Emerging roles of circular RNAs in retinal diseases

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From the Contents

Abstract

Retinal disorders are a group of ocular diseases whose onset is associated with a number of aberrant molecular and cellular processes or physical damages that affect retinal structure and function resulting in neural and vascular degeneration in the retina. Current research has primarily focused on delaying retinal disease with minimal success in preventing or reversing neuronal degeneration. In this review, we explore a relatively new field of research involving circular RNAs, whose potential roles as biomarkers and mediators of retinal disease pathogenesis have only just emerged. While knowledge of circular RNAