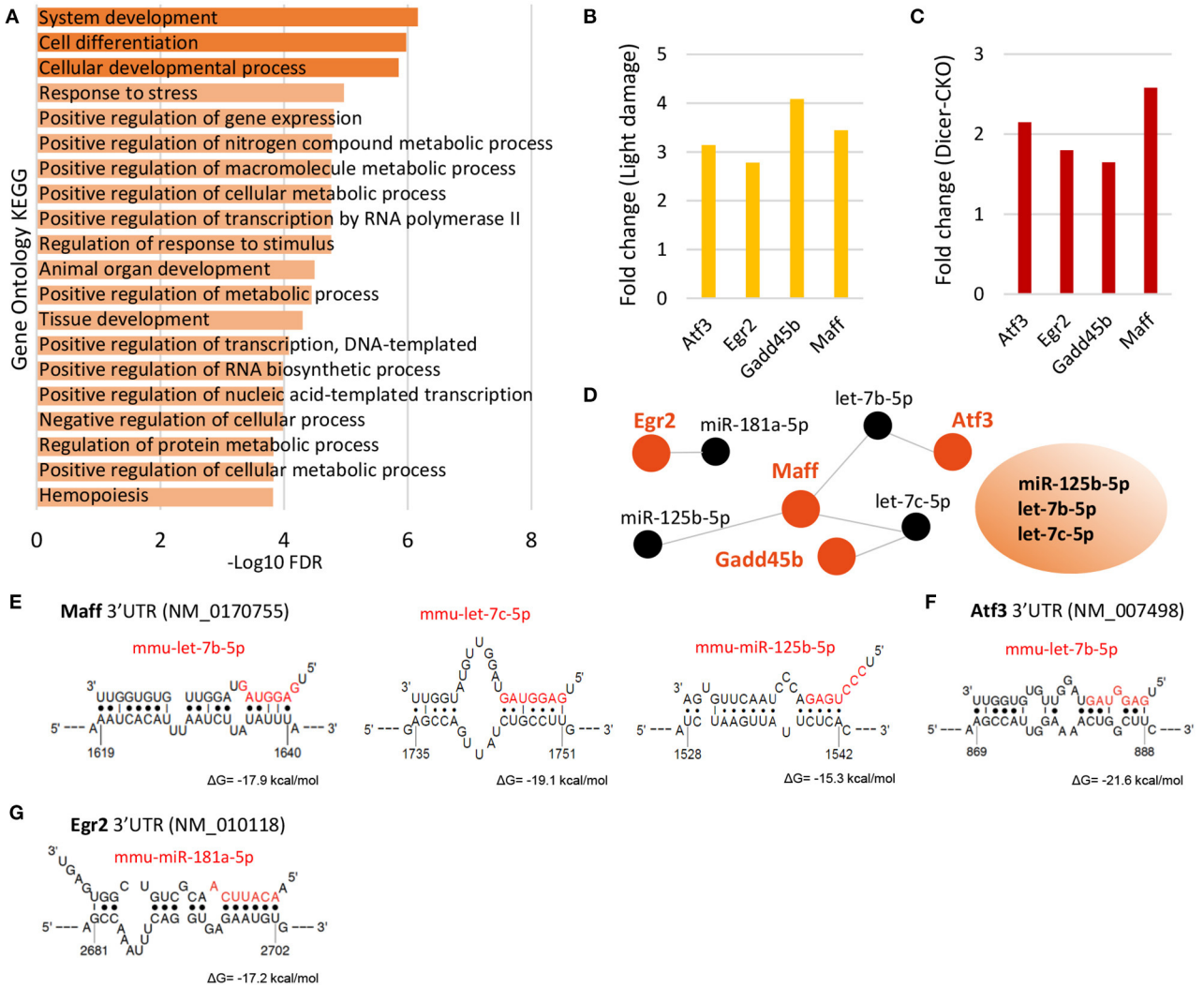

FIGURE 6 | Upregulated genes after light damage and after Dicer deletion and their potential miRNA regulators. **(A)** Gene ontology of the top 20 genes upregulated the most after Dicer deletion and light damage (LD), FDR: false discovery rate. **(B,C)** Top four genes upregulated after light damage (**B**, fold change of $\log_2$ relative expression of light damage vs. controls, five and six biological replicates, respectively, one technical replicate per condition) or Dicer deletion (**C**, fold change of $\log_2$ counts per million of wild type or Dicer-deleted MG, 20 and 14 biological replicates, respectively, 1 technical replicate per condition). **(D)** DIANA-TarBase predicted miRNAs regulating the top four genes (*Atf3*, *Egr2*, *Gadd45b*, and *Maff*) upregulated in the Dicer-cKO and after light damage and miRNAs found to be regulating the top genes after LD and the top 4 genes in the light damage/Dicer-cKO dataset i.e., miR-125b-5p, let-7c-5p, and let-7b-5p. **(E–H)** Binding sites of identified miRNAs from D (DIANA-TarBase) in the 3′UTRs of Maff **(E)**, Atf3 **(F)**, and Egr2 **(G)** using STarMirDB. The seed sequences of the particular miRNA are shown in red. Dots represent complimentary base pairing. ΔG indicates the structural accessibility at the target site.

in response to the injury. Among the top genes upregulated in reactive MG, most were bound to Ago2 in the HITS-CLIP data and many are potential targets of mGliomiRs.

Genes Similarly Upregulated in Reactive and Dicer-Depleted Müller Glia and Their Potential miRNA Regulators

In our previous analysis of the changes in the transcriptome of MG after Dicer-cKO, we identified Bcan as the most highly upregulated gene. Given the similarities in the miRNA reduction in the Dicer-cKO and light damaged MG, we compared the changes in gene expression in these experimental paradigms. We compared all genes that had a minimum of $\log_2$ 5.0 in the light damage dataset and a minimum of $\log_2$ CPM 4.0 in the RNA-Seq dataset. Twenty genes had an at least a 1.3 fold change after light damage or at least a 0.7 fold increase in the Dicer-cKO (**Supplementary Table 9**). We did gene ontology with these 20 genes and found that they are involved in response to developmental, metabolic and stress processes (**Figure 6A**).

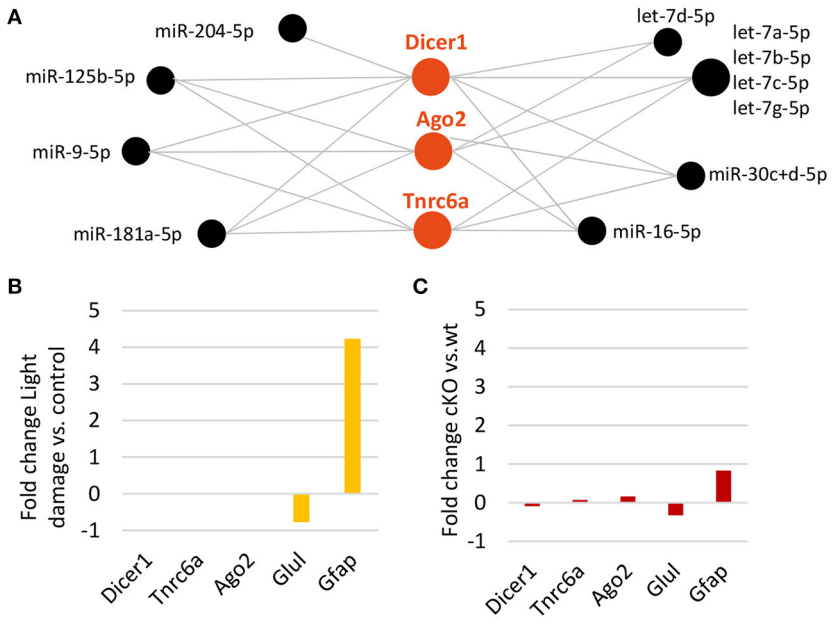

FIGURE 7 | Decline in Müller glia miRNAs does not affect Dicer or RISC. **(A)** DIANA-TarBase predicted/confirmed miRNAs regulating Dicer1, or members of the RNA-induced silencing complex (RISC) such as Argounate2 and or after Dicer deletion. **(B)** Expression levels (fold change of $\log_2$ relative expression) of *Dicer1*, RISC genes, *Glul* and *Gfap* MG genes (reference) after light damage vs. controls (five and six biological replicates, respectively, one technical replicate per condition). **(C)** Expression levels (fold change of $\log_2$ counts per million) from RNA-seq of *Dicer1*, *Tnrc6a*, *Ago2*, *Glul,* and *Gfap* after Dicer deletion vs. wild type (14 and 20 biological replicates respectively, one technical replicate per condition).

Four genes were found in both data sets, which had an at least a 2.7 and 1.4 fold increase after light damage or Dicer-cKO, respectively, which were the stress induced transcription factor *Atf3* (activating transcription factor 3 or also known as cyclic AMP-dependent transcription factor 3), *Egr2* (early growth response 2), *Gadd45b* (growth arrest and DNA damage inducible beta), and *Maff* (MAF BZIP Transcription Factor F) (**Figures 6B,C**). *Atf3* and *Gadd45*b were also bound to Ago2 (**Supplementary Figure 5E**).

In order to identify the miRNAs that potentially regulate the genes, we used DIANA-TarBase and found the miRNAs miR-125b-5p, miR-181-5p, let-7b, and let-7c are potential regulators of these genes (**Figure 6D**). We then compared the miRNA:mRNA network with that after light damage (**Figure 4D**) and found that three microRNAs were present in both networks. These miRNAs were: miR-125-5p and let-7b and let-7c. This suggests that this miRNA trio might play an important role in the changes in gene expression in MG that accompany gliosis. We used STarMirDB to identify the binding sites of the miRNAs in the mRNA molecule and found binding sites in the 3′UTR of the particular mRNA molecule for all DIANA-TarBase predicted miRNAs, with high structural accessibility at the target site ($\Delta G \sim -17$ kcal/mol, **Figures 4F, 6E–G**).

Dicer and the RISC Appear Not to Be Influenced by Light Damage or Dicer Deletion

It is known that Dicer is regulated by miRNAs, in particular by let-7 (Tokumaru et al., 2008). Since we found a significant reduction in MG miRNAs including the let-7 family, we addressed the question whether or not Dicer or members of the RISC complex such as Ago2 or GW182 (encoded by *Tnrc6a*) are affected. DIANA-TarBase revealed eleven miRNAs, which are highly expressed in the MG, have been shown to target all three genes. Among them are the mGliomiRs miR-204, miR-125b, miR-9 as well as five let-7 family members (**Figure 7A**). However, when we compared the expression levels after light damage with undamaged controls, no difference for *Dicer1* or *Tnrc6a* expression levels was found (**Figure 7B**, Ago2 was not found among the genes on the microarray). In addition, none of the three genes were differently expressed after Dicer deletion (**Figure 7C**, Glutamine synthetase and GFAP levels plotted as reference). Ago HITS-CLIP showed that only Ago2 was found to be bound (**Supplementary Figure 5F**). This implies that the loss of miRNAs, at least at these time points of analysis, had no effect on Dicer expression or on the expression of two members of the RISC complex.

DISCUSSION

We previously profiled the miRNAs highly expressed in MG (Wohl and Reh, 2016) and found that they are required for proper MG function, which in turn is required for proper retinal architecture and retinal health (Wohl et al., 2017). In this study, we went on to profile miRNAs in reactive MG after light damage to the rod photoreceptors. We compared the miRNA and mRNA expression pattern in MG from light damaged (7 days) retinas with those from the Dicer-cKO mice (1 month) and found

that the vast majority of miRNAs highly expressed in adult MG declined, while only a few increased. We found that genes upregulated in MG after light damage were the same as reported in other damage studies on whole retina and identified their potential miRNA regulators using DIANA-TarBase. DIANA-TarBase is a tool that combines computational prediction and a database of experimentally validated miRNA:mRNA interactions from over 500 tissues, mostly done by HITS-CLIP (Karagkouni et al., 2018). The comparison with the Dicer-cKO MG revealed striking similarities with regard to miRNAs levels, which implied similar genes to be affected in both conditions. We found four genes, *Maff, Egr2, Atf3,* and *Gadd45b* present in both datasets and three miRNAs, which might be potential key regulators in MG dysfunction, namely miR-125b-5p, let-7b-5p, and let-7c-5p.

Structural, Cellular, and Molecular Similarities and Differences in Retinas After Light Damage and After Müller Glia-Specific Dicer Deletion

We previously reported that deletion of Dicer1 specifically in MG (Dicer-cKO) causes a rod photoreceptor degeneration and retinal disorganization that resembles end stage retinitis pigmentosa (Wohl et al., 2017). At later stages after Dicer deletion, we found a substantial loss of photoreceptor cells that started in the central retina and spreads toward the periphery over time. The retinal remodeling that occurs in later stages of retinitis pigmentosa and other retinal degenerations is dominated by MG hypertrophy that fills the gaps in the tissue as neurons degenerate (Jones et al., 2003; Marc and Jones, 2003; Jones and Marc, 2005; Marc et al., 2007). How reactive gliosis and this glial-driven remodeling are regulated is, however, still unknown. When we compared light damaged retinas with Dicer-cKO^{MG} retinas at later time points (Wohl et al., 2017), the structural and cellular similarities we found were significant thinning of the center of the retina due to photoreceptor loss, MG migration toward the ONL and loss of glutamine synthetase expression as a result of the loss of glutamatergic neurons (Bringmann et al., 2006, 2009). The comparison of miRNA and transcriptomic changes in the MG revealed that under both conditions, all highly expressed MG miRNAs (mGliomiRs and shared miRs) were substantially reduced. Thus, the miRNA profile of reactive MG was similar to that of Dicer depleted MG. However, despite the overall decline, the absolute levels of some miRNAs were different for reactive MG and Dicer-cKO MG. This suggests that specific levels of miRNAs are responsible for particular gene targeting. Since all MG miRNAs were also found to be bound to Ago2, they are indeed regulatory miRNAs in retinal cells (Chi et al., 2009; Chu-Tan et al., 2020). This suggests that MG miRNAs play a role in reactive gliosis.

We also found 5 miRNAs upregulated 7 days after light damage, i.e., miR-720, miR-29a, miR-29b, and miR-1937a/b. Note, miR-720 is no longer considered as a miRNA, but is now known to be a fragment of a tRNA (Schopman et al., 2010). However, it has been reported as a miRNA involved in cell proliferation, differentiation, and migration (Hara et al., 2013; Torres-Martin et al., 2013; Li et al., 2014; Tang et al., 2015). One miRNA upregulated after light damage also showed an increase after Dicer deletion: miR-124. Although there are miRNAs that do not require Dicer for their processing (Cheloufi et al., 2010), miR-124 appears not to be among them (Huyghe et al., 2015). Therefore, this increase is probably due to neuron-glia communication via exosomes, with miR-124 as the predominant miRNA (Morel et al., 2013; Men et al., 2019; Wooff et al., 2020). Increased neuron-glial communication via exosomes has been reported after light damage with miR-124 as the most abundant cargo (Wooff et al., 2020).

Maff, Atf3, Egr2, and Gadd45b and Their Potential Role in Müller Glia

To better understand the common factors that might be driving gliosis, we compared gene expression of reactive MG after light damage with MG from Dicer-cKO mice. We found four genes that had at least 2.8 fold increase after light damage and 1.6 fold increase in Dicer-deleted MG which were *Atf3, Egr2*, *Maff,* and *Gadd45b*. Not much is known about the function of these genes in MG; however, recently, *Atf3, Egr2,* and *Maff* have been identified as murine MG-expressed transcription factors that are involved in TNFα signaling and were also found to be upregulated 36 h after light damage in GlastCre-GFP+ FACS-purified MG (Hoang et al., 2020). TNFα, also known as TNF, is the master pro-inflammatory cytokine, and has been shown to be involved and modulate multiple signaling pathways with wide-ranging downstream effects. It is a multifunctional molecule involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, and apoptosis. Reactive MG release TNFα [see reviews (Bringmann et al., 2006, 2009)] however, we did not find TNFα upregulated in the reactive MG 7 days after light damage, nor after Dicer deletion.

Maff is a member of the small Maf family proteins that can activate or repress transcription, but this transcription factor has not been reported in connection with glia or gliosis. It has been reported to be associated with stress response, which links it to disease pathologies including neurological diseases. These neurological disorders and diseases include schizophrenia (SCZ), bipolar disorder (BD), and major depressive disorder (MDD) (Lanz et al., 2019) as well as Parkinson's disease and Alzheimer's disease (Wang et al., 2017). Interestingly, *Maff* can bind to Nuclear factor erythroid 2-related factor 2 (*Nrf2*, encoded by the *Nfe2l2* gene), a transcription factor that regulates the expression of antioxidant proteins. *Nrf2* has been reported in a variety of studies about retinal injury such as hypoxia, ischemia and light damage, and an activation of NRF2 by different compounds/factors has been shown to reduce the effect of MG gliosis (Tan et al., 2015; Deliyanti et al., 2016; Inoue et al., 2017). We did not find differentially expressed *Nfe2l2* (whose levels are also rather low in MG) after light damage or Dicer deletion. Nevertheless, *Maff* and its role in regulating stress response by binding to *Nfe2l2* could be relevant for regulating gliosis.

Atf3 is a member of the activating transcription factor/cAMP responsive element binding (CREB) protein family of

transcription factors, which share the basic region-leucine zipper (bZip) DNA binding motif. The level of *Atf3* mRNA is low or undetectable in normal mouse tissue and most cell lines but increases significantly after stimulation. *Atf3* is induced by a variety of stress signals including ER stress, stresses that induce integrated stress response, and other stress signals. One striking feature of *Atf3* induction is that it is neither tissue-specific nor stimulus-specific; it can be induced by a broad spectrum of stimuli and can be induced in various tissues or cell types [see (Hai et al., 2011)]. *Atf3* plays a role in metabolic regulation, immune response, and ontogenesis. *Atf3* increases various downstream targets such as *Ccl2* (which is also upregulated after light damage **Figure 4E**), induces cell proliferation but also apoptosis (Ku and Cheng, 2020). In the retina, it has been described in fish regeneration (Saul et al., 2010), as a neuroprotective factor after optic nerve crush in murine neurons (Kole et al., 2020) and as a cyto-protective factor in astrocytes after oxidative stress induction (Kim et al., 2010). For MG, *Atf3* is suggested to be regulated by tumor suppressor protein p53 (Ueki et al., 2012). *Atf3* is also reported to be induced by ciliary neurotrophic factor (CNTF), a member of the interleukin-6 cytokine family that is suggested as an inducer of gliosis (Xue et al., 2011). In the same study, *Atf3* was found to be expressed together with *Egr2* (also termed *Krox20*, which plays an important role in neuronal development). Interestingly, both proteins, Atf3 and Egr2, have also been reported in a study about MG that underwent cyclic mechanical stretching (Wang et al., 2013). The retina in diseases such as myopia or proliferative vitreoretinopathy is often subjected to mechanical forces which lead to tissue deformation and consequently MG stretching. It is interesting that we find two of these genes after Dicer deletion in MG, since the retinas of these mice had puffy/stretched areas. However, we interpreted these areas as a consequence of changes in MG gene expression due the miRNA decline (Wohl et al., 2017). Moreover, it is also very intriguing that these genes are found after light damage, since light damage leads to a significant retinal thinning and causes the opposite of tissue stretching. *Egr2* has also been reported to be upregulated in astrocytes after increased intraocular pressure. This model induces a mechanical stress response that could also be considered as a different kind of cellular stretching (Yang et al., 2004) and implies a common gene for retinal MG and optic nerve head astrocytes. *Gadd45b* (Growth Arrest and DNA-Damage-inducibleprotein45 beta, also known as MyD118) belongs to the highly homologous Gadd45 family of proteins. These proteins are found in the nucleus and are known to act as stress-response genes (Liebermann and Hoffman, 2007). They function in DNA repair, apoptosis, cell survival, growth arrest, and probably DNA demethylation (Barreto et al., 2007). DNA demethylation typically activates gene expression, while DNA methylation is one of the various epigenetic mechanisms for silencing gene expression (Reik, 2007). Altered DNA methylation also occurs in pathological processes, such as silencing of tumor repressor genes in cancer cells. *Gadd45b* was shown to be induced by neuronal activity, which promoted epigenetic DNA demethylation and adult neurogenesis (Ma et al., 2009). However, whether *Gadd45b* has a similar function in glia, in particular in MG, is not known yet.

miR-125b-5p, let-7b-5p and let-7c-5p as Potential Key Regulators in Müller Glia

We found three miRNAs, miR-125b, let-7c, and let-7b, as potential regulators of genes after light damage and in the dataset of light damage/ Dicer-cKO using DIANA-TarBase. This implies that these miRNAs might play an important role for glial function. To our knowledge, none of them has been reported yet in the context of reactive MG. let-7 and miR-125 are known to play an important role during retinogenesis (transition from early to late retinal progenitor cells) and this is a conserved pathway across vertebrates (La Torre et al., 2013).

However, in other regions of the CNS, i.e., the brain or spinal cord, miR-125b has been reported to play a role in glial scar formation. A study analyzing the environment after spinal cord injury by cross-species comparison between salamander and rat revealed that precise levels of miR-125b can recreate a permissive environment for axon regeneration in rat by targeting the semaphorin gene *Sema4* (Diaz Quiroz et al., 2014). It was shown that in normal spinal cords, the radial glia cells in salamander express very high levels of miR-125b. Rat astrocytes, however, have low levels. After spinal cord transection, the levels in the salamander spinal cords drastically decreased by 40%, while the levels in rat astrocytes did not change. When miR-125 was overexpressed in rats using mimics, a reduction of the glial scar (downregulation of genes involved in glial scar formation such as *Gfap, Cspg4* and *Col6A1*) and an increase of the number and length of axons projecting into the scar were observed (Diaz Quiroz et al., 2014). Although the authors showed that this mechanism is conserved across vertebrates, it appears that this mechanism is astrocyte specific. In our dataset, *Sema4, CSPG4,* and *Col6A1* were expressed at very low levels in MG and no change in gene expression was found either after light damage or after the Dicer deletion. This implies that astrogliosis in spinal cord is different from MG gliosis which might be due to the fact that astrocyte miRNAs (low miR-125b) are different from MG miRNAs (high miR-125b).

Another study about normal human brain astrocytes reported that treatment with interleukin IL-6, which induces cellular stress, leads to upregulation of miR-125b and *Gfap* as well as downregulation of cyclin-dependent kinase Cdkn2a, a negative regulator of cell proliferation (Pogue et al., 2010). This correlation was also observed in advanced Alzheimer's disease (Lukiw, 2007). This might suggest that astrogliosis in the human brain is different than astrogliosis in the rat spinal cord since miR-125 levels are different. *Cdkn2a* levels were low in MG and not changing after light damage or Dicer-deletion further implying that MG gliosis and astrogliosis are different. However, this raises the question which miRNAs are found in astrocytes. Rao et al., reported the miRNAs highly expressed in astrocytes of fetal and adult normal brains namely miR-99a, miR-143, and miR-449 (Rao et al., 2016). Interestingly, miR-99a is also among the highly expressed miRNAs in MG (Wohl and Reh, 2016). They

also listed the miRNAs functionally characterized in astrocytes, which included miR-125b and two other miRNAs, which are also found to be expressed in MG, i.e., miR-181a, and miR-100 (Rao et al., 2016). Although levels of miR-125b in astrocytes were reported to be low, Shenoy et al. reported that miR-125b and let-7 are required for astrogliogenesis, meaning the transition from glial progenitor cells into astrocytes (Shenoy et al., 2015). This suggests a role for these miRNAs in glial identity.

The let-7 family members are known to act as tumor suppressors and regulators of developmental processes and that their biogenesis is tightly controlled (Lee and Dutta, 2007; Lee et al., 2011, 2016). Recently, let-7e and let-7i have been described as inhibitors for MG-derived retinal regeneration in Royal College of Surgeon rats (Tao et al., 2016). Let-7 targets Lin-28, a transcription factor, which is essential in fish regeneration (Ramachandran et al., 2010) and Lin-28 itself regulates let-7 expression (feedback loop). Ectopic expression of Lin-28 resulted in decreased accumulation of let-7 miRNAs and promoted MG de-differentiation *in vivo* with subsequent neurogenesis and inhibition of gliogenesis. This confirms the hypothesis of a role in glial identity. However, in our data set, let-7 family members declined but Lin-28 was not found to be increased after let-7 reduction in both of our conditions, light damage and Dicer-cKO.

Similarities and Differences of Müller Glia Gene Expression After Light Damage in Fish

Fish MG have the ability to de-differentiate into a retinal progenitor that can give rise to all retinal neuron types and regenerate the retina (Hitchcock et al., 2004; Bernardos et al., 2007; Kassen et al., 2007; Fausett et al., 2008; Fischer and Bongini, 2010; Karl and Reh, 2010; Ramachandran et al., 2010; Goldman, 2014; Lenkowski and Raymond, 2014; Wan and Goldman, 2016; Otteson, 2017; Elsaeidi et al., 2018). In experiments with fish conduction light damage, MG undergo complex phenotypic changes, enter the cell cycle only within a day and generate new neurons (Yurco and Cameron, 2005; Vihtelic et al., 2006; Kassen et al., 2007; Thummel et al., 2008). Mammals, however, lack that capacity since proliferative and neurogenic competence are both suppressed by a dedicated gene regulatory network in mouse MG that have been recently revealed by Hoang and colleagues (Hoang et al., 2020). The comparison of differentially expressed genes in fish and mouse MG after damage showed that rapidly induced genes in fish were enriched for ribosome biogenesis, protein folding and VEGF signaling pathway. In mouse, however, they included components of the tumor necrosis factor (TNF), nuclear factor kB (NFkB), mitogen-activated protein kinase (MAPK), and Hippo pathways. Slowly induced mouse genes were enriched for ribosome biogenesis and proteasome, whereas in zebrafish, they were enriched for cell cycle| related functions and DNA replication (Hoang et al., 2020). Genes upregulated in both species include *Gfap* and *Stat3* however, the downstream events, especially of the STAT-pathway, are different which is primarily due to epigenetics (Kassen et al., 2007; Ueki et al., 2008; Hong et al., 2014; Hoang et al., 2020; Jorstad et al., 2020). The possibility that this differential regulation could be partially caused by miRNAs has to our knowledge not been explored yet.

Taken together, here we report the miRNA and mRNA profile found in reactive MG 7 days after light damage. We found that the vast majority of MG miRNAs declined and that this profile resembled the MG profile 1 month after MG-specific Dicer deletion, regardless of the extend of the neuronal loss. By analyzing the genes upregulated after light damage, four genes were found to be upregulated in both data sets, *Atf3, Egr2, Gadd45b,* and *Maff*. These genes have not been well-studied in MG yet. Nevertheless, they are associated with stress responses that appear to occur in damage-induced models (direct neuronal damage), and in models of dysregulated MG, here induced by the loss of Dicer1. This implies there is a common unspecific MG stress response that is regulated by miRNAs, potentially regulated by miR-125b, let-7c, and let-7b. This result opens up the possibility that the MG stress response could be altered by manipulating these miRNAs. Subsequent downstream experiments will reveal whether manipulation of these genes has an impact on gliosis, which would have clinical relevance for various types of retinal diseases, independent of the primary cause.

DATA AVAILABILITY STATEMENT

mRNA and miRNA datasets for wild type undamaged control MG and light damaged MG are available at GEO (GSE163754).

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

SW and TR conceived the study. SW, DL, SK, SR, and MA conducted experiments. SW and SR conducted data collection. SW and TR analyzed the data. SW, SK, and DL wrote the manuscript with inputs from co-authors. All authors contributed to the article and approved the submitted version.

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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.2020.620459/full#supplementary-material

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Genome-Wide Analysis of Differentially Expressed miRNAs and Their Associated Regulatory Networks in Lenses Deficient for the Congenital Cataract-Linked Tudor Domain Containing Protein TDRD7

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Mutations/deficiency of *TDRD7*, encoding a tudor domain protein involved in post-transcriptional gene expression control, causes early onset cataract in humans. While Tdrd7 is implicated in the control of key lens mRNAs, the impact of *Tdrd7* deficiency on microRNAs (miRNAs) and how this contributes to transcriptome misexpression and to cataracts, is undefined. We address this critical knowledge-gap by investigating *Tdrd7*-targeted knockout (*Tdrd7-/-*) mice that exhibit fully penetrant juvenile cataracts. We performed Affymetrix miRNA 3.0 microarray analysis on *Tdrd7-/-* mouse lenses at postnatal day (P) 4, a stage preceding cataract formation. This analysis identifies 22 miRNAs [14 over-expressed (miR-15a, miR-19a, miR-138, miR-328, miR-339, miR-345, miR-378b, miR-384, miR-467a, miR-1224, miR-1935, miR-1946a, miR-3102, miR-3107), 8 reduced (let-7b, miR-34c, miR-298, miR-382, miR-409, miR-1198, miR-1947, miR-3092)] to be significantly misexpressed (fold-change $\geq \pm$ 1.2, p-value < 0.05) in *Tdrd7-/-* lenses. To understand how these misexpressed miRNAs impact *Tdrd7-/-* cataract, we predicted their mRNA targets and examined their misexpression upon *Tdrd7*-deficiency by performing comparative transcriptomics analysis on P4 and P30 *Tdrd7-/-* lens. To prioritize these target mRNAs, we used various stringency filters (*e.g.,* fold-change in *Tdrd7-/-* lens, iSyTE-based lens-enriched expression) and identified 98 reduced and 89 elevated mRNA targets for overexpressed and reduced miRNAs, respectively, which were classified as "top-priority" "high-priority," and "promising" candidates. For *Tdrd7-/-* lens overexpressed miRNAs, this approach identified 18 top-priority reduced target mRNAs: *Alad*, *Ankrd46*, *Ceacam10*, *Dgat2*, *Ednrb*, *H2-Eb1*, *Klhl22*, *Lin7a*, *Loxl1*, *Lpin1*, *Npc1*, *Olfm1*, *Ppm1e*, *Ppp1r1a*, *Rgs8*, *Shisa4*, *Snx22* and *Wnk2*. Majority of these targets were also altered in other gene-specific perturbation mouse models (*e.g., Brg1*, *E2f1/E2f2/E2f3*, *Foxe3*, *Hsf4*, *Klf4*, *Mafg/Mafk*, *Notch*) of lens defects/cataract, suggesting their importance to lens biology.

Gene ontology (GO) provided further insight into their relevance to lens pathology. For example, the *Tdrd7*-deficient lens capsule defect may be explained by reduced mRNA targets (*e.g., Col4a3*, *Loxl1*, *Timp2*, *Timp3*) associated with "basement membrane". GO analysis also identified new genes (*e.g., Casz1*, *Rasgrp1*) recently linked to lens biology/pathology. Together, these analyses define a new Tdrd7-downstream miRNA-mRNA network, in turn, uncovering several new mRNA targets and their associated pathways relevant to lens biology and offering molecular insights into the pathology of congenital cataract.

Keywords: cataract, lens aberration, microRNA, microarray, TDRD7, gene regulatory networks, eye development and function

INTRODUCTION

Perturbations in lens development results in congenital cataract in humans and animal models (Graw, 2009; Shiels and Hejtmancik, 2019). Studies over the past several decades have led to a detailed understanding of the key signaling and transcriptional regulatory mechanisms that orchestrate the genetic program of lens development (Donner et al., 2006; Lachke and Maas, 2010; Cvekl and Zhang, 2017). However, compared to signaling and transcription, the impact of post-transcriptional gene expression control to organogenesis, in general, and lens development, in particular, remains relatively understudied (Lachke and Maas, 2011; Blackinton and Keene, 2014; Dash et al., 2016; Cvekl and Zhang, 2017). Post-transcriptional control of gene expression is defined as the regulation of any of the different events from the processing of pre-mRNA to the degradation of mRNA (Singh et al., 2015). Indeed, non-coding RNAs such as microRNAs (miRNAs) as well as RNA-binding proteins (RBPs) are involved in various post-transcriptional regulatory processes, including control over translation or decay of mRNA (Pasquinelli, 2012; Manning and Cooper, 2017; Hentze et al., 2018; O'Brien et al., 2018). Thus far, very few RBPs and post-transcriptional regulatory factors, including miRNAs, have been functionally implicated in lens development and cataract (Lorén et al., 2009; Lachke et al., 2011; Choudhuri et al., 2013; Shaham et al., 2013; Wolf et al., 2013; Xie et al., 2014; Dash et al., 2015, 2020; Siddam et al., 2018; Aryal et al., 2020; Barnum et al., 2020; Nakazawa et al., 2020; Shao et al., 2020). This limited information highlights a substantial knowledge-gap in lens and cataract research because post-transcriptional control represents critical mechanisms that allow precise calibration, in terms of dosage and spatiotemporal pattern, of the cellular proteome. Thus, these regulatory mechanisms may be significant for controlling mRNA and protein abundance in lens fiber cells – a cell fate that faces added challenges to regulate these basic processes as they undergo nuclear degradation in terminal differentiation (Dash et al., 2016).

In embryogenesis, expression of Tdrd7 (Tudor domain containing 7) is highly enriched in lens fiber cells and is conserved between aves and mammals, suggesting its critical function in lens development (Lachke et al., 2011). Indeed, *TDRD7* mutations or deficiency results in congenital cataract in humans, mouse and chicken (Lachke et al., 2011; Tanaka et al., 2011; Chen et al., 2017; Tan et al., 2019). Furthermore, single nucleotide polymorphisms in *TDRD7* are also linked to age-related cataract (Zheng et al., 2014), making it among the select few genes associated with both early- and late-onset cataract (Shiels and Hejtmancik, 2019). Tdrd7 contains three tudor domains and three OST-HTH (Oskar-Tdrd7-Helix turn helix)/LOTUS motifs (Hosokawa et al., 2007; Anantharaman et al., 2010; Callebaut and Mornon, 2010; Tanaka et al., 2011). Tudor domains are considered to facilitate interaction with methylated arginine or lysine residues within other proteins (Chen et al., 2011; Pek et al., 2012; Gan et al., 2019). The OST-HTH/LOTUS domains in Drosophila protein *oskar*, predicted to bind RNA (Anantharaman et al., 2010; Callebaut and Mornon, 2010), has been shown to interact with a dead-box helicase (Jeske et al., 2015, 2017). While Tdrd-family proteins have been implicated in the control of small RNAs, previous studies have primarily focused on their association with piwi-interacting RNAs (piRNAs) in the context of spermatogenesis (Pek et al., 2012; Gan et al., 2019). Interestingly, while its mutation or deficiency is linked to azoospermia in human and mouse (Lachke et al., 2011; Tanaka et al., 2011; Tan et al., 2019), Tdrd7 has been shown to function in repression of Line-1 retrotransposons but is not found to be essential for production of piRNAs in mouse spermatogenesis (Tanaka et al., 2011). However, the effect of *Tdrd7* deletion on other classes of small non-coding RNAs such as miRNAs in the lens, and its impact on lens development and cataract formation has not been addressed.

Previously, we used *Tdrd7*-deficient mice–which exhibit fully penetrant cataracts and re-capitulate features of the human lens defects–to gain insight into Tdrd7's role in lens development (Lachke et al., 2011; Barnum et al., 2020). We showed that removal of *Tdrd7* results in misexpression of several lens expressed mRNAs. We also demonstrated that Tdrd7 protein closely associates with specific mRNAs, for example *Hspb1* mRNA, which may enable it to directly control its abundance in the lens. Here, we sought to examine the impact of Tdrd7 deletion on global miRNA abundance in the lens. We performed microarray-based profiling of miRNAs in *Tdrd7*-/- mouse lens at postnatal day (P) 4, which precedes detectable lens defects and overt cataract formation. We identified misexpressed miRNAs and predicted their mRNA targets in the lens. We then performed comparative analysis with *Tdrd7*-/- lens RNA-seq and microarray datasets to identify inversely associated

mRNA targets of the misexpressed miRNAs. This allowed us to derive a Tdrd7-downstream miRNA-mRNA network that led to the identification of new candidate genes with potential function in the lens. Importantly, iSyTE analysis showed that similar numbers of overexpressed and reduced mRNA targets in *Tdrd7*-/- lens were enriched in normal lens, suggesting that Tdrd7 functions in optimal control of a subset of lens-enriched mRNAs likely via regulating miRNAs. Finally, in addition to informing on *Tdrd7* deficiency, the global lens miRNA profile generated in this study provides independent support for expression of some of the abundant miRNAs in normal lens development that were described in previous studies, involving for example, *in situ* hybridization assays (Conte et al., 2010; Karali et al., 2010; Khan et al., 2016). These are: miR-184, miR-26a, let-7b, let-7c, miR-204 and miR-125b, among others. Together these data identify new high-priority Tdrd7-downstream miRNA and mRNA targets, thereby advancing our understanding of how this conserved Tudor family protein functions to fine-tune lens transcriptome in development and how its misregulation impacts cataract pathology.

MATERIALS AND METHODS

Mouse Studies

Mice were maintained at the University of Delaware Animal Facility. Experimental protocols followed guidelines based on the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of animals in ophthalmic and vision research and were approved by the University of Delaware Institutional Animal Care and Use Committee (IACUC). The present studies were performed on *Tdrd7* targeted germline knockout (KO) mouse line (*Tdrd7*$^{TM1.1Chum}$, hereafter referred as *Tdrd7*-/-) that were genotyped as previously described (Tanaka et al., 2011).

Sample Preparation and miRNA Microarray Analyses

For miRNA microarray, microdissected mouse lenses at postnatal day (P) 4 were collected from *Tdrd7*-/- and control (*Tdrd7*±, which does not develop cataract) in three biological replicates. Total RNA isolation was performed using the *mir*Vana^{TM} RNA isolation kit (Life Technologies, Grand Island, NY). Global expression profiling for miRNAs was performed using Affymetrix miRNA 3.0 arrays. Analysis of the raw expression datasets was performed under 'R' Statistical environment [http://www.r-project.org/index.html] using "Affy" packages. The datasets were background corrected, normalized and summarized using Robust Multi-array Average (RMA) method (Irizarry et al., 2003a,b). The obtained normalized miRNA expression values were subjected to downstream analysis using 'limma' package. Comparisons of control and *Tdrd7*-/- samples was carried out to identify highly and differentially expressed miRNAs with significant p-value (≤ 0.05) and absolute fold change (FC) $\geq \pm$ 1.2. The detailed pipeline for Affymetrix microarray dataset analysis after data normalization is published elsewhere (Anand et al., 2015; Kakrana et al., 2018). The datasets generated in this study were deposited in GSE157061.

qPCR Analysis of miRNAs in the Lens

Selected *Tdrd7*-/- lens misexpressed candidate miRNAs were analyzed by custom-designed PCR primers using miRCURY LNA miRNA PCR system (Qiagen, Germantown, MD). Total RNA was isolated from P4 *Tdrd7*-/- and control lenses in three biological replicates using miRNeasy mini kit (Qiagen Catalog: 217004). First-strand cDNA synthesis was performed using miRCURY LNA Universal RT kit (Qiagen Catalog: 339340) and miRNA expression was quantified using miRCURY LNA SYBR Green PCR kit (Qiagen Catalog: 339345) according to the manufacturer's instructions. qPCR was run on BioRad CFX RT-PCR thermal cycler. MiRNA expression was normalized to miR-17, which exhibits robust expression in the lens and is not altered in *Tdrd7*-/- lens, as well as the housekeeping genes *Gapdh* and *Actb*. Relative expression was estimated using the $2^{--\Delta\Delta CT}$ followed by statistical two-level nested analysis of variance test to calculate p-values.

miRNA Target Prediction and Gene Ontology Enrichment Analysis of miRNA Targets

We performed miRNA target prediction and functional annotation analysis for differentially expressed miRNAs in *Tdrd7*-/- lenses. To retrieve predicted mRNA targets of miRNAs, the miRDB resource (Chen and Wang, 2020) that uses experimental data (*e.g.,* miRNA overexpression, publicly available CLIP-seq data) in their miRNA-mRNA target prediction algorithm, MirTarget, was used. The miRDB-identified mRNA targets with a probability score $\geq$ 50 were retrieved for further downstream analysis and were tested for their functional relevance to lens development using gene ontology (GO) analysis in DAVID bioinformatics tool (https://david.ncifcrf.gov). The top clusters with highest enrichment scores with functionally relevant GO categories at p-value $\leq$ 0.05 were presented. The TargetScan database (www.targetscan.org) was used to search for target mRNA conservation sites that match with the seed region of miRNAs across vertebrates (Lewis et al., 2005).

Identification of Lens Enriched miRNA-mRNA Pairs

The miRNA target mRNAs were curated using lens gene-discovery bioinformatics tool-iSyTE (Lachke et al., 2012; Kakrana et al., 2018) to identify candidate miRNA-mRNA pairs (*i.e.,* miRNA and their predicted mRNA targets) associated with lens function and development. In this study, miRNA-mRNA pairs were tested for lens expression (expression $\geq$ 100) from P4 mRNA microarray dataset in iSyTE (Kakrana et al., 2018). To further assess candidate miRNA-mRNA pairs, we revisited the whole genome RNA-seq (GSE134384) and mRNA microarray datasets on *Tdrd7* null and control lenses at P4 (GSE25775) (Lachke et al., 2011) and performed an analysis to evaluated inverse correlation between differentially expressed

'up' and 'down' miRNA with their mRNA targets. The inverse correlation for elevated and reduced (used in the context of miRNAs) miRNA-mRNA pairs was tested using Pearson method implemented in R-package. Data was presented as miRNA-mRNA plots and miRNA-mRNA regulatory network was visualized as regulatory networks using cytoscape (https://cytoscape.org).

Network Analysis of miRNA Target mRNAs

Candidate target mRNAs of the *Tdrd7-/-* lens differentially miRNAs were examined for differential expression by analyzing lens transcriptomics datasets in various gene-perturbation mouse models that develop lens defects/cataract. These include *Brg1* (dominant negative dnBrg1) at E15.5 (GSE22322), *E2f1:E2f2:E2f3* (triple lens-specific conditional knockout) at P0 (GSE16533), Foxe3 (transgenic mice with *Cryaa*-promoter based over-expression of *Foxe3* in lens fiber cells) at P2 (GSE9711), *Hsf4* (germline knockout) at P0 (GSE22362), *Klf4* (lens-specific conditional knockout) at E16.5 and P56 (GSE47694), *Mafg-/-:Mafk* ± (germline compound knockout) at P60 (GSE65500) and *Notch2* (lens-specific conditional knockout) at E19.5 (GSE31643). The network connectivity for mRNAs with these key lens factors were visualized using an open source tool Cytoscape (https://cytoscape.org).

RESULTS AND DISCUSSION

Global miRNA Profiling of *Tdrd7-/-* Lens Prior to Formation of Cataract

Tdrd7 expression in mouse embryonic lens fiber cells is highly enriched on both the transcript and protein levels (**Figures 1A–C**) and its germline knockout results in severe cataract defects by postnatal day (P) 22 (**Figure 1D**). While the mRNAs misexpressed in *Tdrd7-/-* mouse lens have been examined on the genomic level in previous studies (Lachke et al., 2011; Barnum et al., 2020), there is no information on the impact of Tdrd7 deficiency on lens miRNA expression. Therefore, we sought to examine *Tdrd7-/-* lens by global miRNA profiling (**Figure 2A**). Data on three biological replicates each for *Tdrd7-/-* and control lenses at postnatal day (P) 4 were generated on Affymetrix miRNA 3.0 microarrays. The stage P4 was selected for this analysis because it preceded cataract formation in *Tdrd7-/-* lenses (**Figure 1D**) and therefore was considered to yield information on the early alterations in miRNA expression, prior to the onset of lens defects, in turn reducing the possibility of detecting secondary miRNA changes. The microarray datasets generated were imported in R-statistical environment for systematic analysis of miRNAs expression in the *Tdrd7-/-* and control lens samples. The detailed bioinformatics pipeline is outlined in **Figure 2A**. The quality of miRNA microarray datasets was evaluated using Principal component analysis (PCA) (**Figure 2B**) and boxplot analysis (**Figure 2C**). Normalized miRNA expression intensity in PCA showed that *Tdrd7-/-* sample datasets (T1, T2, T3) were distinct from control

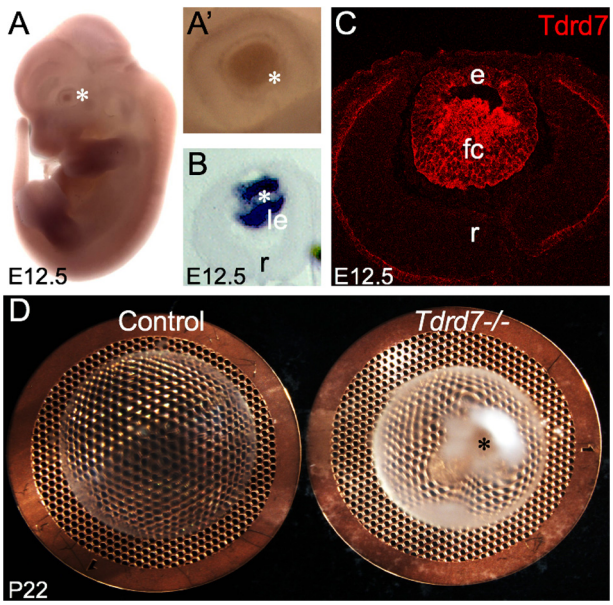

FIGURE 1 | Tdrd7 is expressed in mouse embryonic lens development and its deficiency results in early onset cataract. **(A)** *In situ* hybridization of mouse embryonic day stage E12.5 with *Tdrd7*-specific probe shows Tdrd7 transcript signal in the eye lens (asterisk), **(A')** also clearly observed at high-magnification (asterisk). **(B)** Section *in situ* hybridization confirms *Tdrd7* transcripts to be expressed in the mouse E12.5 lens (le) and demonstrates it to be primarily localized to fiber cells. **(C)** Immunofluorescence staining using antibody against Tdrd7 demonstrates its protein abundance (red) in the cytoplasm of lens fiber cells at E12.5. **(D)** *Tdrd7-/-* germline targeted knockout mouse lens show severe cataract (asterisk) compared to normal control at P22 stage. le, lens; r, retina; e, lens epithelium; fc, lens fiber cells.

datasets (C1, C2, C3) (**Figure 2B**). In P4 lenses, 697 mature miRNAs were detected with expression intensities ranging between 1.2 and 15592 (**Supplementary Table 1**). Further, the expression intensity distribution of these miRNAs showed that the vast majority (n = 503, 72.2%) are detected at expression intensity $\leq$ 5.0 (**Figure 2D**). Further, a majority [481 (95.6%)] of these 503 miRNAs were independently shown to be expressed in the mouse lens (Khan et al., 2015, 2016). The miRNAs found to be differentially expressed between *Tdrd7-/-* and control lens samples were identified in the 5-500 expression intensity range (**Figure 2D**).

Tdrd7-/- Lens Exhibits Differentially Expressed miRNAs

We next compared miRNAs in *Tdrd7-/-* and control lens samples and identified 22 mis-expressed mature miRNAs at significant p-value ($\leq$0.05) and absolute fold change ($\geq$1.2) (**Figure 3A**). These included the following 8 miRNAs that were reduced in *Tdrd7-/-* lens: let-7b, miR-34c, miR-298, miR-382, miR-409, miR-1198, miR-1947, and miR-3092 (**Table 1**). The 14 miRNAs overexpressed in *Tdrd7-/-* lens were: miR-15a, miR-19a, miR-138, miR-328, miR-339, miR-345, miR-378b, miR-384, miR-467a, miR-1224, miR-1935, miR-1946a, miR-3102, and miR-3107.

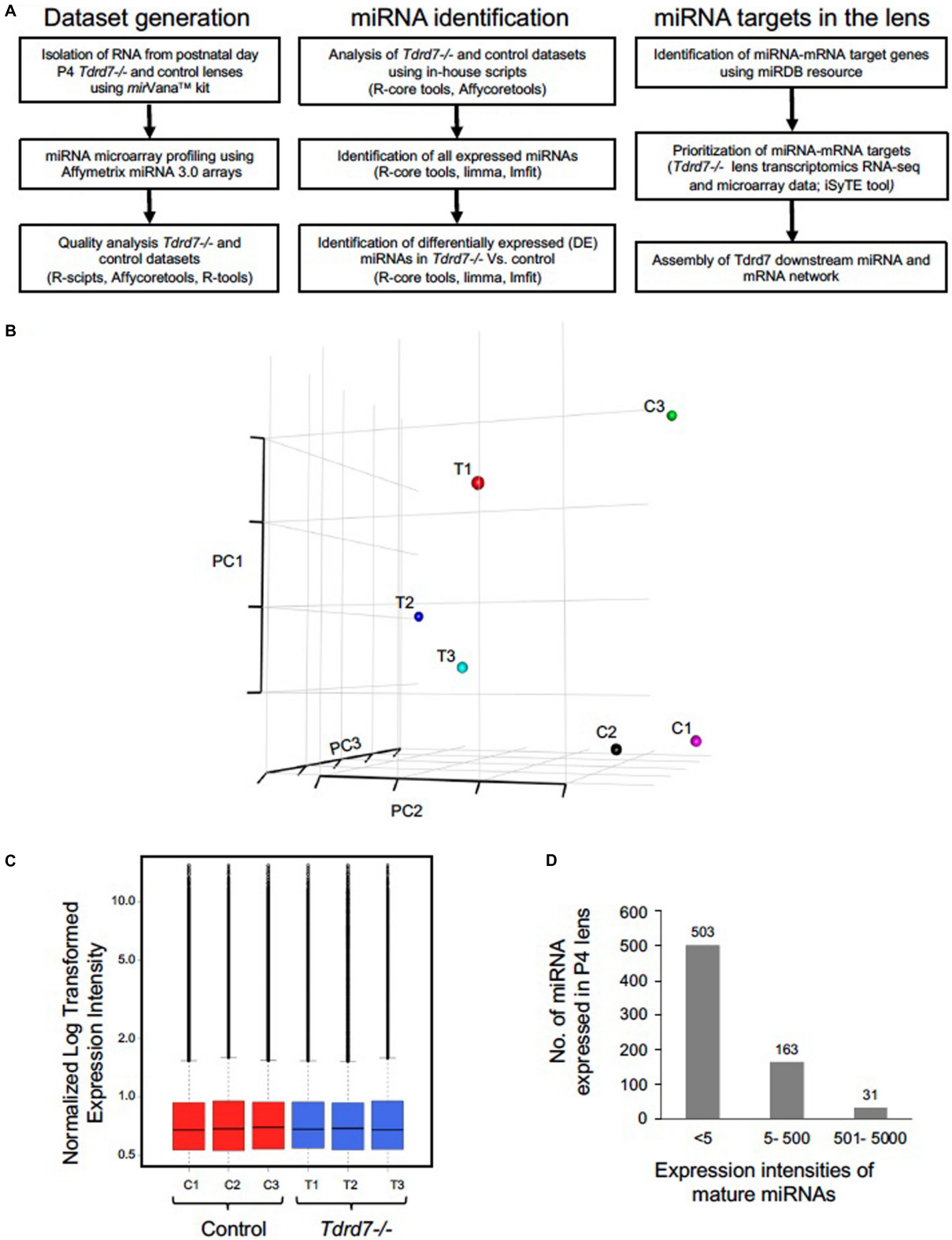

FIGURE 2 | Microarray-based profiling of miRNAs in *Tdrd7-/-* lenses. **(A)** Step-by-step workflow showing bioinformatics tools and R-packages used for generation and analysis of post-natal day (P) 4 *Tdrd7-/-* and control lens microarray datasets, miRNA identification and miRNA-mRNA targets. **(B)** Comparisons of normalized expression intensities for three biological replicates for *Tdrd7-/-* and control using PCA analysis plots and **(C)** boxplot analysis. **(D)** Bar plot shows total number of miRNAs expressed at P4. Distribution of expression intensity for miRNAs is shown by *x*-axis while *y*-axis shows number of miRNAs expressed.

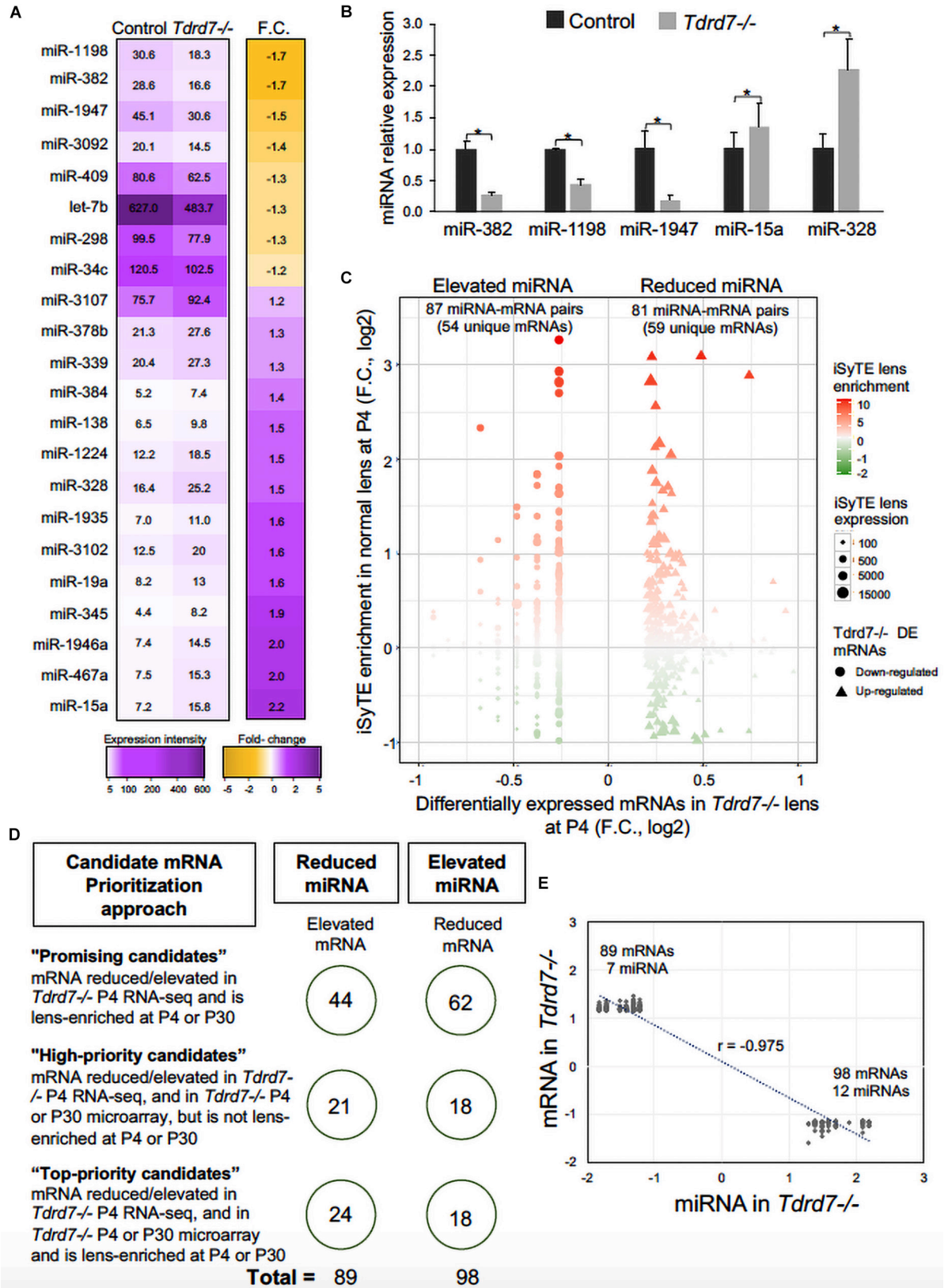

FIGURE 3 | Continued

FIGURE 3 | Mis-expression of miRNAs and their mRNA targets in *Tdrd7*-/- lens. **(A)** Heat-map represents comparison of miRNA expression in *Tdrd7*-/- and control lenses (left column) and mis-expression of twenty-two miRNAs (fold change (F.C.) $\geq \pm$ 1.2 fold-change, $p < 0.05$) in *Tdrd7*-/- vs. control lenses (right column). **(B)** Relative miRNA expression in control and *Tdrd7*-/- lens. qPCR analysis independently validates significant reduction of miRNAs miR382, miR1198 and miR1947, and significant elevation of miRNAs miR15a and miR328. Error bars represents standard error of the mean. Asterisk represents *p*-value < 0.05. NS represents not significant *p*-values. **(C)** Lens-enriched expression of Tdrd7-downstream miRNA target mRNAs. mRNA targets of differentially expressed miRNAs in *Tdrd7*-/- lens (*x*-axis) are plotted against iSyTE enrichment scores at P4 (*y*-axis). Shape gradient represented as circles is for down-regulated mRNAs while triangles represent up-regulated mRNAs. Size gradient represents smaller to higher expression of mRNA. Red and green color gradients represent high and low lens-enrichment, respectively. Similar number of target mRNAs in the *Tdrd7*-/- lens reduced- and elevated-miRNA group exhibit lens enriched expression at stage P4. **(D)** Schematic shows candidate mRNA prioritization approach in the *Tdrd7*-/- lens reduced- and elevated-miRNA group. On the left are shown the rules that were used to prioritize candidate mRNAs in "Top-priority," "High-priority," and "Promising" candidate categories. On the right, shown in circles are the numbers of mRNA in the *Tdrd7*-/- lens reduced- and elevated-miRNA groups. **(E)** Pearson correlation analysis of miRNA and their target mRNAs identifies inverse correlation (r = -0.975) for the *Tdrd7*-/- lens reduced- and elevated-miRNA group.

Prediction of Downstream mRNA Targets of Misexpressed miRNAs in *Tdrd7*-/- Lens

MicroRNAs control the cellular proteome by directly binding to their target mRNAs and channeling them to degradation or by inhibiting their translation into protein (Pasquinelli, 2012; Manning and Cooper, 2017; Hentze et al., 2018). In both cases, direct binding of miRNA to their target mRNA is a critical step. Therefore, to gain insight into the downstream impact of the differentially expressed miRNAs in the *Tdrd7*-/- lens transcriptome, we sought to identify miRNA-mRNA target binding pairs using the miRDB resource (Chen and Wang, 2020). This identified potential 11450 and 7142 mRNA targets for 12 elevated and 7 reduced miRNAs, respectively. The database did not contain information on mRNA targets for the differentially expressed miRNAs miR-3107, miR-1935 and miR-3092, and therefore these miRNAs were not included in further downstream analyses. Next, from the numerous potential target mRNAs, we sought to prioritize key candidates. Therefore, we first analyzed these target mRNAs in the context of genome-wide RNA-seq data on P4 *Tdrd7*-/- lenses (Barnum et al., 2020) and normal lens enriched-expression at P4 using the iSyTE database (Kakrana et al., 2018). At P4, RNA-seq showed *Tdrd7*-/- lens exhibited mis-expression of 1982 reduced and 1832 elevated mRNAs (**Table 2**). Further, among the 1982 mRNAs reduced in the *Tdrd7*-/- lens, 574 unique mRNAs (∼29%) were found to be direct targets of 12 miRNAs elevated in the *Tdrd7*-/- lens (hereafter referred to as miRNA-mRNA pairs). Similarly, among the 1832 elevated mRNAs in the *Tdrd7*-/- lens, 535 (∼29%) mRNAs were found to be direct targets of 7 miRNAs reduced in the *Tdrd7*-/- lens (**Table 2**). Thus, 1109 mRNAs collectively mis-expressed and inversely correlated with 19 miRNAs in *Tdrd7*-/- P4 lenses. Next, the inversely correlated miRNA-regulated mRNAs were tested for lens enriched expression at stage P4 lens using the iSyTE database. We found that the target mRNAs in the 87 miRNA-mRNA pairs (representing 54 unique mRNAs; the reduced number for mRNA targets is due to multiple miRNAs sharing target mRNAs) exhibited lens-enriched expression for miRNAs elevated in the *Tdrd7*-/- lens (**Figure 3B**). The target mRNAs in the 81 miRNA-mRNA pairs (representing 59 unique mRNAs) exhibited lens-enriched expression for miRNAs reduced in the *Tdrd7*-/- lens (**Figure 3B**). These data showed that similar proportions of elevated or reduced miRNA target mRNAs exhibited lens-enriched expression in normal development. We extended this analysis by applying a set of distinct filters and by including lens expression data at a later stage (*i.e.,* P30). This strategy applied differential expression data in *Tdrd7*-/- lens at both P4 and P30 and/or normal lens expression at P4 and P30 to identify miRNA-mRNA pairs as either "top-priority", "high-priority" or "promising" candidates (**Figure 3C**). There were 154 miRNA-mRNA pairs (representing 98 unique mRNA targets) for elevated miRNAs and 123 (representing 89 unique mRNA targets) miRNA-mRNA pairs for reduced miRNA. For the elevated miRNAs, these include 18 top-priority candidates, 18 high-priority candidates and 62 promising candidates. For the reduced miRNAs, these include 24 top-priority candidates, 21 high-priority candidates and 44 promising candidates. Further, the inverse association of 154 elevated (98

TABLE 1 | List of differentially expressed miRNAs in postnatal day 4 *Tdrd7*-/- lens.

miRNA	*Tdrd7* -/-	Control	FC	*P*-value
mmu-mir-1198	18.3	30.6	−1.7	0.024
mmu-mir-382	16.6	28.6	−1.7	0.044
mmu-mir-1947	30.6	45.1	−1.5	0.048
mmu-mir-409	62.5	80.6	−1.4	0.019
mmu-mir-3092	14.5	20.1	−1.4	0.021
mmu-let-7b	483.7	627.0	−1.3	0.031
mmu-mir-298	77.9	99.5	−1.3	0.022
mmu-mir-34c	102.5	120.5	−1.2	0.044
mmu-mir-3107	92.4	75.7	1.2	0.049
mmu-mir-378b	27.6	21.3	1.3	0.046
mmu-mir-339	27.3	20.4	1.4	0.031
mmu-mir-384	7.4	5.2	1.4	0.018
mmu-mir-138	9.8	6.5	1.5	0.042
mmu-mir-1224	18.5	12.2	1.5	0.006
mmu-mir-1935	11.0	7.0	1.6	0.018
mmu-mir-328	25.2	16.4	1.6	0.024
mmu-mir-3102	20.0	12.5	1.6	0.008
mmu-mir-19a	13.0	8.2	1.7	0.049
mmu-mir-345	8.2	4.4	1.8	0.027
mmu-mir-1946a	14.5	7.4	1.9	0.035
mmu-mir-467a	15.3	7.5	2.1	0.010
mmu-mir-15a	15.8	7.2	2.2	0.012

TABLE 2 | miRNA-mRNA pairs differentially expressed in Postnatal day 4 *Tdrd7-/-* lens.

miRNA group	miRNA in *Tdrd7-/-* (FC ± 1.2)	mRNA in *Tdrd7-/-* (FC ± 1.2)	miRNA-mRNA pairs in *Tdrd7-/-* P4 lens	miRNA-mRNA pairs enriched in iSyTE at P4	miRNA-mRNA pairs enriched in iSyTE at P30
Up	14	1982 (down)	915/1982 (46.16%) (representing 574/1982 (28.96%) unique mRNAs)	87 (9.50%) (representing 54 unique mRNAs)	128 (13.98%) (representing 71 unique mRNAs)
Down	8	1832 (up)	781/1832 (42.63%) (representing 535/1832 (29.20%) unique mRNAs)	81 (10.37%) (representing 59 unique mRNAs)	96 (12.29%) (representing 64 unique mRNAs)

unique target mRNAs reduced in *Tdrd7-/-* lens, also termed as belonging to "elevated miRNA group") and 123 reduced miRNA-mRNA pairs (89 unique target mRNAs elevated in *Tdrd7-/-* lens, also termed as belonging to "reduced miRNA group") was validated by performing Pearson correlation analysis. The correlation coefficient (r) for elevated and reduced miRNAs with their target mRNAs was -0.975 at a significant p-value of 0.00001 (**Figure 3D**). The "top-priority", "high-priority" and "promising" mRNAs that are targets of elevated and reduced miRNAs – which are also identified among differentially expressed mRNAs in *Tdrd7-/-* lenses – are presented as heat map in the context of normal lens gene expression (**Figures 4**, **5**).

We next examined the conservation of miRNA seed type in target mRNAs across vertebrates using the TargetScan database. In the elevated miRNA group, the following miRNA target mRNAs were conserved across vertebrates: miR-15a target mRNAs- Egln2 and Aatk; miR-19a target mRNAs- Epn2, Plekhg5 and Timp2; miR-138 target mRNA Nfix. While Egln2 and Plekhg5 have a 7mer conserved seed type, Aatk, Epn2, Timp2 and Nfix have an 8mer conserved seed type. In the reduced miRNA group, the following target mRNAs were conserved across vertebrates: let-7b target mRNAs Sh3glb1, Elovl4, Bcap29; and miR-34c target mRNA Tom1 were conserved at 7mer seed type, while miR-34c target mRNA Tpd52 was conserved at 8mer seed type.

Functional Insights Into mRNA Targets of miRNAs in *Tdrd7-/-* Lens

The prioritized candidates from the above analysis represent numerous miRNA-target mRNA genes whose functions are relevant to the observed lens defects in *Tdrd7-/-* mice. To systematically associate cellular function we imported these unique mRNAs (98 from elevated and 89 from reduced miRNA group, please see above) for analysis using the DAVID bioinformatics tool, and identified functional gene clusters (**Figure 6** and **Supplementary Tables 2, 3**). Notably, several significantly enriched gene ontology (GO) categories (enrichment score > 1.0, $p < 0.05$) for the elevated and reduced miRNA groups are relevant to Tdrd7 function in the lens (**Figures 6A,B**). For example, for elevated miRNA group (*i.e.,* reduced mRNAs in *Tdrd7-/-* lens), GO:0042995 "cell projection" contained ten genes. These candidates were Rgs8, Olfm1 (top-priority), Rilpl2, Ncdn, Anks1 (high-priority) and Zfp385a, Plekhg5, Ctnnd2, Mylk, Aatk (promising category). Further, GO:0005604 "basement membrane" contained four genes including Loxl1 (top-priority), Col4a3, Timp2, Timp3 (promising category). This suggests Tdrd7 functions in downstream control of genes involved in cell-cell interaction or connectivity, in turn suggesting their potential impact on *Tdrd7-/-* cataract pathology, which is associated with vacuole-like gaps in fiber cells and posterior capsule rupture (Lachke et al., 2011; Tanaka et al., 2011; Barnum et al., 2020). Other interesting top clusters with GO categories for elevated miRNA group were GO:0035556 "intracellular signal transduction" (with promising candidates Gna12, Rasgrp1, Rassf5, Plekhm1, Prkcd), UP_keyword "metal binding" (with top-priority candidates Alad,

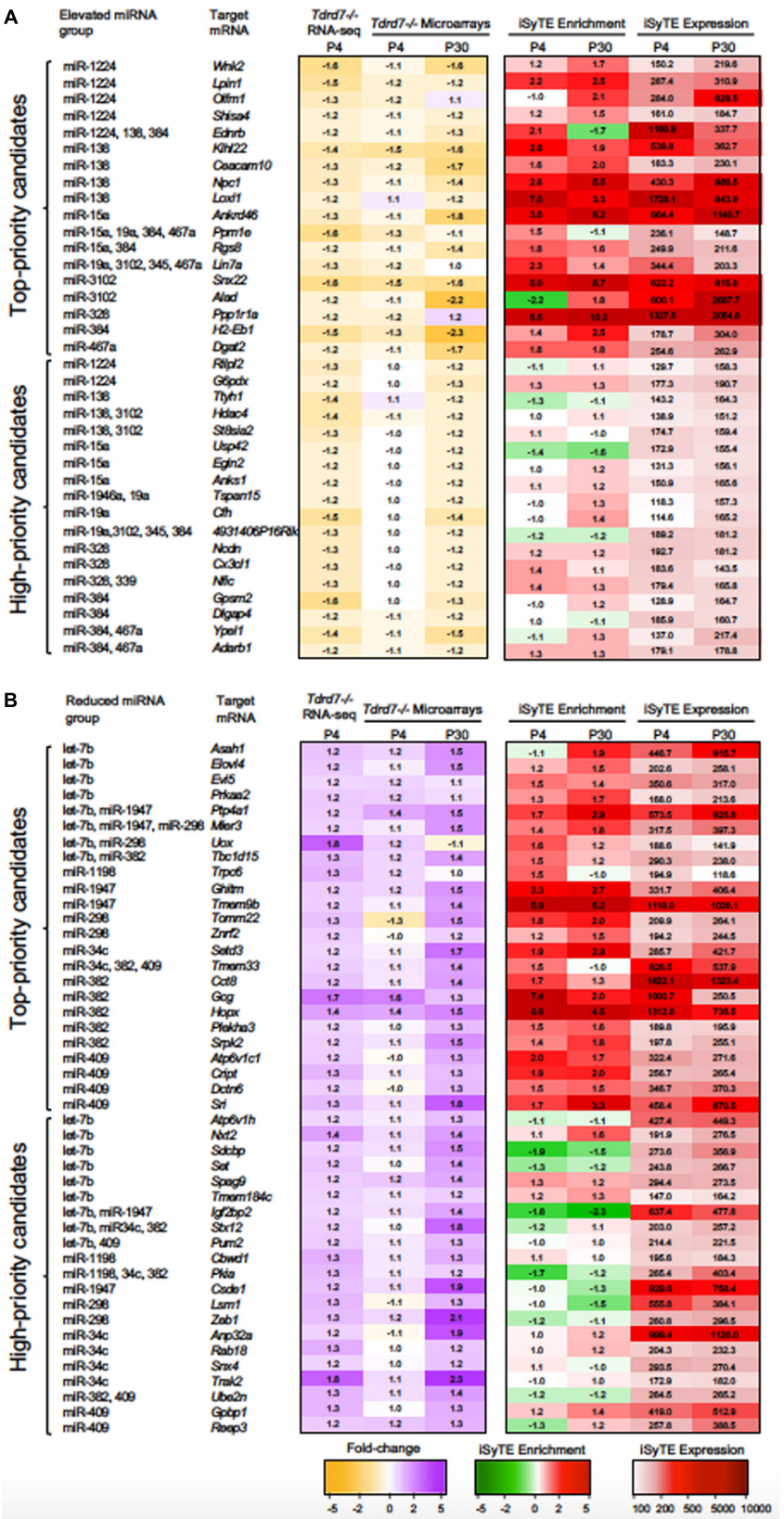

FIGURE 4 | Continued

FIGURE 4 | "Top-priority" and "High-priority" mRNA targets of Tdrd7-downstream miRNAs in the lens. Heat-maps representing expression data of "Top-priority" and "High-priority" mRNA targets of miRNAs identified in **(A)** "Elevated miRNA group" and **(B)** "Reduced miRNA group" in *Tdrd7*-/- lens. Differential expression in P4 (RNA-seq and microarray data) and P30 (microarray data) in *Tdrd7*-/- lens (left) and enriched expression and expression in normal lens as per iSyTE data at postnatal day (P) 4 and P30 (right). "iSyTE Enrichment" represents lens-enriched expression in fold change, while "iSyTE Expression" represents lens expression in fluorescence intensity units at P4 and P30. The mRNA targets are grouped based on the filtering criteria outlined in the text and in **Figure 3C**. Heatmap keys indicate the following: Yellow and purple color gradient represent low to high differential expression in *Tdrd7*-/- compared to control lens (in Fold-change) in RNA-seq data and microarray data. Green and red color gradients represent low to high lens-enrichment (in Fold-change) in iSyTE microarray data. Light-red and red gradients represent low to high lens-expression (in fluorescent intensity units) in iSyTE microarray data.

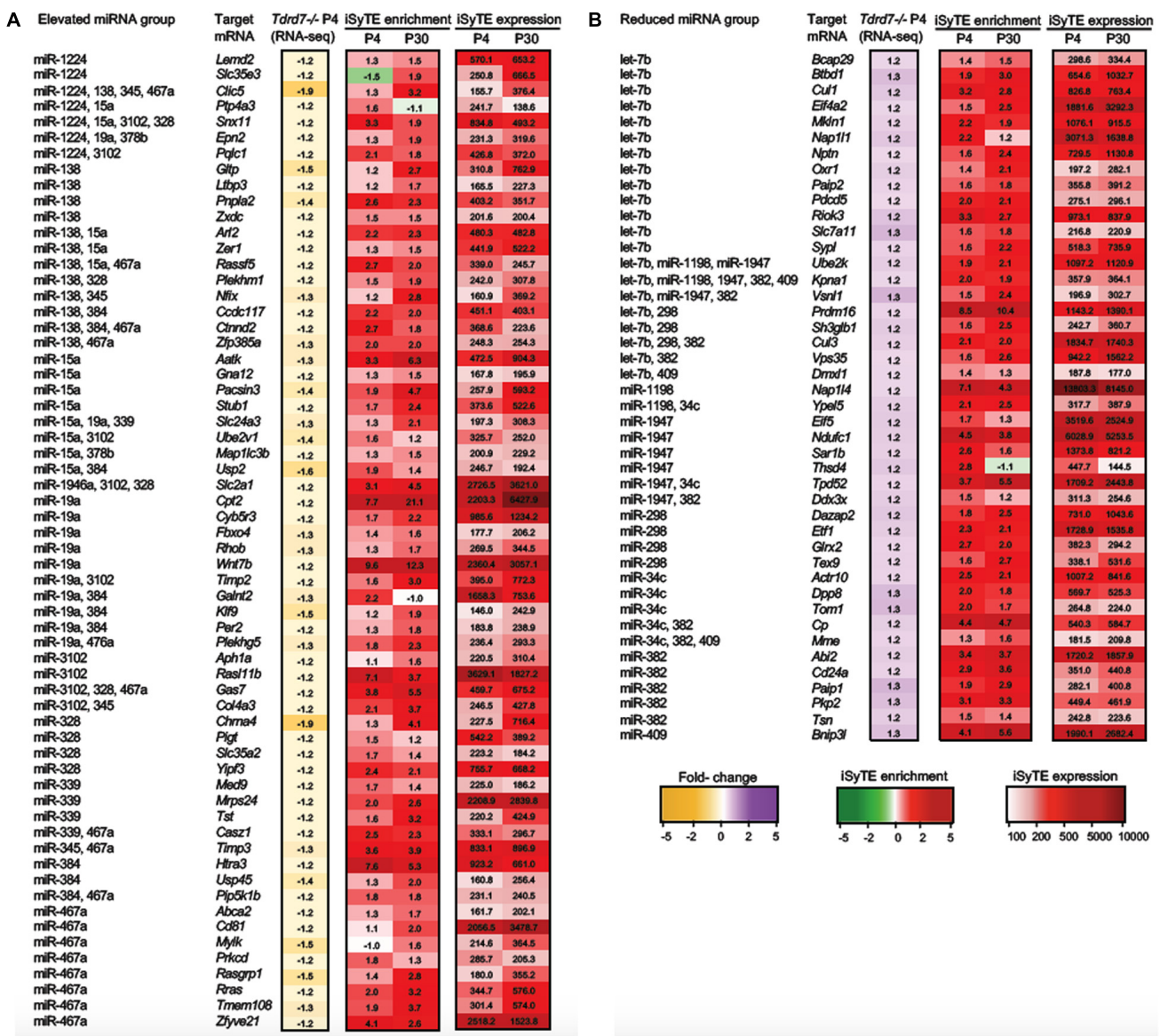

FIGURE 5 | "Promising" mRNA targets of Tdrd7-downstream miRNAs in the lens. Heat-maps representing expression of "Promising" mRNA targets of miRNAs identified in **(A)** "Elevated miRNA group" and **(B)** "Reduced miRNA group" in *Tdrd7*-/- lens. The mRNAs identified as "Promising" targets in the "Elevated miRNA" group are identified as reduced in *Tdrd7*-/- P4 RNA-seq, and are lens-enriched at P4 and/or P30. In the "Reduced miRNA" group the "Promising" targets are identified as elevated in *Tdrd7*-/- P4 RNA-seq and are lens-enriched at P4 and/or P30. Heatmap keys indicate the following: Yellow and purple color gradient represent low to high differential expression in *Tdrd7*-/- lens compared to control lens (in Fold-change) in RNA-seq data. Green and red color gradients represent low to high lens-enrichment (in Fold-change) in iSyTE microarray data at P4 and P30. Light-red and red gradients represent low to high lens-expression (in fluorescent intensity units) in iSyTE microarray data at P4 and P30.

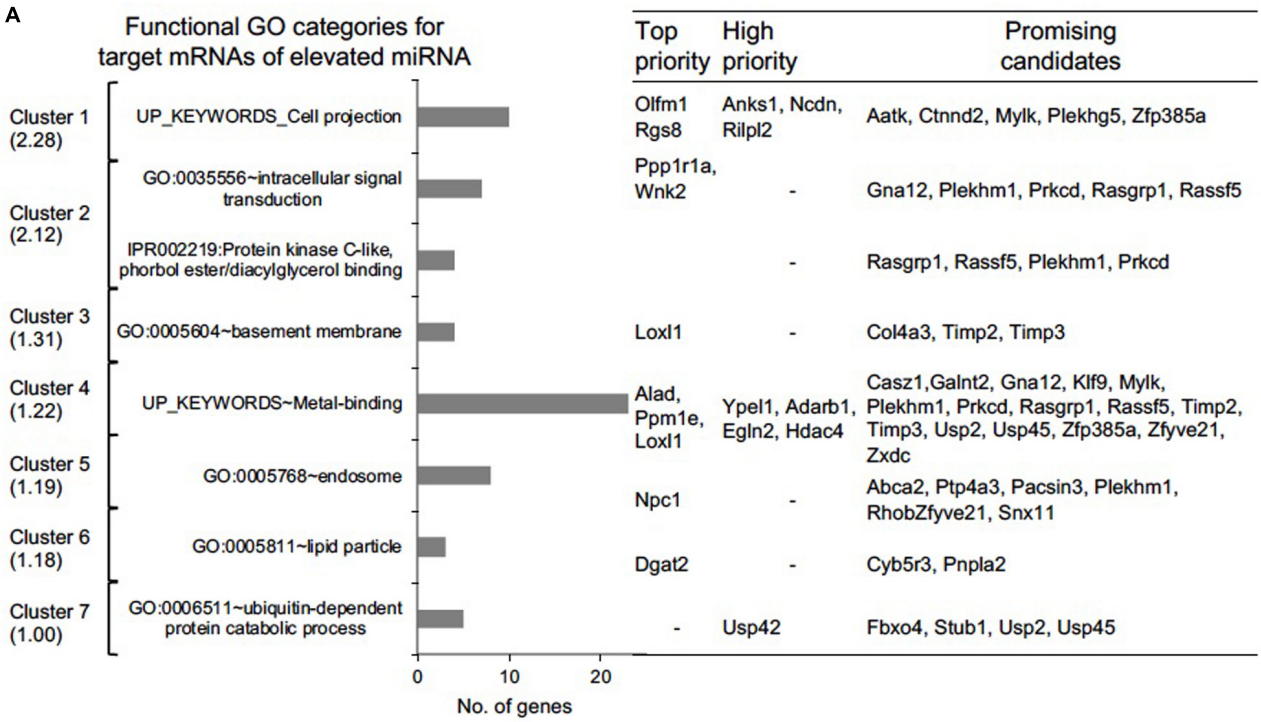

FIGURE 6 | Functional gene ontology (GO) assessment of "Top-priority," "High-priority," and "Promising" mRNA targets of differentially expressed miRNAs in *Tdrd7*-/- lens. Functional GO analysis of **(A)** 154 miRNA-mRNA pairs representing 98 unique mRNA targets for elevated miRNAs and **(B)** 123 miRNA-mRNA pairs representing 89 unique mRNA targets for reduced miRNA using DAVID bioinformatics tool. Candidate mRNAs were grouped in GO clusters, which are presented from high to low cluster enrichment score (left panel). One or more relevant GO category in a cluster is presented as bar graph (middle panel). Number of candidate mRNAs in each cluster are grouped as top, high and promising candidates (right panel).

Ppm1e, Loxl1, high- priority candidates Ypel1, Adarb1, Egln2, Hdac4), GO:0005811 "lipid particle" (with top-priority candidate Dgat2, and promising candidates Cyb5r3 and Pnpla2). On the other hand, the top clusters (enrichment score > 1.2, $p < 0.05$) in reduced miRNA group (*i.e.,* elevated mRNAs in *Tdrd7*-/- lens) were enriched in GO categories such as GO:0044822 "poly(A) RNA binding", GO:0006915 "apoptotic process", GO:0031625 "ubiquitin protein ligase binding", GO:0006810 "transport"

and GO:0006417 "regulation of translation" (**Figure 6B**). The upregulated genes (*i.e.,* reduced miRNA group) such as Pum2, Ddx3x are commonly found in the GO categories of poly(A) RNA binding and regulation of translation and Ddx3x is also present in the GO for apoptotic process. These GO categories defining the differentially expressed target mRNAs could help explain how misregulation of genes result in cataract. For example, these elevated targets could represent an attempt by lens cells to correct for the defects in post-transcriptional control resulting from Tdrd7-deficiency. Furthermore, elevated transcripts for genes associated with apoptotic process could help explain the lens defects such as large "gaps" (sometime referred to as "vacuoles") in the fiber cell compartment of *Tdrd7*-/- mice.

Tdrd7-Downstream miRNA-Based Coordinated Control in the Lens

Notably, miRNAs can function individually or in a coordinated manner to mediate regulation of their target mRNAs. A single miRNA can regulate multiple target mRNAs in a specific pathway or multiple miRNAs can converge on a single target mRNA to mediate its control. To gain insights into such coordinated control events potentially mediated by Tdrd7-downstream miRNAs in the lens, we examined coregulatory relationships among the "top-priority", "high-priority" and "promising" mRNAs (as defined above). An individual miRNA can have multiple targets that are commonly identified in the same GO category. For example, the upregulated miRNA, miR-138, targets the mRNAs encoding Plekhm1 and Rassf5 that are commonly found in GO category "signal transduction", while miR-345 targets Col4a3 and Timp3 mRNAs that are commonly found in the GO category "basal membrane". On the other hand, we found single miRNA targeting mRNAs involved in varying cellular function. For example, miR-1224 targets eight mRNAs with diverse functions, namely, the enzymes G6pdx (dehydrogenase), Lpin1 (phosphohydrolase) and Wnk2 (kinase), an ER-localized protein with unknown function (Olfm1), a member of Shisha family (Shisa4), a lysosome morphology regulator (Rilpl2), a transmembrane protein (Lemd2) and a solute carrier family protein (Slc35e3). Similarly, miR-15a alone targets eight mRNAs such as Aatk (apoptosis associated tyrosine kinase), Ankrd46 and Anks1 (both associated with cytoskeletal regulations), Egln2 (oxygen homeostasis), Gna12 (signaling), Pacsin3 (protein kinase C involved in linking actin cytoskeleton with vesicle formation), Stub1 (ubiquitin ligase/co-chaperone) and Usp42 (de-ubiquitination). Other such miRNAs that alone target multiple mRNAs are miR-19a, miR-138, miR-328, miR-384, miR-3102 and miR-467a. Conversely, in the elevated miRNA group *(i.e.,* with reduced target mRNAs in *Tdrd7*-/- lens), 39 mRNAs were a common target for multiple miRNAs (**Figures 4**, **5**). For example, both miR-384 and miR-15a have two targets in common, namely those encoding a protein phosphatase (Ppm1e) and a G-protein regulator (Rgs8) in the GO categories "cell projection" and "metal binding", respectively. Further, six mRNAs with varying cellular functions such as Clic5 (involved in actin-based cytoskeletal structures), Ednrb (a receptor molecule), Lin7a (involved in maintaining cell membrane receptors and channels), Ppm1e (serine/threonine-protein phosphatases), Snx11 (a member of the sorting nexin family), 4931406P16Rik (unknown function) are common targets of different combinations of four miRNAs that are elevated in the *Tdrd7*-/- lens. Interestingly, for upregulated miRNAs, cohorts of mRNA identified in key GO categories were found to be targets of multiple miRNAs. For example, mRNAs involved in Ras signaling (Rasgrp1, Rasl11b, Rassf5, Rhob, Rras) were targets of multiple combinations of miRNAs (*e.g.,* miR-138, miR-15a, miR-19a, miR-3102 and miR-467a). Similarly, mRNAs encoding proteins in solute carrier family (Slc24a3, Slc2a1, Slc35a2, Slc35e3), metallopeptidases (Timp2, Timp3), proteins related to ubiquitin (Ube2v1, Usp2, Usp45) and zinc-finger proteins (Zfp385a, Zfyve21, Zxdc) were targets of multiple combinations of miRNAs.

In the reduced miRNA group *(i.e.,* with elevated target mRNAs in *Tdrd7*-/- lens), a similar trend in miRNA-mRNA connectivity was identified. For example, 23 mRNAs were a common target for multiple miRNAs (**Figures 4**, **5**). Interestingly, the reduced miRNAs let-7b, miR-1198, miR-1947, miR-382 and miR-409 share a common mRNA target Kpna1[karyopherin (importin) alpha 1]. Further, combinations of multiple reduced miRNAs (*e.g.,* let-7b, miR-34c, miR-382, miR-1198, miR-1947, miR-298 and miR-409) commonly target the following mRNAs: Cul3 (polyubiquitination), Pkia (protein kinase inhibitor alpha), Stx12 (syntaxin), Ube2k (ubiquitin conjugating enzyme) and Vsnl1 (calcium sensor), which are found to be reduced in the *Tdrd7*-/- lens. Conversely, a single reduced miRNA, let-7b, has multiple mRNA targets, namely Cul1, Bcap29, Pdcd5 and Sh3glb1 that are commonly categorized in GO term 'apoptotic process' and are up-regulated in *Tdrd7*-/- lens. This data suggest that upregulated miRNAs have common targets that are involved in similar cellular pathways and are misregulated in *Tdrd7*-/- lens. In the reduced miRNA group, mRNAs encoding translation initiation factors (Eif4a2, Eif5, Etf1), nucleosome assembly proteins (Nap1l1, Nap1l4), polyadenylate binding proteins (Paip1, Paip2) were regulated by one or more different combination of miRNAs (let7b, miR1947, miR-1198, miR298 and miR-382), revealing miRNA-mRNA connectivity and their promising function in *Tdrd7*-/- lens (**Figure 6B**). Together, these analyses uncover the complexity of the Tdrd7-downstream miRNA-based control in the lens.

Derivation of Common Regulatory Networks Between Tdrd7-Downstream miRNA-mRNAs and Other Key Lens Regulators

We next sought to examine which downstream mRNAs are common between Tdrd7-regulated miRNA targets and those of other key regulators implicated in lens development and cataract. We focused on the top-priority Tdrd7-downstream mRNA targets for both elevated and reduced miRNA groups (*i.e.,* mRNA targets of miRNAs elevated or reduced in *Tdrd7*-/- lens; see above) and examined which of these were also misexpressed in different gene-specific perturbation (either gene-specific knockout, dominant negative or overexpression, representing

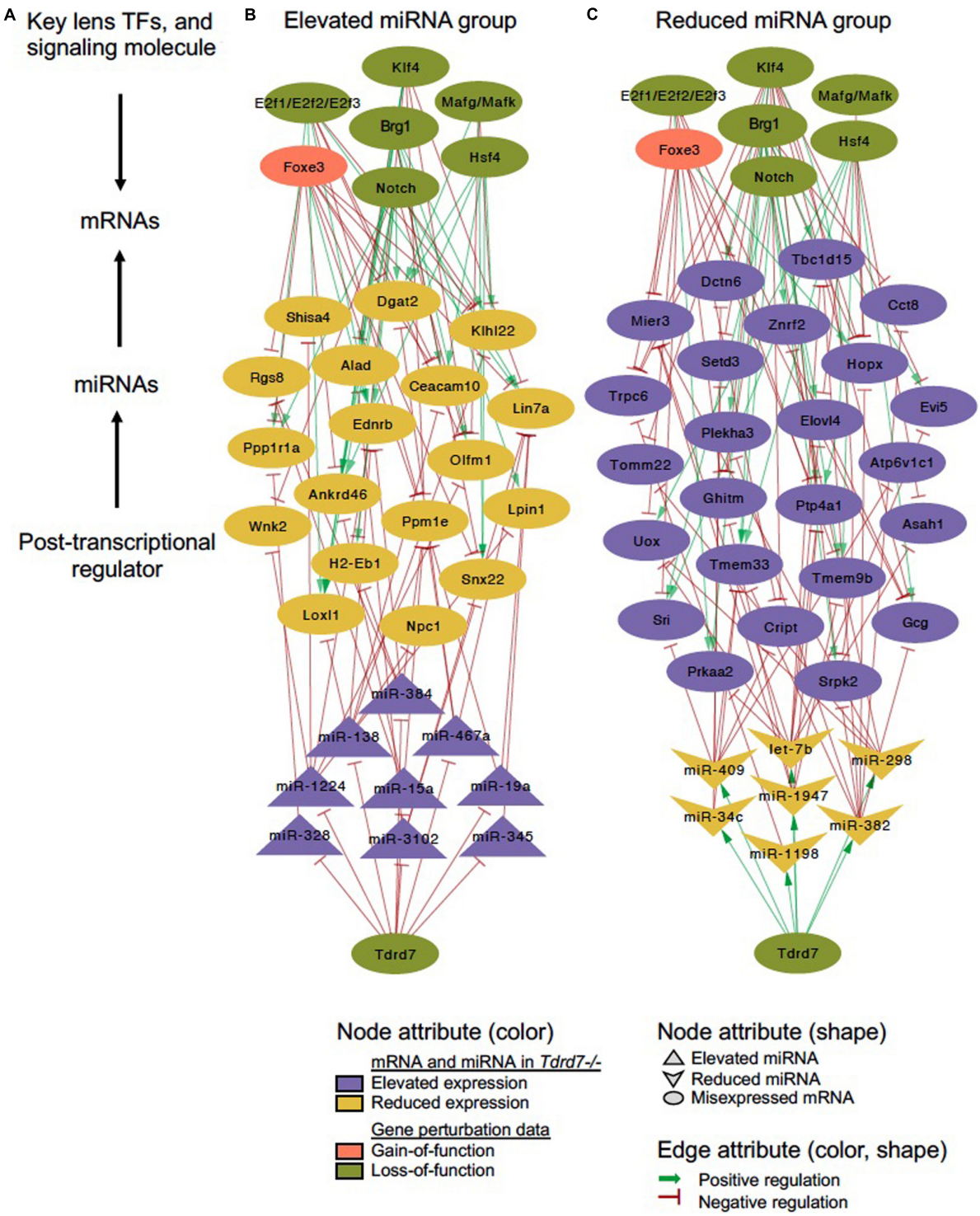

FIGURE 7 | *Tdrd7* downstream global miRNA network and connectivity with key regulators in the lens. Global network analysis of misexpressed miRNA and mRNA targets in *Tdrd7*-/- lens reveals their connectivity with known lens regulators, namely the transcription factors, Brg1, E2f1/E2f2/E2f3, Foxe3, Hsf4, Klf4, Mafg/Mafk, and the signaling molecule, Notch. **(A)** schematic of global network connectivity of key factors in the *Tdrd7*-/- lens. **(B,C)** A regulatory network based on elevated and reduced miRNAs in *Tdrd7*-/- lens and their connectivity with target mRNAs, majority of which are also downstream of several key lens transcription/signaling factors. The edges color/shape represent positive regulation (dark green, arrow) and negative regulation (red, inhibitory sign) between miRNAs or transcription/signaling factors and mRNA targets. The transcription/signaling factor perturbation data in various mouse models is obtained from independent studies using gain-of-function (pink) or loss-of-function (pale green) approaches (details in the Methods section). Node shapes represents miRNA (elevated/reduced), mRNA and transcription/signaling factors.

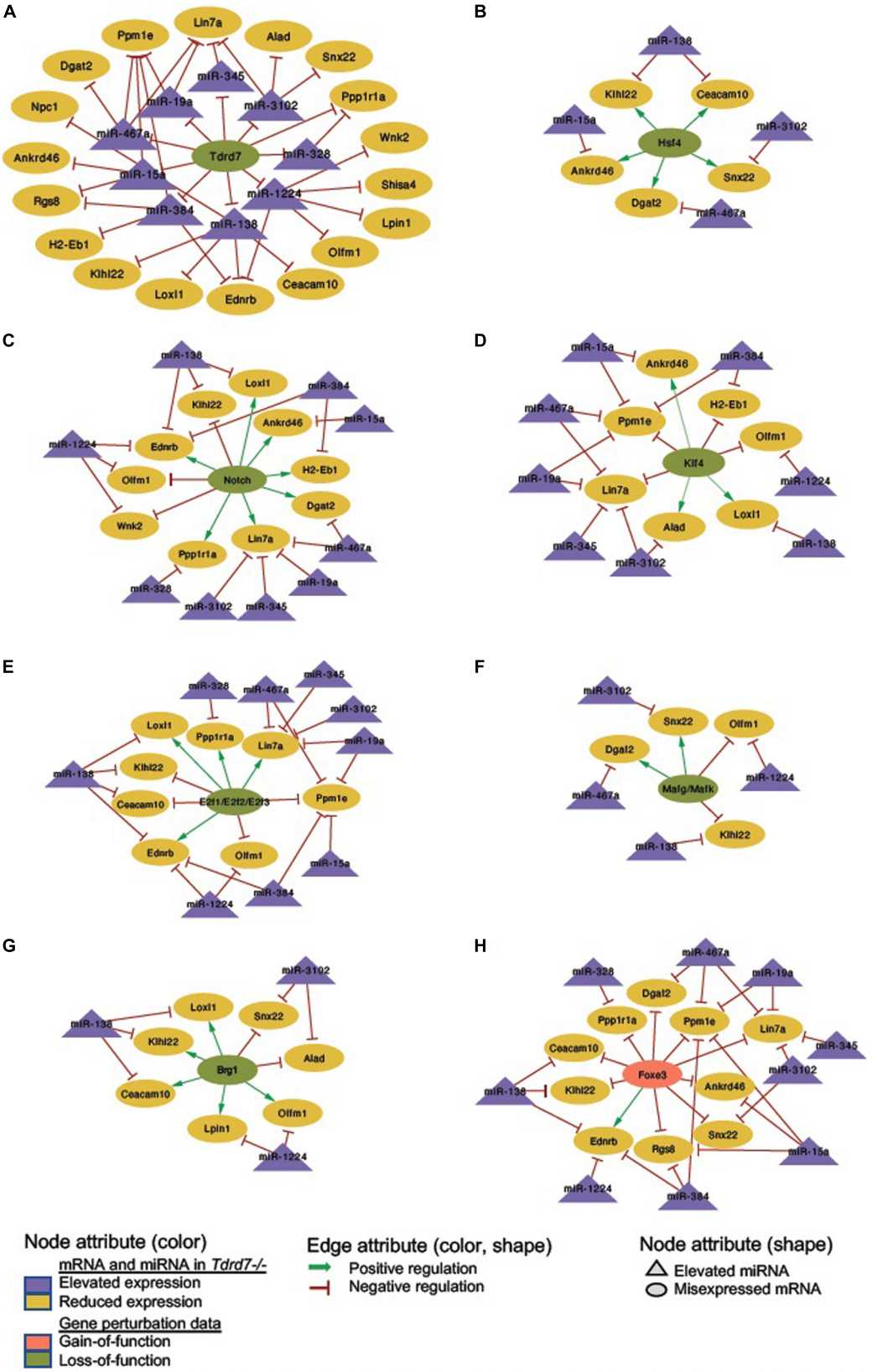

FIGURE 8 | Continued

FIGURE 8 | Regulatory networks for Tdrd7-downstream negatively regulated miRNAs and their mRNA targets and the relationship of these mRNA target with key lens regulators. **(A)** Network depicting Tdrd7 downstream negatively regulated miRNAs and the relationship with their predicted target mRNAs based on interpretation of *Tdrd7-/-* lens transcriptomics data. Deletion of Tdrd7 (pale green node) results in elevation of downstream miRNAs and therefore the relationship between Tdrd7 and these miRNA in normal lens is shown by red color inhibitory edge. In the *Tdrd7-/-* lens, this elevation of miRNAs results in repression of their target mRNAs, which are found to be reduced. Therefore, the relationship between the miRNAs and these target mRNAs in normal lens is indicated by red color inhibitory edge. Similarly derived networks based on lens microarray data on loss-of-function conditions of **(B)** Hsf4, **(C)** Notch, **(D)** Klf4, **(E)** E2f1/E2f2/E2f3, **(F)** Mafg/Mafk, **(G)** Brg1, and gain-of-function (in fiber cells) of **(H)** Foxe3 shows the regulatory relationship between these lens regulators and the Tdrd7-downstream negatively regulated miRNAs in the lens. The key provides information on the Edges, Nodes and gene expression conditions and directionality.

loss-of-function or gain-of-function conditions) mouse models of lens defects/cataract that were subjected to meta-analysis in iSyTE (Kakrana et al., 2018). For key lens regulators, we focused on various transcription factors (*e.g.,* Brg1, E2f1/2/3, Foxe3, Hsf4, Klf4, Mafg, Mafk) and signaling pathways (*e.g.,* Notch) (Landgren et al., 2008; He et al., 2010; Saravanamuthu et al., 2012; Gupta et al., 2013; Agrawal et al., 2015; Anand et al., 2015). Based on this analysis, we derived a regulatory network which demonstrates how signaling, transcription and post-transcriptional regulatory pathways mediate combinatorial control over expression of key genes in the lens (**Figure 7**). To gain insight into the connectivity between Tdrd7-downstream miRNA-mRNA pairs and the above key lens defects/cataract-linked genes, we derived individual regulatory modules that effectively discern the node-edge relationship for individual gene perturbation conditions (**Figures 8**, **9**). The regulatory network module for Tdrd7 shows its relationship with 18 downstream reduced mRNAs (Alad, Ankrd46, Ceacam10, Dgat2, Ednrb, H2-Eb1, Klhl22, Lin7a, Loxl1, Lpin1, Npc1, Olfm1, Ppm1e, Ppp1r1a, Rgs8, Shisa4, Snx22 and Wnk2) (**Figure 8A**). In normal lens, Tdrd7 negatively regulates 9 miRNAs in this network (miR-15a, miR-19a, miR-138, miR-328, miR-345, miR-384, miR-467a, miR-1224, miR-3102), which target these 18 mRNAs (**Figure 8A**). Majority of these mRNAs (16 out of 18; 89%) are also mis-expressed in seven other gene perturbation conditions (**Figures 8B–H**). Out of these 16, majority (81%) have one or more regulatory edge(s) in the same direction as in *Tdrd7-/-* lenses. Interestingly, Ankrd46, Ednrb, Dgat2 and Lox11 are all mis-expressed in multiple gene perturbation conditions and in the same direction as in *Tdrd7-/-* lenses. Next, we derived the regulatory network module for Tdrd7 displaying its relationship with 24 downstream elevated mRNAs (**Figure 9A**). In normal lens, Tdrd7 positively regulates 7 miRNAs in this network (let-7b, miR-34c, miR-298, miR-382, miR-409, miR-1198, miR-1947), which target these 24 mRNAs (**Figure 9A**). Almost all (23 out of 24) of these mRNAs are also mis-expressed in the seven other gene perturbation conditions (**Figures 9B–H**). Out of these 23, a majority (78%) have at least one regulatory edge in the same direction as in *Tdrd7-/-* lenses. Interestingly, Cript1, Gcg, Ghitm, Hopx, Mier3, Ptp4a1, Trpc6 are all mis-expressed in multiple gene perturbation conditions and in the same direction as in *Tdrd7-/-* lenses. Overall, these analyses suggest that many distinct regulatory pathways converge on common targets to mediate precise control over their expression in the lens. Finally, fibroblast growth factor signaling has been shown to control lens fiber cell differentiation (Padula et al., 2019) and Tdrd7 expression and function has been shown to be important in fiber cells as well (Lachke et al., 2011; Barnum et al., 2020). Previously, miRNA profiling has been performed on FGF2-induced rat lens explants (Wolf et al., 2013). To identify miRNA common to these pathways, we next compared Tdrd7-downstream miRNAs (present study) with the FGF2-induced lens explant miRNAs dataset (Wolf et al., 2013). In both studies, miRNAs miR-138, miR-328, miR-345 were commonly found to be significantly elevated, indicating that Fgf and Tdrd7 regulatory pathways potentially converge to mediate control over select downstream miRNAs in lens fiber cells.

Microarray Profiling Identifies Highly Expressed miRNAs in Early Postnatal Lens

Finally, the miRNA profiling by microarray allows an opportunity to assemble a global catalog of the different miRNAs that are robustly expressed in the early postnatal mouse lens. Therefore, we next examined the highly expressed miRNAs (n = 31) defined as those having expression intensity $\geq$ 500 ($p \leq 0.05$) in the control P4 lens (**Table 3**). This analysis identified 26 new highly expressed miRNAs in the lens while also validating the high expression of previously identified miRNAs (**Supplementary Table 4**). For example, the miRNAs miR-5105, miR-5109, miR-1298 and miR-378 were newly identified to be highly expressed in the lens. Further, this work offered independent support for the high expression of numerous miRNAs that were previously described in the lens. For example, the present study validates miRNA expression in the mouse lens in agreement with previous studies using microarrays, RNA-sequencing, and *in situ* hybridization (**Supplementary Table 4**) (Conte et al., 2010; Karali et al., 2010; Khan et al., 2016). Interestingly this analyses also identified several miRNAs (miR-184, miR-26a, miR-204, let-7b and let-7c) (**Table 3**) that were previously found to be misexpressed in human cataractous lenses, again offering independent support that these miRNAs are of significance to lens biology and cataract (Wu et al., 2012, 2017). Further, many FGF2-regulated miRNAs that were described in a previous study (Wolf et al., 2013) were also found to be highly expressed in the lens in the present study, offering independent support that miRNA function is important in lens development. Thus, this study identifies many miRNAs with high expression in the lens, which can be candidates for future investigations in lens development and cataract pathology.

CONCLUSION

These findings suggest that Tdrd7-downstream miRNAs function to maintain optimal levels and specificity of the mRNA

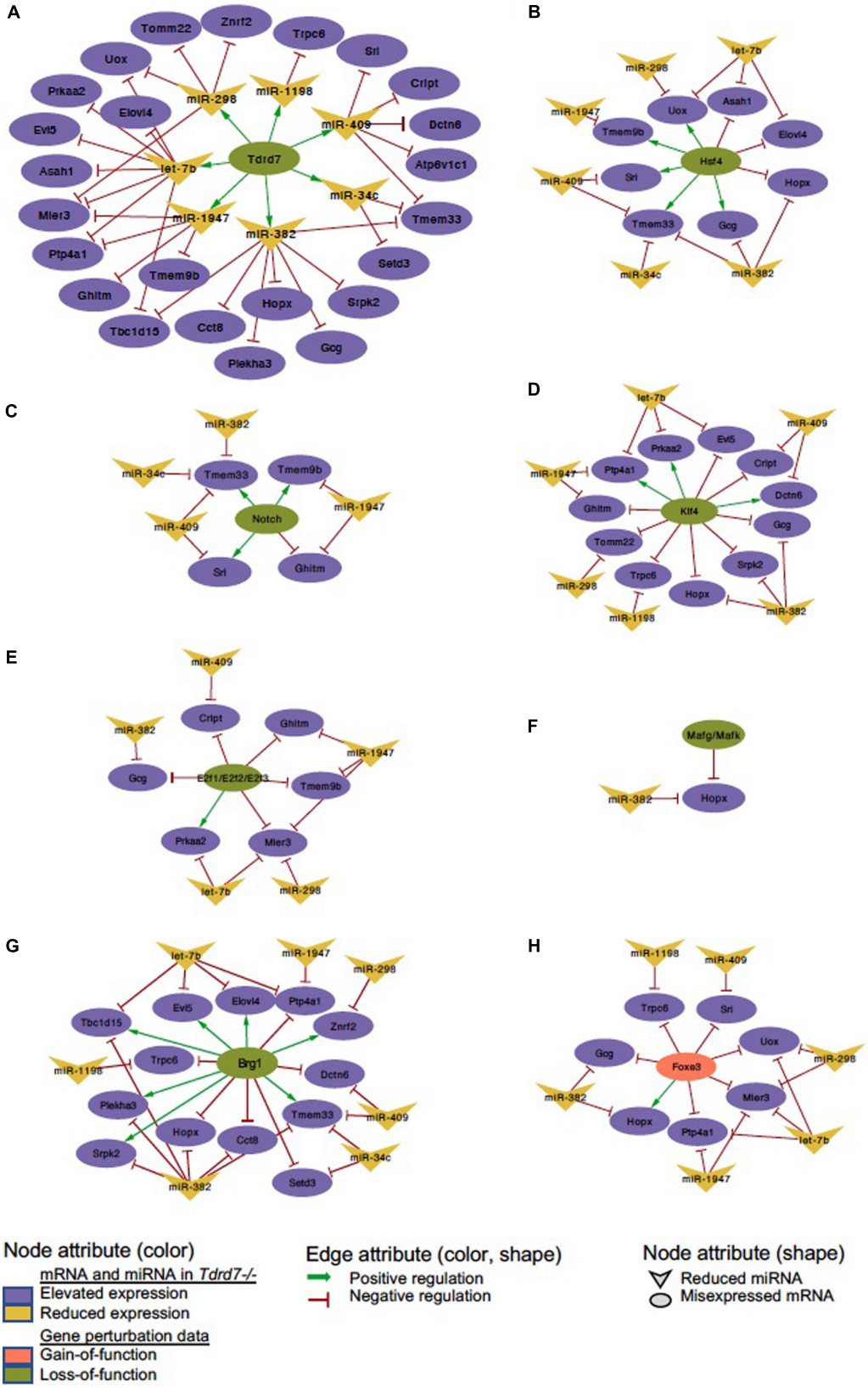

FIGURE 9 | Continued

FIGURE 9 | Regulatory network for Tdrd7-downstream positively regulated miRNAs and their mRNA targets and the relationship of these mRNA target with key lens regulators. **(A)** Network depicting Tdrd7 downstream positively regulated miRNAs and the relationship with their predicted target mRNAs based on interpretation of *Tdrd7-/-* lens transcriptomics data. Deletion of Tdrd7 (pale green node) results in reduction of downstream miRNAs, thus representing positive control by Tdrd7 in normal lens (therefore indicated by green arrow edge). This results in release of repression of the predicted target mRNAs of these miRNAs in the *Tdrd7-/-* lens (where these mRNA are found to be elevated) and thus the relationship between miRNA and these mRNAs in normal lens is indicated by red color inhibitory edge. Similarly derived networks based on lens microarray data on loss-of-function conditions of **(B)** Hsf4, **(C)** Notch, **(D)** Klf4, **(E)** E2f1/E2f2/E2f3, **(F)** Mafg/Mafk, **(G)** Brg1, and gain-of-function (in fiber cells) of **(H)** Foxe3 shows the regulatory relationship between these lens regulators and the Tdrd7-downstream positively regulated miRNAs in the lens. The key provides information on the Edges, Nodes and gene expression conditions and directionality.

transcriptome in the lens, the misexpression of which may contribute to cataract pathology. This is in line with the notion that many small changes orchestrated by several miRNAs may contribute toward fine-tuning gene expression in a cell/tissue (Lim et al., 2005). Regulatory connections between many genes relevant to lens biology and pathology were identified in this study. For example, candidates associated with cell projection, such as Mylk, Olfm1, Plekhg5 and Rgs8, among others, were in the reduced mRNAs (elevated miRNAs) category in the *Tdrd7-/-* lens. Further, in "intracellular signal transduction" category, Rasgrp1 was identified among other candidates. This is interesting because in an independent study we recently found Rasgrp1 to be involved in the rescue of fiber cell defects in *Fgfr2:Pten* compound null lenses (Padula et al., 2019). In the GO category "basement membrane", Col4a3, Loxl1, Timp2 and Timp3 were identified as reduced mRNA candidates, which is relevant to the lens capsular defects observed in *Tdrd7*-defcient lenses, especially because *COL4A3* (Collagen Type IV Alpha-3) and LOXL1 (lysyl oxidase-like 1) mutations are associated with cataract and glaucoma in humans (Thorleifsson et al., 2007; Uzak et al., 2013). Further, this analysis also identified – among the promising candidates – the transcription factor *CASZ1*, which was previously predicted by iSyTE as potentially important in lens (Kakrana et al., 2018) and recently found in a genome-wide association study (GWAS) to be associated with cataract in humans (Choquet et al., 2020). Finally, several genes in the GO category "apoptotic process" were found to be elevated (reduced miRNAs) in the *Tdrd7-/-* lens, offering an explanation for the large vacuole-like gap defects observed in the fiber cells of Tdrd7-defecient cataractous lenses. To test individual or combinatorial contributions in the lens of the cohort of miRNAs described here, simultaneous gain/loss of function experiments involving these miRNAs will have be to performed in the future. Also, the present study does not assess the impact of these miRNAs on the level of proteins in the lens. This can be addressed in future studies determining the proteome profile of *Tdrd7-/-* lens at a stage prior to the onset of lens defects. It will also be interesting to examine the nature of control (whether direct or indirect) that is mediated by Tdrd7 over miRNAs in the lens. In sum, the data presented here indicate that Tdrd7 coordinates distinct downstream regulatory events–either through miRNA-mRNA interactions or through protein-mRNA interactions–to mediate post-transcriptional gene expression control in lens development, misregulation of which causes lens defects and congenital cataract.

TABLE 3 | Highly expressed miRNAs in Postnatal day 4 mouse lens.

miRNA ID	miRNA expression intensity at P4
mir-184	15592.3
mir-709	8631.6
mir-31	5871.0
let-7e	3255.7
mir-26a	3129.2
mir-17	2697.1
mir-181a	2298.5
mir-181b	2227.2
mir-125a	2226.9
mir-99b	2181.6
mir-20a	1956.3
let-7c-1	1708.8
mir-24	1397.8
mir-125b	1339.0
mir-103	1280.4
mir-23b	1133.3
mir-23a	1088.0
mir-191	947.9
mir-93	939.8
mir-107	910.4
let-7a	889.8
mir-5105	752.1
mir-16	727.8
mir-5109	684.1
mir-92a	634.0
let-7b	627.0
mir-106a	601.0
mir-130a	594.7
mir-5126	590.7
let-7d	590.4
mir-204	515.4

DATA AVAILABILITY STATEMENT

The miRNA microarray datasets generated for this study can be found in the Gene Expression Omnibus database under accession number GSE157061.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Delaware Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

DA, SA, CB, SS, SC, and SL contributed to the generation of the data. DA, SA, and SL analyzed the data. DA and SL wrote the manuscript. All the authors contributed to the revision 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.2021.615761/full#supplementary-material

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MicroRNA Signatures of the Developing Primate Fovea

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Rod and cone photoreceptors differ in their shape, photopigment expression, synaptic connection patterns, light sensitivity, and distribution across the retina. Although rods greatly outnumber cones, human vision is mostly dependent on cone photoreceptors since cones are essential for our sharp visual acuity and color discrimination. In humans and other primates, the *fovea centralis* (fovea), a specialized region of the central retina, contains the highest density of cones. Despite the vast importance of the fovea for human vision, the molecular mechanisms guiding the development of this region are largely unknown. MicroRNAs (miRNAs) are small post-transcriptional regulators known to orchestrate developmental transitions and cell fate specification in the retina. Here, we have characterized the transcriptional landscape of the developing rhesus monkey retina. Our data indicates that non-human primate fovea development is significantly accelerated compared to the equivalent retinal region at the other side of the optic nerve head, as described previously. Notably, we also identify several miRNAs differentially expressed in the presumptive fovea, including miR-15b-5p, miR-342-5p, miR-30b-5p, miR-103-3p, miR-93-5p as well as the miRNA cluster miR-183/-96/-182. Interestingly, miR-342-5p is enriched in the nasal primate retina and in the peripheral developing mouse retina, while miR-15b is enriched in the temporal primate retina and increases over time in the mouse retina in a central-to-periphery gradient. Together our data constitutes the first characterization of the developing rhesus monkey retinal miRNome and provides novel datasets to attain a more comprehensive understanding of foveal development.

Keywords: microRNAs, retinal development, fovea, miR-342-5p, miR-15b, rhesus monkey

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INTRODUCTION

Sight is often considered our most fundamental sense to perceive and navigate the world and, as a result, vision loss has a devastating impact on everyday life. Visual perception begins when photons of light enter the eye and are absorbed by the photoreceptors, the light-sensitive cells of the retina. There are two classes of photoreceptors named rods and cones because of their distinctive morphologies. While both populations contribute to the information transmitted to the visual centers of the brain by the optic nerve, these two cell types serve different purposes: rods

are highly sensitive to light and provide relatively coarse, colorless images, while cones require considerably brighter light and are responsible for our sharp chromatic vision and spatial acuity (Arshavsky and Burns, 2012).

All photoreceptors are localized in the outer nuclear layer of the retina and are organized in a mosaic pattern that varies in different organisms to fit their environments and behaviors (Raymond et al., 1995; Bruhn and Cepko, 1996; Szel et al., 1996; Fadool, 2003; Viets et al., 2016). In most mammals, rods outnumber cones by orders of magnitude; in the mouse retina, rods constitute 97.2% and cones are 2.8% of all photoreceptors [38:1 rod to cone ratio, (Carter-Dawson and LaVail, 1979; Jeon et al., 1998)], while the human retina contains an average of 92 million rods and 4.6 million cones [20:1 rod to cone ratio, (Curcio et al., 1987)]. In humans and other primates, most of the cones are confined to a small region of the central temporal retina called the *macula lutea* (macula), where the cone concentration is about 200-fold higher than the most eccentric retinal regions (Curcio et al., 1987, 1990; Hendrickson et al., 2012; Hendrickson, 2016). At the center of the macula, a small indentation marks the location of the *fovea centralis* (fovea) corresponding with the center of the visual field. At the foveal pit, all photoreceptors are densely packed cones and there are virtually no rods (O'Brien et al., 2004; Springer and Hendrickson, 2005; Dubis et al., 2012; Provis et al., 2013). Despite the overall predominance of rod photoreceptors, primates have evolved to primarily utilize cone pathways, and most of our useful photopic vision depends on the cones in the fovea such that a 2-millimeter lesion in this area will result in legal blindness.

Age-related macular degeneration (AMD), one of the most prevalent types of photoreceptor degeneration, affects millions of people worldwide, and causes irreversible vision loss from the selective degeneration of the photoreceptors of the fovea (Wong et al., 2014). It has been estimated that AMD affects up to 25% of the United States population over the age of 80 (Friedman et al., 2004), illustrating the urgent need for novel treatments to restore the cones of the fovea. Efforts to develop therapies aimed at cone replacement will inevitably require preclinical studies using non-human primates, but our understanding of primate retinogenesis is still incomplete. Similarly, despite the fundamental importance of the fovea for human vision, the molecular mechanisms that guide the development of this region as well as the pathways that regulate the higher ratios of cone production remain largely unresolved.

During retinal development, different classes of retinal populations are consecutively added in a well-known sequence that is conserved in all vertebrates (Sidman, 1961; Young, 1985; Cepko et al., 1996; Livesey and Cepko, 2001): Retinal ganglion cells (RGCs), cone photoreceptors, and horizontal cells are the first cell populations to be born, followed by amacrine cells and rod photoreceptors, and finally, bipolar cells and Müller glia are born last. Classic lineage-tracing studies showed that retinal progenitor cells are multipotent such that one single type of progenitor cell has the ability to differentiate into multiple postmitotic cell types. Evidence from heterochronic transplants (McConnell, 1985; Watanabe and Raff, 1990; Belliveau et al., 2000; Rapaport et al., 2001), in which neural progenitors were transplanted into an environment of a different age and, more recently, single-cell transcriptomics (Clark et al., 2019; Lu et al., 2020; Sridhar et al., 2020) has revealed that (1) retinal progenitors are intrinsically restricted, and (2) retinal progenitors pass through waves of competence to acquire and lose the ability to make specific cell types at different developmental stages.

MicroRNAs (miRNAs) are small RNA molecules known to regulate several aspects of development. To date, over 2,000 miRNAs have been recorded in miRbase (miRbase.org) (Kozomara and Griffiths-Jones, 2014) and both computational and experimental analyses indicate that most protein-coding genes are regulated by one or more miRNAs (Baek et al., 2008; Selbach et al., 2008). The essential roles of miRNAs in cell fate acquisition and central nervous system (CNS) patterning are well established. miRNAs are known to regulate neural progenitor competence *in vivo* (Georgi and Reh, 2011; La Torre et al., 2013; Saurat et al., 2013; Shu et al., 2019; Wohl et al., 2019) and *in vitro* (Andersson et al., 2010; Patterson et al., 2014), and some miRNAs have been associated with the production of specific cell types (Bian et al., 2013; Nowakowski et al., 2013; Patterson et al., 2014; Wohl and Reh, 2016).

Given the vast importance of miRNAs as developmental regulators, we have sought to characterize the miRNome of the early developing non-human primate retina, specifically the rhesus monkey (*Macaca mulatta*), an Old World non-human primate. We have generated transcriptomic profiles of rhesus retinas at three developmental time points, spanning the major stages of development, and we have used miRNA-sequencing technologies to identify miRNAs differentially expressed in the presumptive fovea (temporal posterior side of the retina) compared to its equivalent region at the other side of the optic nerve head (nasal posterior) at early stages of retinal development. In addition, we have chosen miRNAs with significant differential expression between retinal regions and we have validated their expression using *in situ* hybridization in mouse and human samples. Together, our data provides invaluable resources for studies aimed at understanding the role of miRNAs in retinal development as well as datasets to broaden our knowledge of foveal development.

RESULTS

Transcriptomic Characterization of the Developing Rhesus Monkey Retina

Total RNA was obtained from retinal punches (approximately 2.5 mm in diameter) from the prospective fovea (temporal side) and the equivalent region at the other side of the optic nerve head (nasal side), from three different time points spanning the three trimesters [50 days gestational age (late first trimester), 90 days (second trimester), and 150 days (third trimester); term 165 ± 10 days]. Rhesus monkey trimesters are divided by 55-day increments (0–55, 56–110, and 111–165 days) (Tarantal, 2005). We performed Next Generation Sequencing (NGS) analyses (50 days: 6 samples, 3 temporal, and 3 nasal, 90 days and 150 days: 2 samples each, 1 temporal, and 1 nasal for each ontogenic stage). After the pre-processing pipeline and quality

controls, more than 89% of the reads were aligned with the rhesus monkey genome (reference genome: Mmul_1; annotation reference: Ensembl_75) for each sample. On average, 74.9 million reads were obtained from each sample, and genome mapping was on average 90%.

We used the expression of cell type-enriched genes as a read-out of the timing of retinal histogenesis (**Figures 1A–E** and **Supplementary Table1**). As expected, by 50 days gestational age, several well-known progenitor genes are highly expressed (e.g., PRTG, FOXP1) but not all progenitor genes reach the highest expression point at these early stages and several progenitor genes such as bHLH transcription factors (e.g., ASCL1, NEUROG2) and genes associated with active proliferation (e.g., CCND1, CDK4, E2F1, and E2F2) do not peak until 90 days gestational age (**Figure 1A** and **Supplementary Figure 1A**). Previous reports have identified clear transcriptional differences between early and late retinal progenitor competence states in mouse and human retinas (Clark et al., 2019; Lu et al., 2020; Sridhar et al., 2020), including a progressive increase in Notch signaling. The activation of the Notch pathway maintains cells in a proliferative state ensuring that a subset of progenitors remains for the consecutive waves of neurogenesis (Perron and Harris, 2000; Louvi and Artavanis-Tsakonas, 2006). Notch also regulates fate decisions through the regulation of neurogenic genes (Kageyama et al., 2008; Maurer et al., 2014). Correspondingly, many genes involved in the Notch signaling pathway show their highest levels of expression at 90 days gestational age in our screening

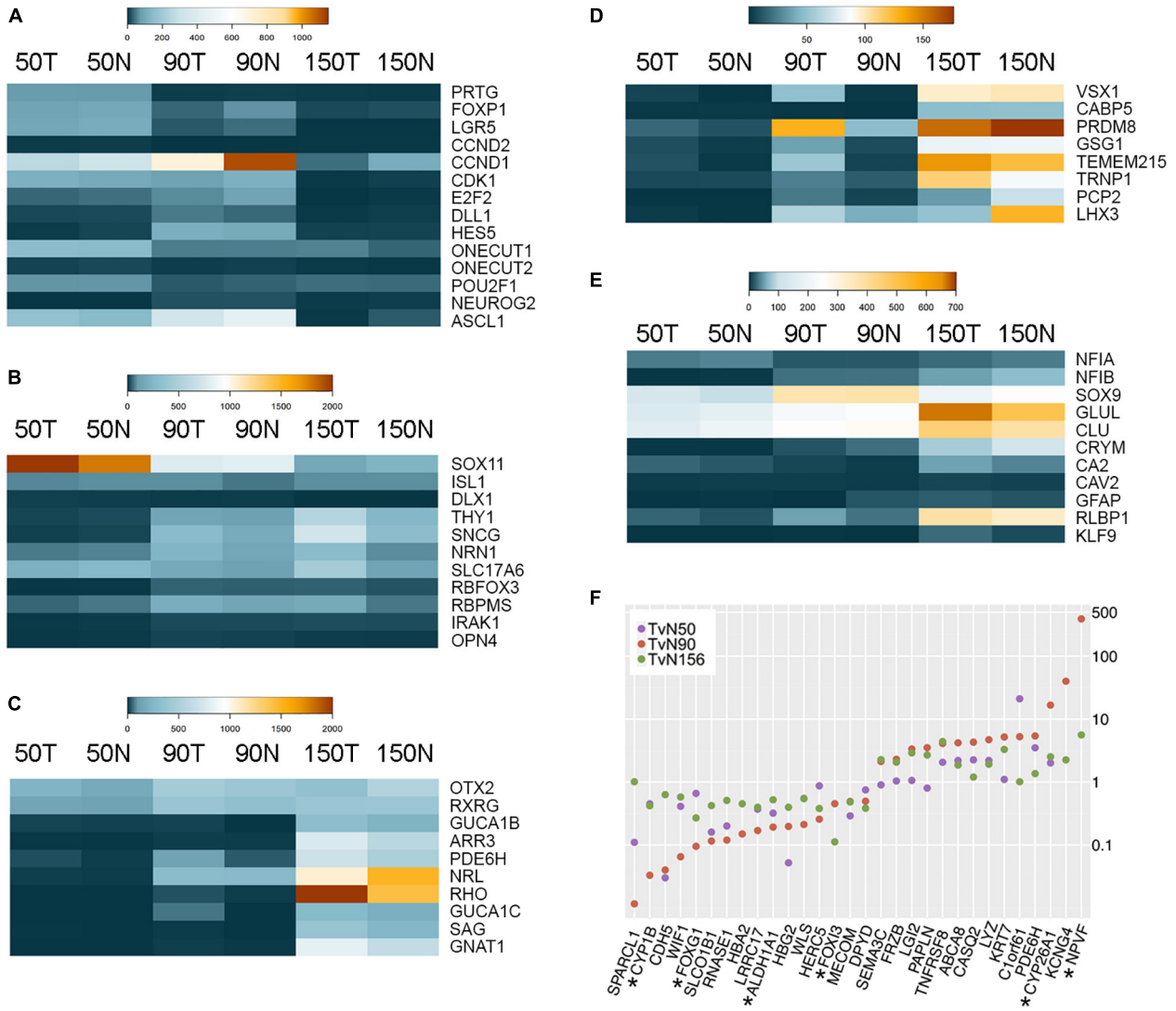

FIGURE 1 | RNA-sequencing of *Macaca mulatta* retinas. **(A–E)** Heatmaps showing expression of cell-specific markers during *Macaca mulatta* retinal development. Comparisons between Temporal (T) and Nasal (N) data is shown at 50, 90, and 150 days gestational age. **(A)** Retinal Progenitor Cell markers. **(B)** Retinal Ganglion Cell markers. **(C)** Photoreceptor cell markers. **(D)** Bipolar cell markers. **(E)** Muller Glia cell markers. All data is shown as CPM (counts per million). **(F)** Scatter plot of genes showing differences between temporal and nasal expression. TvN: Temporal vs Nasal. Stars indicate genes previously identified as macula-enriched.

(**Supplementary Figure 1B**), with NOTCH1, NOTCH3, DLL1, DLL3, and HES5 peaking at this time.

Similarly, genes known to be expressed in both mouse and human RGCs exhibit specific expression at different time points (**Figure 1B**). For example, several transcription factors such as ISL1 and SOX11 are highly expressed at early stages of development corresponding with their expression in other species (Mu et al., 2008; Pan et al., 2008; Jiang et al., 2013), while genes associated with RGC synaptic maturation (e.g., NRN1 or SNCG) increase over developmental time and peak in the third trimester. Interestingly, many photoreceptor-specific (Brzezinski and Reh, 2015) and bipolar cell-specific markers (Park et al., 2017) are detected first in the temporal samples before the nasal samples starting from 50 days gestational age (**Figures 1C,D**). Accordingly, by this time, the temporal samples exhibit higher levels of cone genes (e.g., PDE6H, 3.5-fold enrichment; **Figure1C**) and early bipolar genes (e.g., VSX1 shows a 28-fold enrichment, GSG1 shows a 3.1-fold enrichment, and TMEM215 shows an 8.3-fold enrichment; **Figure 1D**). These differences between temporal and nasal regions are more prominent in the second trimester (90 days gestational age), reflecting a vast developmental acceleration in the presumptive fovea. Thus, by this time, the expression of GUCA1B is 14.5-fold higher in the temporal samples, PDE6H shows an enrichment of 5.8-fold (photoreceptor markers), and the bipolar markers VSX1, CABP5, PRDM8, GSG1, TMEM215 are enriched 39.8-, 2.6-, 2.8-, 4.5- and 10.3-fold, respectively. Correspondingly, many Müller glia-specific genes are up-regulated over developmental time, including NFIX, GLUL, CA2, and RLBP1 (**Figure 1E**).

In addition to the cell-specific markers, other genes also exhibit transcriptional differences between the temporal and nasal regions of the developing rhesus eye (**Figure 1F** and **Supplementary Tables 1, 2**). Notably, many of these genes have been previously shown to be differentially expressed in the developing macula or the high-acuity area of other species. For example, FOXG1 is a transcription factor exclusively expressed in the nasal portion of the retina in fish, chicken, mouse, and human (Shintani et al., 2004; Zhao et al., 2009; Fotaki et al., 2013; Hernandez-Bejarano et al., 2015; Hoshino et al., 2017; Smith et al., 2017). Hoshino and collaborators demonstrated that CYP1B1 is enriched in the periphery of the human fetal retina (Hoshino et al., 2017). Notably, CYP26A1 is higher in the temporal retina at all the ages analyzed and ALDH1A1 is enriched in the nasal retina. CYP26A1 and ALDH1A1 are negative and positive regulators of retinoic acid (RA) levels, respectively, and downregulation in RA signaling correlates with the development of a rod-free area in the avian retina (da Silva and Cepko, 2017). CYP26A1 and NPVF have also been previously identified as developing macula markers in human samples by different reports (Hoshino et al., 2017; Lu et al., 2020). Additionally, our analyses also identify novel genes such as CROC4 (C1orf61), CASQ2, SPARCL1, and WIF1 as genes presenting strong differential expression signatures between the presumptive fovea and the nasal side at different gestational time points (**Figure 1F**). Collectively, these results show that the rhesus monkey presumptive fovea is developmentally advanced relative to the opposite nasal region, confirming the utility of these data as a tool to analyze differences between temporal and nasal expression.

miRNA-Sequencing and Differential Expression Profiles Between Temporal and Nasal Fetal Rhesus Monkey Retinas

miRNA libraries were obtained from retinal punches from the temporal side of the retina (presumptive fovea) and the nasal side of the optic nerve head as described above, at 50 days gestational age (n = 3 samples for each anatomical region, six samples total). After NGS profiling, an average of 29.9 million reads were obtained per sample, and the data was mapped to miRBase (release 20) and normalized. Principal Component Analysis (PCA) was performed by including the top 50 microRNAs that varied the most across all samples using normalized reads. As shown in **Figure 2A**, the foveal/temporal samples form a relatively robust cluster indicating that the biological differences between these samples are pronounced despite the nasal samples exhibiting larger intra-group variability.

Importantly, our experiments indicate that several miRNAs are differentially expressed in the different regions of the developing primate retina (**Figures 2B,C**). For example, miR-183, miR-96, and miR-182 are significantly enriched in the developing fovea (3.3-fold, 5.21-fold, and 5.11-fold, respectively). miR-183/-96/-182 are co-expressed together as a single primary transcript, are highly expressed in developing photoreceptors in mouse and zebrafish, and play essential roles in photoreceptor development and maintenance (Zhu et al., 2011; Xiang et al., 2017; Fogerty et al., 2019). Since the temporal region of the developing primate eye exhibits both a developmental acceleration and higher percentage of photoreceptors, it is not surprising that this family is expressed at greater levels in the temporal samples. Additionally, several other miRNAs are significantly enriched in the presumptive fovea compared to the nasal samples, including miR-369-3p (4.86-fold enrichment with a p-value of 2.45E-7), miR-15b-5p (3.85-fold enrichment with p-value of 5.6E-7), miR-30b-5p (3.96-fold enrichment with a p-value of 8.99E-6), miR-103-3p (2.69-fold enrichment with a p-value of 0.0009), and miR-93-5p (2.57-fold enrichment with a p-value of 0.0019). However, the expression levels of miR-369-3p are fairly low (**Figure 2B**). In contrast, miR-342-5p is significantly enriched in the nasal site (2.71-fold with a p-value of 1.96E-10).

Interestingly, miRNAs belonging to the same families often show similar expression profiles: all members of the miR-17/-20/-93/-106/-519 family are enriched over 2-fold in the temporal samples, miR-15b and miR-16 are enriched 3.85-fold and 2.65-fold, respectively, and miR-130a/-130b/-454/-301 are also all expressed at higher levels in the temporal region of the eye (**Supplementary Table 3**). This suggests that these miRNA families are frequently regulated as a whole, perhaps at the primary transcript stage.

Previous studies have indicated that miRNAs coordinately regulate protein levels and thus, miRNAs that target the same complexes are often co-expressed (Sass et al., 2011). We

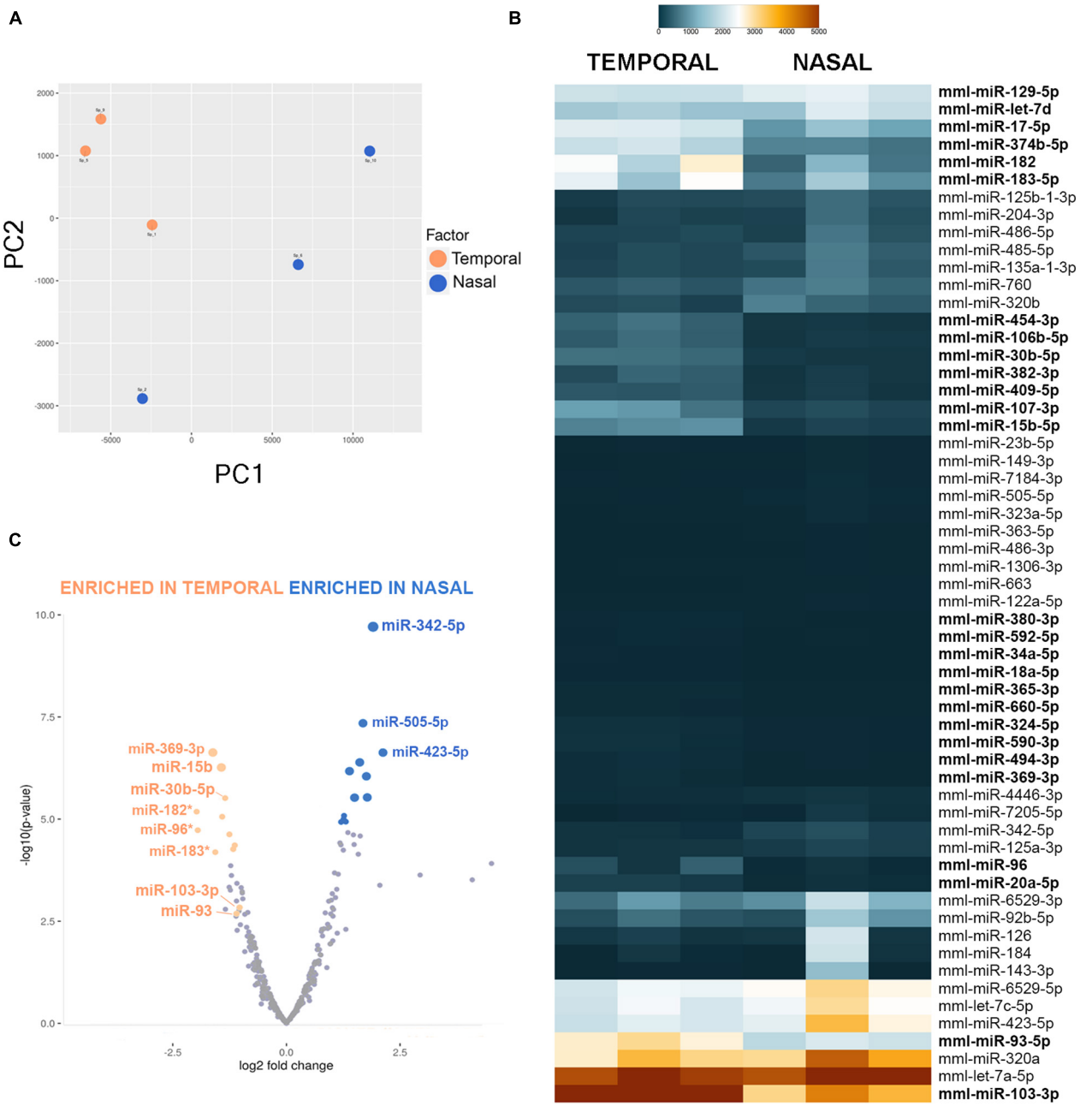

FIGURE 2 | MiRNA-sequencing of *Macaca mulatta* retinas. **(A)** Two-dimensional Principal Component Analysis was used to visualize sample variance between six retinal tissue samples collected at 50 days gestational age. **(B)** Heatmap shows different miRNA expression between temporal and nasal samples. Only miRNAs with a False Discovery Rate (FDR) value of < 0.012 between nasal and temporal samples are shown. miRNAs enriched in the temporal samples are shown in bold. **(C)** Volcano plot of miRNA plotted on fold change (log2, x-axis) and *P* value [−log10(*p*-value), y-axis] shows difference in expression between nasal and temporal retina. Stars indicate the photoreceptor enriched miR-183/96/182 cluster.

have used MIENTURNET [MicroRNA ENrichment TURned NETwork, (Licursi et al., 2019)] to gain insight into the possible miRNA networks in the different regions of the primate retina (**Supplementary Figure 2**). The network analyses of some of the highest expressed miRNAs for each region suggest possible differences in cell cycle regulation as several cell cycle genes including CCND1, CDKN1A, TP53, and CCNE1 are potentially regulated by differentially expressed miRNAs (**Supplementary Tables 4, 5**). Similarly, FOXG1 is potentially targeted by miR-30b-5p and miR-103-3p while NFIA and NFIB, two transcription factors involved in fate specification in the retina (Clark et al., 2019), are potentially targeted by miR-30b-5p, miR-103-3p, and miR-93 (**Supplementary Figure 2** and **Supplementary Table 5**). Additionally, several genes involved

in the NOTCH (DLL1), WNT (WNT3A, AXIN2), FGF (FGF4, FGF18), and RA (RORB, RORA) pathways are also targeted by the temporal miRNA network.

Notably, our analyses also reveal several miRNAs that were not previously annotated in the *Macaca mulatta* database but known in other species (**Figure 3A**) as well as putative novel miRNAs (**Figure 3B**), based on counts and putative secondary precursor hairpin structures identified using the miRPara software (Wu et al., 2011).

miRNA Expression in the Developing Mouse Retina

The miRNAs identified in our screening could be differentially expressed in the developing fovea for various reasons; for example, since the fovea is developmentally accelerated, temporally-regulated miRNAs are expected to increase first in the temporal side of the retina. Similarly, miRNAs enriched in cell populations found in higher percentages in the fovea (e.g., cones) could also exhibit higher expression levels in the temporal samples. Finally, the progenitors of the fovea could possess unique properties and miRNA signatures.

Since miRNA-seq technologies do not offer cellular resolution, and given the scarcity of primate samples, we first attempted to validate the developmental expression and cellular resolution of the top miRNA candidates using mouse tissue at three different time points: embryonic day 13.5 (E13.5), E16.5, and postnatal day 3 (P3) by *in situ* hybridization (ISH) (**Figure 4**). All the miRNAs tested show some level of expression in the murine samples and, in all cases, the expression detected was above the labeling threshold in negative controls (scrambled probe, **Figures 4S–U**). miR-15b, miR-30b, and miR-103-3p are up-regulated over the time points analyzed and show the highest levels of expression at P3 (**Figures 4A–F,J–L**). Interestingly, miR-15b exhibits a clear center-to-periphery pattern and it is first detected in the central retina at E13.5 (black arrows, **Figure 4A** and **Supplementary Figure 3**). By E16.5, most of the retina expresses miR-15b, but we found lower levels of expression in the peripheral tips (**Figure 4B**, white arrows) and the whole retina expresses high levels of miR-15b by P3. miR-30b is expressed throughout the thickness of the retina at P3 but we observed a moderate enrichment in the ganglion cell layer (GCL) and the basal part of the inner nuclear layer, suggesting higher expression in amacrine cells and possibly RGCs (**Figure 4F** and **Supplementary 3**). Interestingly, miR-93 expression is missing from the apical side of the retina at P3, suggesting that this miRNA may be expressed at lower levels in developing murine photoreceptors (**Figure 4I** and **Supplementary 3**). Finally, miR-342-5p shows higher expression levels in the peripheral retina from E16.5 onward (**Figure 4N** and **Supplementary Figure 3**), and this pattern of expression is maintained by P3 (**Figure 4O**, black arrows). In contrast, positive control experiments (U6 probe, **Figures 4P–R**) show neither regional differences nor changes in expression coordinated with the stage of development.

Expression in the Developing Human Retina

The experiments using murine samples indicate that several of the miRNAs identified in our miRNA-seq screening are

A

ID	Chr	Strand	Start	Stop	Sequence	Counts
hsa-miR-744-5p	16	+	11829814	11829835	TGCGGGGCTAGGGCTAACAGCA	27596
hsa-miR-4684-5p	1	+	25309179	25309200	CTCTCTACTGACTTGCAACATA	27
bmo-miR-2779	4	+	26617823	26617838	ATCCGGCTCGAAGGAC	14
mmu-miR-7689-3p	5	+	1472793	1472815	TTAGAGCCAGACTGCCTGGGTTT	9
ggo-miR-1291	8	+	56485909	56485923	GTGGCCCTGACTGAA	11
bta-miR-2424	11	+	6658740	6658760	ATCTTTGGTAATCTGATGGCT	140
hsa-miR-1248	2	+	1.79E+08	1.79E+08	ACCTTCTTGTATAAGCACTGTGC	155
hsa-miR-4508	18	+	71266455	71266471	GCGGGGCTGGGCGCGCG	3

B

ID	Chr	Strand	Start	Stop	Sequence	Counts
put-miR-349	2	+	117300472	117300497	TCTGTGGGATTATGACTGAACGCCTC	11196
put-miR-348	2	+	117300369	117300395	GATGTGTTGTTGCCATGGTAATCCTGC	4114
put-miR-92	1	+	95623473	95623499	GTCCGATGGTAGTGGGTTATCAGAACT	3564
put-miR-91	1	+	47709617	47709643	GTAAGTGACGATAAAGTGTGTCTGAGG	3253
put-miR-42	14	+	83661692	83661717	AACGCGCCCGATCTCGTCTGATCTCG	3149
put-miR-66	19	+	34152595	34152621	AGCTACCATCTGTGGGATTATGACTGA	2056
put-miR-300	X	+	39631265	39631288	CGTCTGATCTCGGAAGCTAAGCAG	1819
put-miR-214	6	+	125875024	125875047	CGTCTGATCTCGGAAGCTAAGCAG	1806
put-miR-27	14	+	11231916	11231942	AATGTGACTGAAAGGTATTTTCTGAGC	1571
put-miR-65	19	+	22514233	22514256	CCCAGTGCTCTGAATGTCAAAGTG	1443
put-miR-224	7	+	3968825	3968842	TCATCGGAACTGAGGTCC	1301
put-miR-93	1	+	95830029	95830052	AATTGTTTCAAGACGGGACTGATG	1016
put-miR-40	14	+	73584717	73584743	GATTGATTTAGAGGCATTTGTCTGAGA	908
put-miR-15	12	+	63966580	63966606	GATGGTAGTGGGTTATCAGAACTTATT	846
put-miR-4	10	+	51653988	51654013	ATGGTAGTGGGTTATCAGAACTTATT	817

FIGURE 3 | Novel *Macaca mulatta* miRNAs at 50 days gestational age. **(A)** miRNAs previously discovered in other species but unknown to be expressed in *Macaca mulatta* prior to this study. **(B)** Putative novel miRNAs based on counts and secondary precursor hairpin structure.

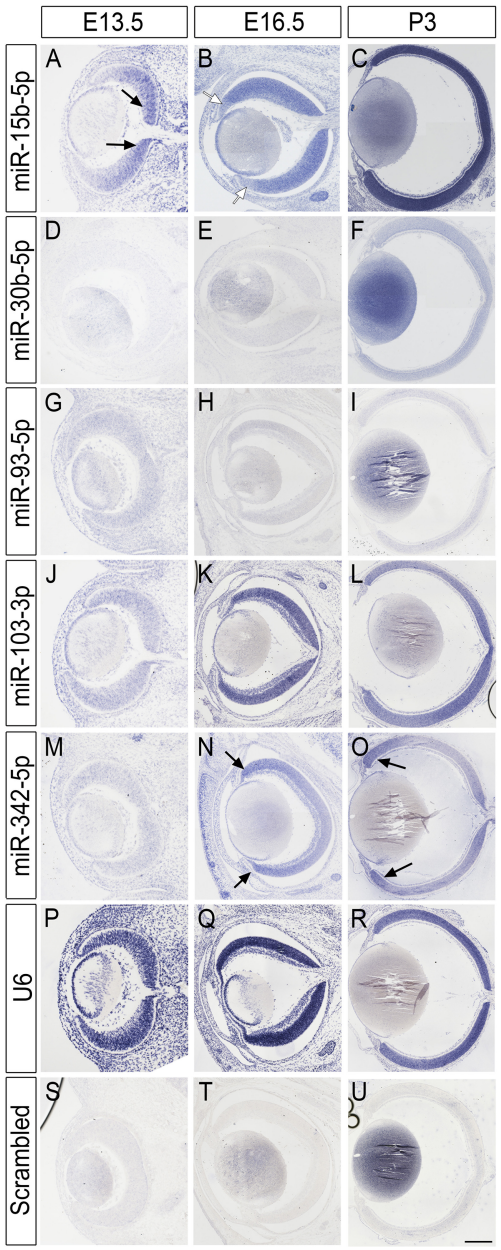

FIGURE 4 | miRNA *in situ* hybridization in the mouse retina. **(A–U)** miRNA expression in the murine retina at E13.5, E16.5, and P3. Rows are labeled with the miRNA probe used, including U6 positive control and scrambled negative control. Each column shows a different developmental time point. Black arrows indicate increased expression in central **(A)** or peripheral **(N,O)** retina regions. White arrows indicate lower expression **(B)**. Scale bars: 40 microns for panels **(A,D,G,J,M,P,S)** (first column), 100 microns for panels **(B,E,H,K,N,Q,T)** (second column), and 500 microns in panels **(C,F,I,L,O,R,U)** (last column).

developmentally regulated and that miR-15b and miR-342-5p also show compartmentalized expression with central-to-peripheral differences. To further assess whether these expression patterns are conserved in primates and relevant to human biology, we used human fetal retina tissue to test miRNA expression of our top candidates (**Figure 5** and **Supplementary Figure 6**). Since the rhesus samples were obtained at 50 days gestational age (30% gestation), we collected human fetal samples at gestational ages estimated to be between 77–83 gestational days (28–31% of gestation, **Supplementary Figure 4**). In order to obtain additional data on the developmental stage of the samples assessed, we performed immunohistochemistry using known markers and Hematoxylin and Eosin staining (**Supplementary Figure 5**). At the stage analyzed, there are PCNA+ retinal progenitors in all the quadrants of the retina, but the thickness of the neuroblastic layer where the retinal progenitors reside is thinner on the temporal side (NbL, **Supplementary Figures 5A–C′**). Similarly, we detected fewer PH3+ mitotic cells on the temporal site of the retina compared to the nasal side (arrows, **Supplementary Figures 5B–C′,F**), indicating that more progenitors have already exited the cell cycle in this region. Correspondingly, we also detected increased

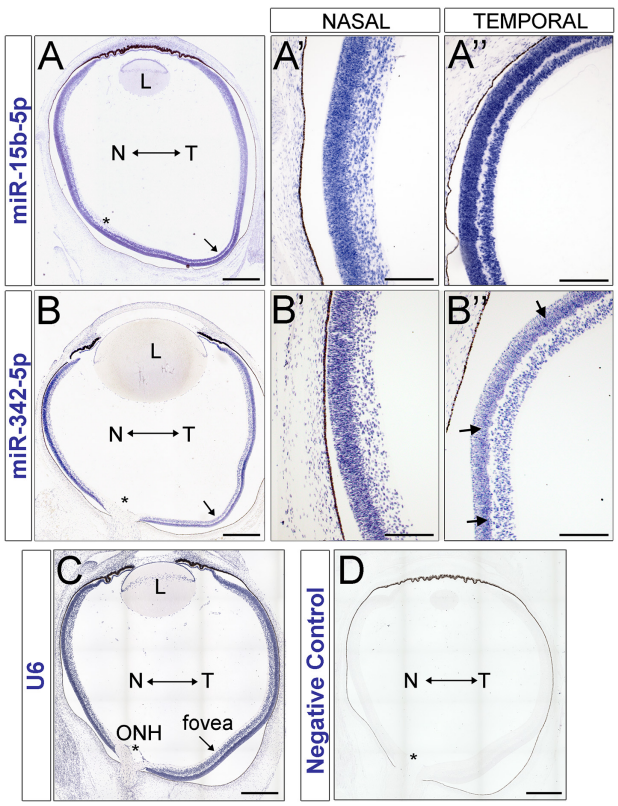

FIGURE 5 | miRNA *in situ* hybridization in the human retina. **(A–D)** miRNA expression in the human fetal retina at 70–82 days gestation. miR-15b **(A–A″)** and miR-342-5p **(B–B″)** expression in the developing human retina. **(A,B)** tiled montage of the whole eye. **(A′,B′)** inset of nasal retina. **(A″,B″)** inset of temporal retina at the presumptive fovea. **(C)** U6 positive control. **(D)** ISH negative control. L, Lens; N, Nasal; T, Temporal; ONH, Optic Nerve Head. Asterisks indicate the location of the optic nerve head and the arrows indicate the presumptive foveal region. Scale bars: 500 microns in A, B, C and D, 200 microns in panels **(A′,A″,B′,B″)**.

numbers of OTX2+ photoreceptors on the temporal side of the eye (**Supplementary Figures 5D–G′**).

Remarkably, miR-15b is expressed at higher levels in the temporal side of the optic nerve head (**Figures 5A–A″**) and miR-342-5p shows higher labeling signals in the nasal side of the eye (**Figures 5B–B″**) as predicted by our miRNA-seq analyses (**Figures 2B,C**). Both miRNAs display stronger signal in the neuroblast layer compared to other regions of the retina, including the GCL and the most apical side of the retina where the developing photoreceptors reside (arrows in **Figure 5B″** and **Supplementary Figure 7**). In contrast, we did not detect significant miR-93 or miR-30b differences between the temporal and nasal retina (**Supplementary Figure 6**). As shown previously, our U6 positive control is ubiquitously expressed (**Figure 5C**) and our negative controls (**Figure 5D**) show very low levels of non-specific labeling.

DISCUSSION

Our most advanced visual abilities such as reading and recognizing faces are dependent on the highly-specialized structure of the fovea. Unfortunately, the current understanding of retinal development is primarily based on mouse studies. Since the mouse retina does not contain a fovea and the rod-to-cone ratio in rodents resembles the most eccentric regions of the human retina, the molecular events that lead to the formation of the macula and the cone-dominated fovea remain largely unknown.

Previous studies have shown that the primate retina develops over many months and, in fact, the human fovea is not fully developed until 4 years of age (Hendrickson et al., 2012). Histological data using human and non-human primate samples has revealed that retinal development takes place in a dramatically compartmentalized manner such that two regions separated by a few millimeters may be at vastly different ontogenic stages (Yuodelis and Hendrickson, 1986; Xiao and Hendrickson, 2000; Hendrickson et al., 2008; Hendrickson, 2016). Moreover, primate retinal development does not progress in a central-to-peripheral gradient similar to mice but advances in a fovea-to-periphery manner. Consistent with this species-specific difference in patterning, the expression of S-Opsin and L/M-Opsin is first detected in the fovea (Cornish et al., 2004a,b). Recently, the first transcriptional profiling datasets of the developing human retina have been published using both human fetal tissue and stem cell organoids (Hoshino et al., 2017; Lu et al., 2020; Sridhar et al., 2020). However, obtaining human tissue at very early or late stages of development is challenging and these resources are subject to ethical and political issues (Ledford, 2017). In contrast, non-human primate models offer a unique opportunity to decipher some of the molecular mechanisms that dictate foveal development. The genus *Macaca*, probably the most extensively used non-human primate model (Roska and Sahel, 2018; Picaud et al., 2019), shares with humans susceptibility genes for AMD (Francis et al., 2008; Pahl et al., 2012; Yiu et al., 2017) and for other photoreceptor pathologies such as achromatopsia (Moshiri et al., 2019). Consequently, a comprehensive characterization of the specific mechanisms that regulate rhesus retinal development could facilitate the study of the pathophysiological events that lead to these diseases and enable the development of clinical approaches aimed at vision restoration.

Here, we provide the first spatio-temporal transcriptional datasets of the developing rhesus monkey retina obtained from temporal and nasal regions at three different gestational time points spanning all trimesters. The current study has limitations, including a modest sample size at some developmental time-points. Additionally, the incomplete annotation of the *Macaca mulatta* genome poses some challenges as the assembly contains many gaps, sequencing errors and misassembled scaffolds (Zhang et al., 2012; Norgren, 2013).

By analyzing the expression of cell-specific markers, our data offers insights into the timing of retinal histogenesis and indicates that by 50 days gestational age, the temporal side of the retina is already more developmentally advanced when compared to the nasal side (**Figure 1**). A model developed by Finlay (Finlay and Darlington, 1995), Clancy (Clancy et al., 2001), and Workman (Workman et al., 2013) and available at translatingtime.org explores the idea that timing of many neurodevelopmental events—such as the timing of retinal neurogenesis—is highly conserved among species and thus, can be predicted with high accuracy taking into account the growth rates for the different species. According to this model (**Supplementary Figure 4**), by 50 days gestational age (end of the first trimester), the rhesus monkey retina is at the peak of cone genesis and approximately at the onset of bipolar cell genesis. Assuming that neurogenesis for all the different cell types begins at the foveal region, these predictions appropriately fit our RNA-seq data. Indeed, by 50 days, we detect higher temporal expression of several bipolar genes, including GSG1 and TMEM215 (**Figure 1D**), two genes identified by the Brzezinski group as cone bipolar markers (Park et al., 2017). Our data also indicate that the maturation of photoreceptor cells follows a fovea-to-periphery gradient (**Figure 1C**), in agreement with histological evidence (Hendrickson and Zhang, 2019). Importantly, we also distinguish other genes differentially expressed in the presumptive developing fovea (**Figure 1F**), including genes previously identified in the human macula and in the avian high-acuity area as well as novel genes, such as the gene encoding for the calcium-binding binding protein Calsequestrin-2 and SPARCL1/Hevin. Future studies will shed light on the role of these genes in retinal development.

It has been proposed that the accelerated developmental timing of the fovea may be partially responsible for its unique cellular composition. During the sequence of retinal cell specification, cones are generated earlier than rods and thus, precocious cell cycle exit from the retinal precursor pool would result in increased representation of early cell types (e.g., cones). Comparison between diurnal (foveated) and nocturnal (afoveated) New World primates suggested that alterations in cell cycle kinetics could explain some of the differences between these models, including the higher production of cones in foveated species (Dyer et al., 2009). However, molecules associated with rod photoreceptor differentiation such as NRL and NR2E3 are

never detected in the foveal region while other late cell types (e.g., bipolar cells and Müller glia) are present in the presumptive fovea before the cell movements that lead to pit formation (Yanni et al., 2012; Hoshino et al., 2017). Thus, it is feasible that the progenitors of the fovea possess unique characteristics that result in the stark difference in cell composition.

Prior studies have revealed that miRNAs are key regulators of the temporal changes that allow progenitors to produce different cell populations as development proceeds (Georgi and Reh, 2010; La Torre et al., 2013; Wohl et al., 2019). Similarly, we have also shown that miRNAs coordinate cell cycle kinetics (Fairchild et al., 2019). Given that the fovea exhibits both different cell composition and perhaps different cell cycle dynamics, we have characterized the miRNome of the early developing primate retina with the goal to pinpoint miRNAs differentially expressed in the progenitors of the fovea. We identified several miRNAs with different temporal and nasal expression levels. Among these, miRNA-183/96/182, a miRNA cluster highly expressed in photoreceptors and vital in maintaining cone photoreceptor outer segments (Busskamp et al., 2014; Zuzic et al., 2019) is significantly enriched in the temporal samples. Similarly, other miRNAs including miR-15b and miR-342-5p also showed significant differences in our datasets and we utilized ISH to further validate these differences using mouse and human fetal samples. According to our assessment, the human samples used in this study are in a developmental stage comparable to the rhesus monkey samples we used for the miRNA-seq (**Supplementary Figure 4**). Remarkably, miR-15b showed higher labeling in the temporal retina while miR-342-5p exhibited lower expression in the temporal side of the retina. Past studies in different models and contexts have revealed that miR-15b plays roles in cell cycle regulation and survival (Cimmino et al., 2005) while miR-342–5p acts downstream of Notch to regulate neural stem cell fate choices (Gao et al., 2017). This raises the possibility that one or both of these miRNAs may contribute to the molecular events that lead to the development of the central primate retina. Future studies aimed at the identification of the miRNA-mediated networks in conjunction with the existing human and primate expression datasets may shed light on the regulatory events that orchestrate the cytoarchitecture of the primate fovea.

MATERIALS AND METHODS

Experimental Models and Subject Details

Rhesus Monkeys

All animal procedures conformed to the requirements of the Animal Welfare Act and protocols were approved prior to implementation by the Institutional Animal Care and Use Committee (IACUC) at the University of California at Davis. Normal, healthy adult female rhesus monkeys (*Macaca mulatta*) were bred and identified as pregnant using established methods (Tarantal, 2005). Pregnancy in the rhesus monkey is divided into trimesters by 55-day increments, with 0–55 days representing the first trimester, 56–110 days representing the second trimester, and 111–165 days gestational age the third trimester (term 165 ± 10 days). Normal embryonic/fetal growth and development were confirmed by ultrasound across gestation and until tissue collection (Tarantal, 2005). Dams were scheduled for hysterotomy (e.g., approximately 50, 90, or 150 days gestational age) for fetal tissue collection. Dams were returned to the breeding colony post-hysterotomy.

The fetal eyes were collected in cold PBS and the retinas were immediately dissected. With the cornea facing up, we made a small puncture in the center of the cornea with an 18 gauge needle. Using spring scissors (10 mm tip), we slowly cut the cornea from the puncture toward the corneo-scleral junction. We successively rotated the eye 90° and made three more cuts and we gently removed the lens. Then, using one of the cuts, we carefully inserted the lower blade of the scissors between the sclera/RPE and the retina and we cut all the way to the optic nerve head being careful not to damage the retina. We repeated using the other cuts at the corneo-scleral divide. Next, the sclera, RPE, and choroid were carefully removed with fine forceps (World Precision Instruments, Dumont tweezers 0.05 mm × 0.01 mm tips) to dissect the retina away from the rest of the tissues. We performed two cuts in the dorsal and ventral part of the retina to open its cup shape and the temporal and nasal samples were obtained using 2.5 mm biopsy punches (World Precision Instruments) at equidistant regions about 0.5 mm from the ONH. As the total size of the retina changes during development, the percentage of retina captured at the different stages varied in the different samples. At 50 days gestational age, the biopsy captured more than half of the retina from the ONH to the ora serrata, thus extending beyond the foveal anlage.

Mice

Pregnant CD-1 IGS females were obtained from Charles River and housed until embryos or neonates were at the proper developmental stage for dissection and fixation. All animals were used with approval from the University of California Davis IACUC. Dams were euthanized and embryos were dissected and fixed for ISH as described below.

Human Fetal Samples

Eyes (n = 6) were obtained from discarded de-identified human fetal tissue with permission of the University of California, Davis Institutional Review Board. The age for the human specimens was estimated by clinic intakes.

RNA and miRNA Sequencing

Library Preparation and Next Generation Sequencing

Upon dissection, all the tissues were preserved in RNAlater (Thermo Fisher) at −80°C. Then, total RNA was obtained from all the samples using the Total RNA Purification plus micro kit (Cat #48500, Norgen), and we used an Agilent Bioanalyzer 2100 to evaluate the quality of the RNA obtained.

The sequencing experiments were conducted by Exiqon (Denmark). The library preparation was performed using Illumina TruSeq® Stranded Total RNA (with Ribo-Zero Gold) preparation kit.

The starting material (1,000 ng) of total RNA was depleted of rRNAs using ribo-zero gold (to remove both cytoplasmic

and mitochondrial rRNA) magnetic bead-based capture-probe system (Illumina Inc.). The remaining RNA (including mRNAs, lincRNAs and other RNA species) was subsequently purified (RNAcleanXP) and fragmented using enzymatic fragmentation. Then, first strand synthesis and second strand synthesis were performed, and the double stranded cDNA was purified (AMPure XP). The cDNA was end repaired, 3′ adenylated and Illumina sequencing adaptors ligated onto the fragments ends, and the library was purified (AMPure XP). The stranded libraries were amplified with PCR and purified (AMPure XP). The libraries size distribution was validated and quality inspected on a Bioanalyzer (high sensitivity DNA chip). High quality libraries were quantified using qPCR, the concentration normalized, and the samples pooled. The library pool(s) were re-quantified with qPCR and optimal concentration of the library pool used to generate the clusters on the surface of a flowcell before sequencing on a Nextseq500/High Output sequencing kit (51 cycles according to the manufacturer instructions (Illumina Inc.) using 50-bp single-end reads and 30 million reads.

Sequence Analyses

Our data analysis pipeline is based on the Tuxedo software package, including Bowtie2 (v. 2.2.2), Tophat (v2.0.11), and Cufflinks (v2.2.1). CummeRbund was used for post-processing Cufflinks and Cuffdiff results. The heatmap.2 function contained within the ggplot2 R package was used to produce all heat maps. Transcriptomic heat maps were produced by selecting genes that represent specific retinal cell types based on established literature using normalized CPM values.

miRNA-Sequencing: Library Preparation and Next Generation Sequencing

For miRNA-sequencing, we used the same samples that were used for RNA-seq. A total of 500 ng of total RNA was converted into microRNA NGS libraries using NEBNEXT library generation kit (New England Biolabs Inc.) according to the manufacturer's instructions. Each individual RNA sample had adaptors ligated to its 3′ and 5′ ends and converted into cDNA. Then the cDNA was pre-amplified with specific primers containing sample specific indexes. After 18 PCR cycles the libraries were purified on QiaQuick columns and the insert efficiency evaluated by a Bioanalyzer 2100 instrument on high sensitivity DNA chip (Agilent Inc.). The microRNA cDNA libraries were size fractionated on a LabChip XT (Caliper Inc.) and a band representing adaptors and 15–40 bp insert excised using the manufacturer's instructions. Samples were then quantified using qPCR and concentration standards. Based on quality of the inserts and the concentration measurements the libraries were pooled in equimolar concentrations (libraries to be pooled are of the same concentration). The library pool(s) were finally quantified again with qPCR and optimal concentration of the library pool used to generate the clusters on the surface of a flowcell before sequencing using v2 sequencing methodology according to the manufacturer instructions (Illumina Inc.). Samples were sequenced on the Illumina NextSeq 500 system.

Analyses of RNA-seq and miRNA-seq Data

Following sequencing, intensity correction and base calling (into BCL files), FASTQ files were generated using the appropriate bcl2fastq software (Illumina Inc.) which includes quality scoring of each individual base in a read. We found that the vast majority of the data has a Q score greater than 30 (>99.9% correct), indicating that high quality data was obtained for all samples.

Principal Component Analysis was performed on miRNA samples using the base R function. To produce the hierarchically clustered heat map, the miRNA-seq data were initially filtered by removing any miRNAs that had a False Discovery Rate (FDR) of greater than 0.001 to improve readability of the heat map. All miRNAs with an FDR of < 0.001 were then hierarchically clustered using the built-in hierarchical clustering algorithm in the heatmap.2 function. The color-key for each heat map was created using predetermined break points to bin the TMM value into colors for each marker. The volcano plot was also obtained using the base R volcano plot function.

In situ Hybridization

All samples were collected and quickly fixed in a modified Carnoy's fixative overnight at 4°C. For the mouse embryonic samples, we fixed whole heads while postnatal day 3 and human fetal samples were fixed as whole eyes. A small hole was made with an 18 gauge needle at the corneal limbus to facilitate the fixation. After fixation, samples were dehydrated and embedded in paraffin as described elsewhere (Fairchild et al., 2018). Horizontal sections of whole embryo heads (mouse E13.5 and E16.5) and sagittal sections of whole eyes (mouse P3 and human 77–83 days) were prepared at 5 μm, collected onto SuperFrost slides, and air dried overnight at room temperature. Paraffin wax-embedded sections were baked for 45 min at 60°C, deparaffinized using xylene, rehydrated with ethanol (stepwise) and PBS, and treated with Proteinase K for 10 min at 37°C. A double digoxigenin (DIG)-labeled locked nucleic acid (LNA) ISH probe (miRCURY LNA Detection probe) was purchased from Exiqon/Qiagen. ISH was performed using the miRCURY LNA microRNA Detection FFPE microRNA ISH Optimization Kit 4 (Exiqon), which includes hybridization buffers and control probes (LNA scramble microRNA and LNA U6 snRNA control probe), according to manufacturer's protocol. The following LNA miRNA probes were used for ISH: miR-15b-5p (Qiagen, Cat#YD00611174-BEG, 1:500), miR-30b-5p (YD00610927-BCG, miR-30b, 1:500), miR-93-5p (Qiagen, Cat#YD00611038, miR-93-5b, 1:300), miR-103-3p (Qiagen Cat#YD00612004, 1:500), miR-342-5p (Qiagen, Cat#YD00611489, 1:625), U6 (Qiagen, Cat#YD00699002-BEG, 1:500), scrambled (Qiagen Cat#YD00699004, 1:300). LNA probes were hybridized for 1 h at 55°C and rinsed with SSC buffer (stepwise from 5× to 0.2×). Sections were blocked in 2% sheep serum/1% bovine serum albumin/PBS-0.01% Tween for 30 min at room temperature. Detection was performed using an alkaline phosphatase conjugated anti-DIG secondary antibody (Roche) in 1% sheep serum/1% bovine serum albumin/PBS-0.05% Tween for 1 h at room temperature. Following rinsing in

PBS-0.1% Tween, sections were incubated in developing solution of sodium chloride 0.1 M/tris pH 9.5 0.1M/magnesium chloride 10 mM/0.1% Tween-20 and NBT (nitroblue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl phosphate) stock solution (Roche). After the reactions were deemed complete (1–4 days), sections were fixed with 4% paraformaldehyde and mounted for microscopy using Fluoromount-G (Southern Biotech).

Immunofluorescence

Sections were prepared as described previously (Fairchild et al., 2018; Leger et al., 2019). Sections were then deparaffinized using xylene, rehydrated with ethanol (stepwise), rinsed with PBS-0.3% Triton X-100, and antigen retrieval was performed by treating the slides with 0.1 M sodium citrate. All sections were then blocked in 10% normal donkey serum/PBS-0.1% Triton X-100 in PBS for 1 h at room temperature and incubated in primary antibody in blocking solution overnight at 4°C. The following antibodies were used for immunofluorescence: goat anti-OTX2 (R&D Systems Cat#BAF1979), 1:500; rabbit anti-RBPMS (Phosphosolutions Cat#1832-RBPMS, 1:400, and anti-PCNA (Abcam Cat#ab18197, 1:500), and anti-PH3 (Thermo Fisher Cat#PA5-17869, 1:300). After primary antibody incubation, sections were rinsed in PBS and incubated with appropriate Alexa Fluor-conjugated secondary antibodies (Invitrogen, 1:300) in blocking solution for 1 h at 4°C. Cell nuclei were counterstained with DAPI. The sections were rinsed with PBS and mounted for microscopy using a Fluoromount-G (Southern Biotech).

Hematoxylin and Eosin Staining

Samples were prepared as described previously (Fischer et al., 2008). Next, sections were deparaffinized using xylene, rehydrated with ethanol (stepwise) and water, and stained with hematoxylin and eosin, and dehydrated with ethanol (stepwise). The sections were then rinsed in xylene and mounted for microscopy using a Fluoromount-G (Southern Biotech).

Microscopy

In situ hybridization were imaged using an Axio Imager M2 with ApoTome2 microscope system (Zeiss) using tile scan options (ZEN imaging software), and immunolabeling experiments were documented using a Fluoview FV3000 confocal microscope (Olympus). Images were processed using Fiji (ImageJ software), and figures were prepared in Adobe Photoshop 2000.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI Gene Expression Omnibus, accession no: GSE168475

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of California Davis Institutional Review Board, University of California, Davis. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by Institutional Animal Care and Use Committee; University of California, Davis.

AUTHOR CONTRIBUTIONS

EF, ML, AMi, SC, JW, and NS conducted experiments and/or analyses. AMo conducted sample collection, contributed to the study design, and revised the manuscript. AFT identified, selected, and monitored the pregnancies sonographically, and collected the specimens for analysis, contributed to the study design, and revised the manuscript. SS and ALT supervised and designed the study, conducted experiments, and wrote the manuscript. All authors contributed to the manuscript and approved the submitted version.

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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.2021.654385/full#supplementary-material

Supplementary Figure 1 | (related to **Figure 1**). **(A,B)** Heatmaps showing expression of cell-specific markers during *Macaca mulatta* retinal development. Comparisons between Temporal (T) and Nasal (N) data is shown at 50, 90, and 150 days gestational age. Expression of genes involved in panel **(A)** cell cycle and **(B)** Notch signaling pathway. All the data is expressed as CPMs.

Supplementary Figure 2 | (related to **Figure 2**). MiENTURNET network analysis of differentially expressed miRNAs. **(A)** mRNA-miRNAs network of miRNAs enriched in the temporal samples **(B)** mRNA-miRNAs network of miRNAs enriched in the nasal samples. miRNAs are indicated as blue dots, while target genes are yellow dots. Relevant targets genes are indicated.

Supplementary Figure 3 | (related to **Figure 4**). Close-ups of miRNA *in situ* hybridization in the mouse retina. Black arrows indicate regions with higher expression level while white arrows indicate areas that display lower levels of

expression. Scale bar: 100 microns for the top panel (miR-15b-5p) and 200 microns for the other panels.

Supplementary Figure 4 | Comparison between developmental timing in mouse, rhesus monkey, and human. The timing of key events during retinal histogenesis has been calculated using a prediction model previously published (translatingtime.org).

Supplementary Figure 5 | (related to **Figure 5**). Hematoxylin and Eosin (H&E) staining and immunohistochemistry of human fetal retinas. **(A–A″)** H&E staining of human fetal retina at 77 days of gestation (H&E) staining. **(B–C′)** Immunohistochemistry using PH3 [green, white arrows in panels **(B,C)**], PCNA (red), RBPMS (gray) antibodies and counterstained with DAPI. **(D–E′)** OTX2 staining (green). The samples were also counterstained with DAPI (blue). **(F–G)** Quantification of the number of PH3+ **(F)** and OTX2+ cells **(G)** per 250 μm of retina in the temporal and nasal regions of the retina. L, lens; N, Nasal; T, Temporal; ON, optic nerve; NbL, neuroblastic layer; GCL, Ganglion cell layer; * indicates the localization of the optic nerve head. Scale bars: 500 microns in panel **(A)**, 200 microns in panels **(A′–E″)**. Error bars indicate standard deviation.

Supplementary Figure 6 | (related to **Figure 5**). miRNA *in situ* hybridization in the human retina. **(A–D)** miRNA expression in the human fetal retina at 70–82 days gestation. miR-93 **(A–A″)** and miR-30b **(B–B″)** expression in the developing human retina. **(A′,B′)** Inset pictures of the nasal retina. **(A″,B″)** Inset pictures of the temporal retina at the foveal anlage. Scale bars: 500 microns in panels **(A,B)**, and 200 microns in panels **(A′, A″, B′,B″)**.

Supplementary Figure 7 | (related to **Figure 5**). Close-ups of miRNA *in situ* hybridization in the human fetal retina. miR-15b expression in the human fetal retina at 95 days gestation. miR-15b is enriched in the temporal progenitors (black arrows). Immunolabeling experiments using OTX2 (blue), PAX6 (green) and PCNA (red) using consecutive sections. Scale bar: 50 microns.

Supplementary Table 1 | (related to **Figure 1**). mRNA-sequencing results of *Macaca mulatta* retinas.

Supplementary Table 2 | (related to **Figure 2**). miRNA-sequencing results of *Macaca mulatta* retinas

Supplementary Table 3 | (related to **Supplementary Figure 2**). MIRTURNET target enrichment for the temporal network.

Supplementary Table 4 | (related to **Supplementary Figure 2**). MIRTURNET target enrichment for the nasal network.

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MINI REVIEW
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The Role of MicroRNAs in Mitochondria-Mediated Eye Diseases

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The retina is among the most metabolically active tissues with high-energy demands. The peculiar distribution of mitochondria in cells of retinal layers is necessary to assure the appropriate energy supply for the transmission of the light signal. Photoreceptor cells (PRs), retinal pigment epithelium (RPE), and retinal ganglion cells (RGCs) present a great concentration of mitochondria, which makes them particularly sensitive to mitochondrial dysfunction. To date, visual loss has been extensively correlated to defective mitochondrial functions. Many mitochondrial diseases (MDs) show indeed neuro-ophthalmic manifestations, including retinal and optic nerve phenotypes. Moreover, abnormal mitochondrial functions are frequently found in the most common retinal pathologies, i.e., glaucoma, age-related macular degeneration (AMD), and diabetic retinopathy (DR), that share clinical similarities with the hereditary primary MDs. MicroRNAs (miRNAs) are established as key regulators of several developmental, physiological, and pathological processes. Dysregulated miRNA expression profiles in retinal degeneration models and in patients underline the potentiality of miRNA modulation as a possible gene/mutation-independent strategy in retinal diseases and highlight their promising role as disease predictive or prognostic biomarkers. In this review, we will summarize the current knowledge about the participation of miRNAs in both rare and common mitochondria-mediated eye diseases. Definitely, given the involvement of miRNAs in retina pathologies and therapy as well as their use as molecular biomarkers, they represent a determining target for clinical applications.

Keywords: microRNA, retina, mitochondria, mitochondrial diseases, glaucoma, AMD, diabetic retinopathy, MitomiR

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INTRODUCTION

Mitochondria are key players in different cellular processes, and their dysfunction contributes to the pathogenesis of neurodegenerative disorders (NDs), including many retinal diseases. To date, a connection between vision and defective mitochondrial functions has been extensively described (Yu-Wai-Man et al., 2011; Gueven et al., 2017). Mitochondrial diseases (MDs) are a heterogeneous group of rare disorders caused by mutations in nuclear or mitochondrial genes that affect proteins essential for mitochondrial structure and function. Although they are highly genetically and clinically heterogeneous, several MDs, such as Leber hereditary optic neuropathy (LHON), autosomal dominant optic atrophy (ADOA), and neuropathy, ataxia, and retinitis

pigmentosa (NARP), show some form of vision impairment and can be classified as primary mitochondrial eye diseases (PMEDs). Moreover, mitochondrial dysfunctions represent a common denominator and a common cause of neuronal death involved in the pathogenesis of many NDs due to mutations in genes encoding non-mitochondrial proteins or characterized by more complex pathogenetic events (Niyazov et al., 2016).

The great concentration of mitochondria in metabolically active tissues with high-energy demands, such as the retina, makes them particularly sensitive to mitochondrial dysfunction. The retina comprises different cell types organized in layers that form neuronal circuits working in parallel and in combination to produce a complex visual output (**Figure 1**) (Carrella et al., 2020). The outer nuclear layer (ONL) is composed of photoreceptor cells (PRs), subdivided into rods and cones. They synapse with interneurons of the inner nuclear layer (INL), namely, bipolar cells, amacrine cells, and horizontal cells, which in turn contact RGCs in the RGC layer. Retinal layers show a peculiar distribution of mitochondria to guarantee the energy supply for the conversion and propagation of the light signal (**Figure 1**). PRs, which capture photons and generate electrophysiological signals, display many mitochondria in the inner segment. In RPE, mitochondria are located at the basal region, that is, in contact with PRs. Instead, in the inner retina, mitochondria are predominantly concentrated in the unmyelinated proximal axons of RGCs, which transmit visual information to the brain. It is thus not surprising that the most common retinal disorders, i.e., glaucoma, age-related macular degeneration (AMD), and diabetic retinopathy (DR), show mitochondrial dysfunction and share some clinical similarities with PMEDs (Carelli et al., 2004; Yu-Wai-Man et al., 2011; Gueven et al., 2017; Ferrington et al., 2020). Interestingly, many studies also reported vision impairment and retinal abnormalities in the majority of Alzheimer's and Parkinson's disease patients and animal models, highlighting the involvement of mitochondrial anomalies in the development of visual defects (Colligris et al., 2018; Indrieri et al., 2020b; Marrocco et al., 2020; Mirzaei et al., 2020).

MicroRNAs (miRNAs) are a class of non-coding RNAs able to post-transcriptionally regulate gene expression through a powerful mechanism of sequence-specific recognition. Each miRNA is predicted to recognize about 200 mRNA targets, guaranteeing a pleiotropic fine-tuning of correlated transcripts that confers robustness to pathway regulation (Bartel, 2018).

Soon, their relevant role in different retina pathologies (Karali and Banfi, 2019; Zuzic et al., 2019) and the possibility to exploit their modulation as a possible gene/mutation-independent strategy for these disorders became evident (Carrella et al., 2020). The extensive genetic heterogeneity of many inherited retinal disorders, including PMEDs, indeed represents a significant limitation to the development and application of gene-replacement therapy in most of patients. Moreover, gene replacement cannot be applied in disorders caused by gain-of-function mutations and too complex multifactorial diseases such as AMD, glaucoma, and DR (Carrella et al., 2020). In this respect, miRNAs represent interesting therapeutic targets able to regulate common dysregulated pathways underlying retinal damage.

Moreover, dysregulated miRNA expression profiles in retinal degeneration models and in patients indicate that they may represent reliable biomarkers for the diagnosis of these disorders or to predict the onset and the progression of the disease, and the evaluation of the response to treatments. Circulating miRNAs and exosomal miRNAs can be indeed easily detected, thus representing promising disease predictive/diagnostic/prognostic biomarkers (Saxena et al., 2015; Palfi et al., 2016; Anasagasti et al., 2018).

MicroRNAs can localize to different subcellular compartments (i.e., mitochondria, endoplasmic reticulum, and exosomes) (Leung, 2015); and an increasing interest is growing about miRNAs, called MitomiRs, that regulate mitochondrial function. MitomiRs can be divided into two subgroups: those binding to nuclear-transcribed mRNA encoding mitochondrial proteins and those imported into mitochondria targeting mitochondrial-encoded mRNAs (Purohit and Saini, 2021). Moreover, some MitomiRs (i.e., miR-1974, miR-1977, and miR-1978) may be transcribed by the mitochondrial DNA (mtDNA) (Bandiera et al., 2011); however, more data are necessary to validate these findings.

Modulation of miRNAs has been recently applied as therapy to different disorders reaching preclinical and clinical stages (Bajan and Hutvagner, 2020). However, investigations on the role of miRNAs, and specifically MitomiRs, in mitochondrial-mediated disorders are few. In this review, we will summarize the current knowledge about the involvement of miRNAs in mitochondria-mediated eye diseases, including both rare PMEDs and the most common retinal disorders, i.e., glaucoma, AMD, and DR. In particular, their role in retina pathologies and therapy, as well as their role as biomarkers in these disorders, will be analyzed, highlighting their huge potential in clinical medicine.

MITOCHONDRIA-MEDIATED EYE DISEASES

Primary Mitochondrial Eye Diseases

Leber Hereditary Optic Neuropathy

Leber hereditary optic neuropathy is one of the most frequent PMEDs with a prevalence of between 1/15,000 and 1/50,000 people worldwide. LHON is an organ-specific disease, characterized by death of RGCs leading to degeneration of the optic nerve (ON) and bilateral or unilateral loss of vision, which typically occurs between the ages of 20 and 40 (Meyerson et al., 2015). It shows maternal inheritance, and it results more commonly in men, with variable disease penetrance. Approximately 95% of LHON cases are associated with three mtDNA point mutations (m.11778G > A, m.3460G > A, and m.14484T > C) that primarily affect mitochondrial respiratory chain (MRC) complex I genes (*ND1*, *ND4*, and *ND6*) (Yu-Wai-Man et al., 2002; Newman, 2005).

The molecular mechanism underlying death of RGCs is still not clear, even if it has been correlated to a reduction of ATP, an increase of reactive oxygen species (ROS) production

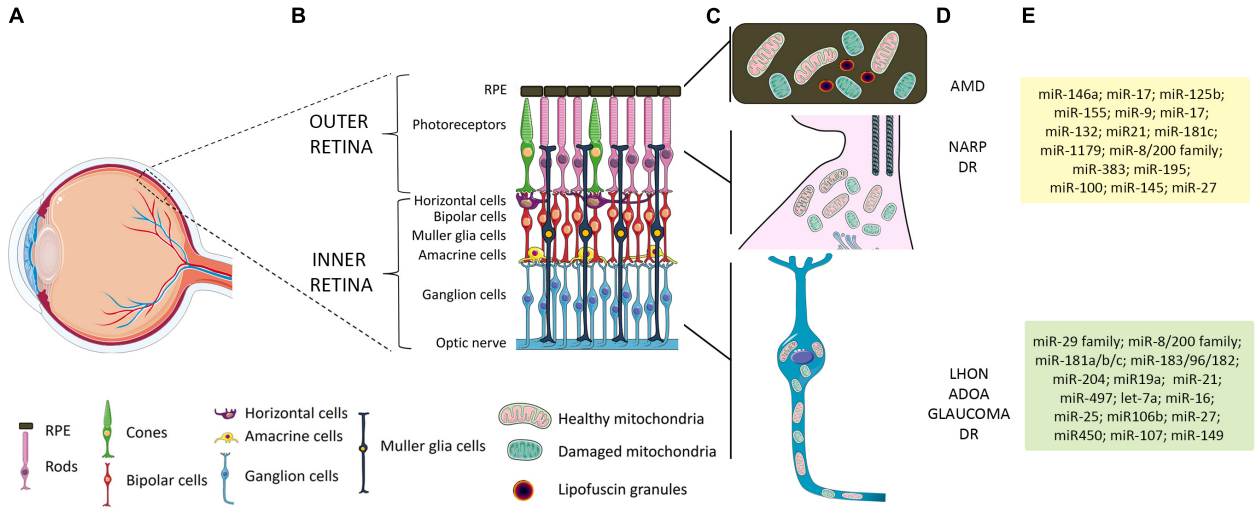

FIGURE 1 | Schematic representation of retinal layers, mitochondrial distribution, and related retinal pathologies and miRNAs. **(A)** Schematic representation of the human eye. The box identifies the position of the retinal layers in the posterior part of the eye. **(B)** Magnification of the retina and retinal layers in healthy conditions. **(C)** A peculiar distribution of mitochondria is graphically represented: in RPE, which overlays the PRs, mitochondria are located at the basal region; in PRs, a high number of mitochondria are present in the inner segment; in the inner retina, mitochondria are predominantly concentrated in the unmyelinated proximal axons of RGCs. **(D)** Primary or secondary defect in mitochondrial functionality in RPE and PRs has been linked to AMD, NARP, and other forms of visual loss, such as DR. Primary or secondary impairment in mitochondrial function leading to RGC degeneration is considered a major cause of retinal diseases such as LHON, ADOA, glaucoma, and DR. **(E)** List of miRNAs expressed in specific cell retinal layers whose function or alteration in expression has been linked to mitochondrial dysfunction and retinal diseases. Yellow box shows relevant miRNAs related to AMD and DR; green box, LHON and glaucoma-related miRNAs. RPE, retinal pigment epithelium; PRs, photoreceptors; RGCs, retinal ganglion cells; AMD, age-related macular degeneration; NARP, neuropathy, ataxia, and retinitis pigmentosa; DR, diabetic retinopathy; LHON, Leber hereditary optic neuropathy; ADOA, autosomal dominant optic atrophy.

due to defective MRC, and a significantly impaired mitophagy (Sharma et al., 2019).

Autosomal Dominant Optic Atrophy

With a prevalence of 1/10,000–1/35,000, ADOA is the most common form of PMEDs due to nuclear DNA mutations. Bilaterally symmetric progressive deterioration of the central visual acuity, ON pallor, dyschromatopsia, and blindness are the main symptoms, usually beginning in childhood (Fraser et al., 2010; Yu-Wai-Man et al., 2014). As in LHON, the disease primarily affects the RGCs and their axons, even if the ADOA progression with age is highly variable (Lenaers et al., 2012). In about 50–60% of the cases, patients harbor mutations in the *OPA1* (Alexander et al., 2000; Ferre et al., 2005). In addition, other mutated genes include *OPA2*, *OPA3*, *OPA4*, *OPA5*, *OPA8*, *WFS1*, and *SSBP1* (Finsterer et al., 2018; Piro-Megy et al., 2020).

OPA1 is a crucial component of the mitochondrial fusion machinery and also controls crista biogenesis and remodeling, impacting apoptosis and mitochondrial respiration (Cogliati et al., 2016). In accordance, Opa1 deficiency induces a significant fragmentation of the mitochondrial network and impairs ON structure and visual function in a mouse model of ADOA (Davies et al., 2007).

Recently, the role of autophagy in the regulation of mitochondrial distribution in axons of RGC and in visual loss in an ADOA mouse model (Zaninello et al., 2020) has also been demonstrated, indicating an important patho-mechanism contribution of mitophagy.

Neuropathy, Ataxia, and Retinitis Pigmentosa

Neuropathy, ataxia, and retinitis pigmentosa is an inherited neurologic/metabolic syndrome whose clinical hallmarks are (i) sensory neuropathy including progressive motor weakness and lethargy, (ii) ataxia, which affects the balance and coordination, and (iii) ophthalmologic findings including retinitis pigmentosa, optic atrophy, and eye movement disorders. Usually, the retina defects worsen over time, leading to severe vision loss and blindness. NARP typically begins in childhood or early adulthood. The clinical expression of the NARP syndrome is very variable, and the predominant ocular manifestation is characterized by an initial RPE degeneration and a rod/cone dysfunction in different families (Gelfand et al., 2011).

neuropathy, ataxia, and retinitis pigmentosa results from mtDNA heteroplasmic mutations in *ATP6* gene (predominantly m.8993T > G/C), coding for the mitochondrial ATP synthase subunit 6 (Holt et al., 1990; Duno et al., 2013; Miyawaki et al., 2015). ATP synthase impairment affects oxidative phosphorylation, causing energy deprivation and overproduction of ROS (Nijtmans et al., 2001; Baracca et al., 2007).

Eye Diseases Associated With Mitochondrial Dysfunctions

Glaucoma

With about 70 million patients worldwide, glaucoma is the leading cause of blindness and a major economic burden (Quigley and Broman, 2006). The term glaucoma describes a group of complex multifactorial diseases characterized by ON damage and

loss of RGCs resulting in progressive loss of vision. Age, genetics, and elevated intraocular pressure (IOP) are prominent risk factors; however, about one-third of cases have ON degeneration despite IOP in the normal range (Almasieh and Levin, 2017).

Interestingly, similarities between glaucoma and PMEDs have been described, and defects in mitochondria have been connected to glaucomatous neurodegeneration (Kong et al., 2009; Sundaresan et al., 2015; Shim et al., 2016; Williams et al., 2017; Singh et al., 2018; Tribble et al., 2019). Moreover, mutations in *OPTN* encoding for the mitophagy adaptor protein optineurin (Rezaie et al., 2002; Wong and Holzbaur, 2014), *TBK1* encoding the serine threonine protein kinase TANK-binding kinase 1 involved in autophagy (Sears et al., 2019), and *OPA1* (Aung et al., 2002; Yu-Wai-Man et al., 2010; Guo et al., 2012) have been associated with glaucoma, thus highlighting a crucial role for mitochondrial dynamics and mitophagy pathways in glaucoma pathogenesis (Ito and Di Polo, 2017). In addition, numerous studies reported mtDNA mutations, decrease in the mitochondrial respiratory activity, and oxidative stress in glaucoma patients and in animal models of this disease (Lee et al., 2011; Tseng et al., 2015; Hondur et al., 2017; Williams et al., 2017; Tribble et al., 2019). Notably, mitochondrial dysfunction can be detected before RGC death occurs in glaucoma animal models (Kong et al., 2009; Williams et al., 2017; Singh et al., 2018), suggesting a primary effect for mitochondrial abnormalities in glaucoma onset.

Age-Related Macular Degeneration

Mitochondrial dysfunction has been implicated in the pathophysiology of several age-related diseases including those that involve PRs and RPE cells (Lukiw et al., 2012; Lefevere et al., 2017; Ferrington et al., 2020). Aging and oxidative stress have been recognized as primary risk factors for AMD (Liang and Godley, 2003; Jarrett and Boulton, 2012; Lefevere et al., 2017), a complex degenerative and progressive disease.

There are two forms of AMD: the "wet" form that is associated with abnormal growth of blood vessels into the retina and the "dry" form with primary pathogenic event involving RPE degeneration causing PR cell death (Liang and Godley, 2003). RPE cells engulf photoreceptor outer segments (POSs) that are shed daily during renewal of PRs. RPE accumulation of lipofuscin, a product of POS turnover, has been hypothesized to be the primary source of ROS responsible for oxidative damage of the RPE resulting in impaired metabolism and apoptosis (Liang and Godley, 2003; Vives-Bauza et al., 2008; Jarrett and Boulton, 2012). Several studies have provided evidence that impaired autophagy (Mitter et al., 2014; Hyttinen et al., 2017) and mitochondrial dysfunction (Barron et al., 2001; Feher et al., 2006), in both RPE and PRs, exacerbate oxidative stress and contribute to the pathogenesis of AMD.

Diabetic Retinopathy

Diabetic retinopathy represents one of the most common slow-progressing microvascular complications of diabetes. In diabetic patients, damaged blood vessel of the retina leads to retinal detachment and reduction in the visual field and blindness (Frank, 2004). The retinal neurodegeneration is associated with retinal electrophysiological dysfunction and thinning of RGC and PR layers (Carbonell et al., 2019). Accelerated apoptosis of both neuronal and vascular cells (Mizutani et al., 1996; Barber et al., 2011) indicates apoptotic cell death as a contributing process to DR.

Although the detailed mechanisms of action in the development of DR are still unknown, involvement of mitochondrial dysfunctions with ROS formation and a decrease of the mitochondrial fusion protein mitofusin 2 (Mfn2) have been found in experimental models of this retinopathy (Eshaq et al., 2014; Duraisamy et al., 2019).

MicroRNAs IN MITOCHONDRIA-MEDIATED EYE DISEASES

As reported before, an increasing number of miRNAs have been shown to be involved in the regulation of mitochondrial metabolism, although there is no evidence, to date, that mitochondrial disorders affect their expression or are directly caused by their dysregulation. Recently, miR-181a and miR-181b (miR-181a/b) were shown to directly target genes involved in mitochondrial biogenesis and function, and ROS detoxification (Indrieri et al., 2019). Inactivation of miR-181a/b leads to increased levels of mitochondrial biogenesis and mitophagy leading to a significant amelioration of the disease phenotype in LHON mouse models. These data suggest that miR−181a/b may represent gene−independent therapeutic targets for mitochondrial-related eye diseases (Indrieri et al., 2019). In accordance with the pervasive and pleiotropic roles of the miR-181 family (Indrieri et al., 2020a), miR-181c might be associated with vascular proliferation in high glucose diabetic-like environment (Qing et al., 2014; Zitman-Gal et al., 2014).

Large-scale studies have been performed to identify glaucoma-relevant miRNAs (Li et al., 2009; Liu et al., 2018; Hindle et al., 2019). Among the 159 miRNAs identified, many were differentially expressed in the aqueous humor (AH) and/or tear of glaucoma patients and controls. MiRNA-29 family controls extracellular matrix (ECM) homeostasis in trabecular meshwork (TM) cells, by negatively regulating collagens, fibrillins, and elastin (Luna et al., 2009; Villarreal et al., 2011). Moreover, a specific crosstalk between TGFβ, whose alteration are often observed in glaucoma, and miR-29 levels highlighted miR-29-family implication in glaucoma (Luna et al., 2011). The expression profile of miR-8/miR-200 family is upregulated in transgenic mice carrying a mutation in *OPTN* (Chi et al., 2010; Gao et al., 2016). Moreover, miR-200c can decrease trabecular contraction and IOP by regulating genes associated with TM cell contraction regulation (Luna et al., 2012). The miR-183/96/182 cluster is highly expressed in retina and implicated in several aspects of retinal cell development and maintenance (Amini-Farsani and Asgharzade, 2020). In particular, miR-182 was found to be the most abundant miRNA also in the axons of developing RGC where it regulates axon guidance (Bellon et al., 2017). Interestingly, a case–control study conducted on patients with primary open-angle glaucoma (POAG) concludes that the

TABLE 1 | Summary of miRNAs involved in mitochondria-mediated eye diseases.

Pathology	Etiology	Mitochondrial dysfunction	Related miRNAs as biomarkers	Related miRNAs as therapeutic target	References
PMEDS					
Leber hereditary optic neuropathy (**LHON**) (*OMIM #535000*)	Mutations in MRC complex I genes (*ND1*, *ND4*, and *ND6* of mtDNA)	– Reduction in ATP production – Increase of free radical production – Impaired mitophagy		Mir-181a; miR-181b	Indrieri et al., 2019
Autosomal dominant optic atrophy (**ADOA**) (*OMIM* #165500)	nDNA mutations (*OPA1*, *OPA2*, *OPA3*, *OPA4*, *OPA5*, *OPA8*, *WFS1*, and *SSBP1*)	– Disorganized cristae – Fragmentation of mitochondrial network – Apoptosis – Impaired respiratory functions – Abnormal mitophagy			
NARP (*OMIM #551500*)	MtDNA mutations in *ATP6* gene	– Malfunctioning of ATP synthase – Energy deprivation – Overproduction of ROS			
EDAMDs					
Glaucoma	Age, genetics (*Optn*, *Opa1*, and *Tbk1*), and elevated IOP	– Decreased mitochondrial respiratory activity – Oxidative stress – Impaired mitochondrial dynamics – Defective mitophagy	MiR-8/200 family; miR-16; **miR-21**; miR-25; miR-27a; miR-29 family; miR-106b; **miR-107**; **miR-149;** miR-181c; miR-183/96/182; **miR-204**; **miR-450**; miR-497; **let-7a**;	**MiR-19a**; **miR-21;** **miR-204**	Luna et al., 2009; Chi et al., 2010; Luna et al., 2011; Villarreal et al., 2011; Huang et al., 2013; Izzotti et al., 2015; Jayaram et al., 2015; Gao et al., 2016; Bellon et al., 2017; Wang et al., 2018; Moschos et al., 2020
Age-related macular degeneration (**AMD**)	Aging and oxidative stress	– Increased ROS production – Oxidative damage resulting in impaired metabolism	**MiR-17**; **miR-125b**; miR-146a; **miR-155**	MiR-9; miR-146a; **miR-155**	Lukiw et al., 2012; Yan et al., 2015; Berber et al., 2017; Natoli and Fernando, 2018; Pogue and Lukiw, 2018; Martinez and Peplow, 2021
Diabetic retinopathy (**DR**)	Diabetes-caused damage of retinal blood vessel leads to retinal detachment	– Mitochondrial dysfunctions with ROS formation – Decreased mitochondrial fusion protein Mfn2	MiR-8/200 family; miR-19a; **miR-21;** miR-27; miR-31; **miR-34a/c;** miR-100; **miR-126**; miR-132; miR-145; **miR-146**; miR-155; miR-181c; miR-1179	MiR-34a; miR-195; miR-383; miR-451a	Sugioka et al., 2004; Zheng and Xiao, 2010; Wu et al., 2012; Li et al., 2013; Ragusa et al., 2013; Kong et al., 2014; Mastropasqua et al., 2014; Qing et al., 2014; Wang Y. G. et al., 2014; Zitman-Gal et al., 2014; Wang et al., 2017; Li J. et al., 2018; Shafabakhsh et al., 2019; Shao et al., 2019; Thounaojam et al., 2019

MitomiRs are highlighted in red. The most promising miRNAs for clinical development are in bold.
mtDNA, mitochondrial DNA; ATP, adenosine triphosphate; nDNA, nuclear DNA; ROS, reactive oxygen species; IOP, intraocular pressure; Mfn2, mitofusin 2; PMEDs, primary mitochondrial eye diseases; NARP, neuropathy, ataxia, and retinitis pigmentosa; EDAMDs, eye diseases associated with mitochondria dysfunctions.

carriers of polymorphism in miR-182 and *CDKN2B* genes have an increased risk of developing POAG (Moschos et al., 2020). MiR-204 caused reduced expression of *FOXC1*, implicated in glaucoma development, and its target genes (Paylakhi et al., 2013). Moreover, it has been shown that in ON injury, miR-204 can downregulate *GAP43*, which plays an important role

TABLE 2 | Mitochondrial-related targets and pathways modulated by MitomiR.

MiRNA	Targets	Mitochondria-related pathways	References
MiR-8/miR-200 family	MFF	Mitochondrial dynamics	Eades et al., 2011; Zhu et al., 2012; Yao et al., 2014; Lee et al., 2017
	TFAM	Mitochondrial biogenesis	
	KEAP1	Oxidative stress response	
	BCL2 and XIAP	Mitochondria-mediated apoptosis	
MiR-9	BCL2L11	Mitochondria-mediated apoptosis	Wei et al., 2016
MiR-16	BCL2	Mitochondria-mediated apoptosis	Cimmino et al., 2005; Nishi et al., 2010
	ARL2	Mitochondrial ADP/ATP	
MiR-17	SOD2, TRXR2, and GPX2	Antioxidant response	Xu et al., 2010; Weng et al., 2014; Lu et al., 2016
	BIM-S	Mitochondria-mediated apoptosis	
	MFN2	Mitochondrial dynamics	
MiR-19a	PTEN	Mitochondria-mediated apoptosis	Zhao et al., 2017
MiR-21	BCL2	Mitochondria-mediated apoptosis	Dong et al., 2011
MiR-25	MCU	Mitochondrial Ca^{2+} uptake	Zhang et al., 2012; Marchi et al., 2013; Wu et al., 2015, 2017; Feng et al., 2016
	MOAP1; PTEN; BIM	Mitochondria-mediated apoptosis	
	NCOA3	Release of mitochondrial DNA	
MiR-27	PHB	Mitochondrial dynamics	Kang et al., 2013; Kim et al., 2016; Shen et al., 2016; Li H. et al., 2017
	PINK	Mitophagy	
	FOXJ3	Mitochondrial biogenesis	
	BAX	Mitochondria-mediated apoptosis	
MiR-29 family	MCL 1 and BAX	Mitochondria-mediated apoptosis	Mott et al., 2007; Garzon et al., 2009; Xue et al., 2016; Muluhngwi et al., 2017; Caravia et al., 2018; Jing et al., 2018
	PGC1α	Mitochondrial biogenesis	
	ATP5G1 and ATPIF1	Mitochondrial bioenergetics	
MiR-31	SIRT3	Oxidative stress response	Lee et al., 2016; Kao et al., 2019
	SDHA	Mitochondrial metabolism	
MiR-34a/c	BMF; CYC	Mitochondria-mediated apoptosis	Catuogno et al., 2013; Hu et al., 2020
	TXNRD2; SOD2	Antioxidant response	Bai et al., 2011; Fan et al., 2017; Zhu et al., 2017; Thounaojam et al., 2019
	SIRT1	Mitochondrial biogenesis	
	Notch2	Mitochondria-mediated apoptosis	
MiR-96	CASP9	Mitochondria-mediated apoptosis	Iwai et al., 2018
MiR-106b	MFN2	Mitochondrial dynamics	Wu H. et al., 2016; Li P. et al., 2017; Xu et al., 2017; Zhang C. et al., 2021
	OPTN, MFN2, and NDP52	Mitophagy	
	MCL1; DR4	Mitochondria-mediated apoptosis	
MiR-125b	BIK	Mitochondrial metabolism	Xie et al., 2015; Duroux-Richard et al., 2016; Hu et al., 2018
	MTP18	Mitochondrial dynamics	
	MCL1; HAX1	Mitochondria-mediated apoptosis	
MiR-145	BNIP3	Mitochondria-mediated apoptosis	Li et al., 2012
MiR-146a	CypD	Mitochondria-mediated apoptosis	Su et al., 2021; Heggermont et al., 2017
	DLST	Oxidative metabolism	
MiR-149	PARP-2	NAD^+ content and mitochondrial biogenesis	Mohamed et al., 2014
MiR-155	TFAM	Mitochondrial biogenesis	Quinones-Lombrana and Blanco, 2015; Tsujimoto et al., 2020
	BAG5	Mitophagy	
MiR-181a/b/c	PINK1 and Parkin	Mitophagy	Cheng et al., 2016
	BCL2, MCL1, BCL2L11, and XIAP	Mitochondria-mediated apoptosis	Indrieri et al., 2019
	SIRT1, TFAM, and NRF1	Mitochondrial biogenesis	Das et al., 2012; Ouyang et al., 2012; Rivetti di Val Cervo et al., 2012; Wang L. et al., 2014; Wang et al., 2015; Barbato et al., 2021
	MT-COI, COX11, and COQ10B	OXPHOS	
	SIRT1 and PRDX3	Antioxidant response	
	GPX1	Oxidative stress	
MiR-183	IDH2	TCA cycle	Tanaka et al., 2013
MiR-195	MICU1	Mitochondrial Ca^{2+} uptake	Singh and Saini, 2012; Zhou et al., 2013; Zhang et al., 2018; Rao et al., 2020
	ARL2	Mitochondria-mediated apoptosis	
	MFN2	Mitochondrial dynamics	
	BCL2	Mitochondria-mediated apoptosis	
	SIRT3	Mitochondrial energy metabolism	

(Continued)

TABLE 2 | Continued

MiRNA	Targets	Mitochondria-related pathways	References
MiR-204	PGC1a	Mitochondrial biogenesis	Hwang et al., 2016; Houzelle et al., 2020; Zhang L. et al., 2021
	BCL2	Mitochondria-mediated apoptosis	
	TRPML1	Mitophagy and ROS production	
MiR-383	PRDX3	Antioxidant response	Li et al., 2013
MiR-497	BCL2	Mitochondria-mediated apoptosis	Yadav et al., 2011; Wu R. et al., 2016

TCA, tricarboxylic acid; ROS, reactive oxygen species.

in axonal growth and in experimental chronic glaucomatous injury (Huang et al., 2013; Wang et al., 2018). Moreover, overexpression of miR-19a augments axon regeneration via miR-19a–PTEN axis, underscoring the therapeutic potential of local administration of miRNAs via intravitreal injection (Mak et al., 2020). Another interesting miRNA is miR-21, whose inhibition in a model of ON crush promotes axonal regeneration and RGC survival and function (Li H. J. et al., 2018; Li et al., 2019). In the retina of rats with advanced nerve damage induced by elevated IOP, eight miRNAs were significantly downregulated as compared with those in controls (miR-181c, miR-497, miR-204, let-7a, miR-29b, miR-16, miR-106b, and miR-25) and miR-27a was significantly upregulated. Observed miRNA level alterations caused enrichment of targets associated with ECM/cell proliferation, immune system, and regulation of apoptosis (Jayaram et al., 2015). Several miRNAs have been also found to be released in extracellular space in glaucomatous AH. Released miRNAs include miR-21 (apoptosis), miR-450 (cell aging and maintenance of contractile tone), miR-107 (nestin expression and apoptosis), and miR-149 (endothelia and ECM homeostasis) (Tanaka et al., 2014; Izzotti et al., 2015).

Few dysregulated miRNAs in multiple studies have been identified in the blood and vitreous humor of AMD patients. The serum profiles of patients with both wet and dry AMD have shown differences and partial overlap in several miRNAs (Szemraj et al., 2015; Berber et al., 2017), reflecting the difficulty of reducing biomarkers for AMD to one common group (Natoli and Fernando, 2018). A group of dysregulated miRNAs were reported in mouse models of distinct AMD features and demonstrated some similarities with the human AMD findings, including miR-146a, miR-9, miR-17, miR-125b, and miR-155 (Lukiw et al., 2012; Berber et al., 2017; Natoli and Fernando, 2018; Pogue and Lukiw, 2018; Martinez and Peplow, 2021). Those miRNAs can be considered as potential biomarkers and as possible therapeutic targets for AMD. MiR-146a has been found in the plasma (Ertekin et al., 2014; Menard et al., 2016) and retinas (Bhattacharjee et al., 2016) of AMD patients and was modulated in human monocytes stimulated with lipopolysaccharide (Taganov et al., 2006). MiR-146a and miR-9 are upregulated by NF-κB and present indirect correlation with complement factor H (CFH) levels, a key repressor of the innate immune response and a key player in AMD pathogenesis, indicating their modulation as a therapeutic strategy (Lukiw et al., 2012). MiR-17, a regulator of angiogenesis (Doebele et al., 2010) and anti-apoptotic genes as well (Song et al., 2015), is upregulated in an oxidative-induced retina model, an oxidative stress model in RPE cells, and neovascularization AMD plasma. MiR-155 has a role in angiogenesis, complement activation, and inflammation, making it a candidate for therapeutic interventions for AMD. The expression of miR-155 is also induced by AMD-related inflammatory cytokines (O'Connell et al., 2007). In an animal model of AMD, miR-155 has been shown to be upregulated in correlation with increased cell death and inflammation (Saxena et al., 2015), and its downregulation reduced retinal neovascularization (Zhuang et al., 2015). In addition, miR-155 depletion correlates with decreased levels of the mitochondrial translocator protein (TSPO), a selective marker of microglia in their highly reactive state (Yan et al., 2015). Interestingly, miR-146a and miR-155 recognize an overlapping 3′ UTR in CFH, to which both miRNAs may interact (Lukiw et al., 2012).

Several miRNAs, related to DR, are involved in vasculature regulation (miR-126, miR-200b, and miR-31), chronic inflammation pathway (miR-146, miR-155, miR-132, and miR-21), and oxidative stress (miR-21, miR-181c, miR-1179, and miR-8/miR-200 family); other miRNAs present altered expression in DR, but their role is not yet defined (Wu et al., 2012; Andreeva and Cooper, 2014; Mastropasqua et al., 2014; Pusparajah et al., 2016; Shafabakhsh et al., 2019). MiR-383 presents an increased expression in hyperglycemic conditions and targets the mitochondrial peroxiredoxin 3 involved in ROS detoxification and apoptosis (Li et al., 2013). Indeed, miR-383 inhibition diminished ROS and cell death in RPE treated with high glucose (Jiang et al., 2017), representing one of the major keys for the treatment of DR. The expression of miR-451a was found downregulated in diabetic conditions. MiR-451a mimic overexpression showed a protective effect on mitochondrial function in diabetic conditions, probably via the downregulation of activating transcription factor 2 (ATF2) and its downstream target genes CyclinA1, CyclinD1, and MMP2, providing new perspectives for developing effective therapies for proliferative DR (Shao et al., 2019). In both experimental and human diabetes, miR-34a showed increased expression. It promotes mitochondrial dysfunction and retinal microvascular endothelial cell senescence by suppressing the SIRT1–PGC-1α axis as well as the mitochondrial antioxidants TrxR2 and SOD2 (Thounaojam et al., 2019). MiR-195 acts as a regulator for *Mfn2*, which is reduced in the retina of diabetic patients and is involved in maintaining mitochondrial morphology, fusion, and ROS metabolism (Sugioka et al., 2004; Zheng and Xiao, 2010). Oxidative stress-induced overexpression of miR-195 can result in the downregulation of Mfn2 leading to tube formation and to increased blood–retinal barrier permeability, which are two

common pathogenic events of DR (Zhang et al., 2017). Therefore, miR-195 could be considered as a potential therapeutic target for DR (Zhong and Kowluru, 2011). Another miRNA increased upon oxidative stress is miR-100, able to downregulate AKT pathway, extracellular-signal regulated kinase pathway, and TrkB pathway (Kong et al., 2014). MiR-145 overexpression reduced ROS production and increased the activity of SOD (Hui and Yin, 2018). Finally, miR-27 reduces ROS generation and downregulates the P13K/AKT/mTOR signaling pathway by inhibition of Nox2 (Li J. et al., 2018) implicated in ROS induction and neovascularization (Chan et al., 2013, 2015).

Overall, the positive effect of miR-19a, miR-204, and miR-21 modulation on glaucoma murine models, as well as downregulation of miR-155 in AMD mice, highlights the possibility of their rapid translation into clinical application as therapeutic molecules for these eye diseases (bold miRNAs in **Table 1**). However, other preclinical validation steps are required for most of the previously mentioned miRNAs, thus underlining the need and importance of this emerging field of research.

Systematic expression profiling of miRNAs in retinal cells could be of benefit to identify possible involvement of their function in specific retinal cell types, in physiological and pathological conditions. Although novel strategies are under development to study miRNA expression in single-cell transcriptomic conditions (Liu and Shomron, 2021), there are no data reported for such analysis in the retina. However, systematic analysis of miRNA expression and variability in the mouse (Soundara Pandi et al., 2013) and human neural retina and RPE/choroid tissues (Karali et al., 2016) have been reported. Interestingly, among the top 30 expressed miRNAs in retina are reported several miRNAs that present a role in mitochondrial-mediated eye diseases (i.e., miR-181a/b, miR-182, miR-183, miR-204, let-7a, miR-9, miR-96, miR-125b, miR-100, and miR-181c; see **Table 1**). Notably, many of the miRNAs here described and associated with mitochondria-mediated eye diseases can be classified as MitomiR (Purohit and Saini, 2021) (**Table 1**) since they regulate important transcripts impacting different mitochondrial pathways (**Table 2**), thus suggesting an additional possible role of these miRNAs in the pathogenesis and therapy of these disorders.

CONCLUSION

MicroRNAs are promising therapeutic tools due to their capability to simultaneously modulate multiple pathways involved in disease pathogenesis and progression. Moreover, they also represent a class of interesting molecules useful as disease predictive/prognostic biomarkers. Indeed, several miRNAs (let-7a, miR-450, miR-107, miR-204, miR-21, and miR-149 for glaucoma; miR-17 and miR-125b for AMD; miR-126, miR-146a, miR-155, miR-132, miR-21, and miR-34a/c for DR) differentially expressed in body fluids (i.e., serum, plasma, and vitreous liquid or tears) of eye diseases associated with mitochondria dysfunctions (EDAMDs) human patients may be already considered as clinically relevant biomarkers (bold miRNAs in **Table 1**).

Recently, an increasing interest is growing about MitomiRs, which regulate mitochondrial function. As described before, many MitomiRs have been linked to mitochondria-mediated eye diseases, including both rare PMEDs and common retinal diseases (**Tables 1**, **2**). Due to the genetic heterogeneity that characterizes PMEDs and to the big complexity that underlies the most common retinal disorders (e.g., glaucoma, AMD, and DR), no effective treatments are still available. For the above-mentioned reasons, miRNA-based gene/mutation-independent therapeutic strategies may represent a great promise. By targeting common dysregulated pathways that play a key effector role in retinal damage (e.g., mitochondrial dysfunction, oxidative stress, inflammation, and neovascularization), miRNA modulation can protect retinal cells regardless of the primary etiology of the addressed disorder. Considering that the retina is an easily accessible tissue, we believe that the potential application of miRNA therapeutics in retinal disorders could rapidly move to the clinic.

AUTHOR CONTRIBUTIONS

SC and AI conceived the study. SC, FM, and AI wrote the manuscript. All authors contributed to the article and approved the submitted version.

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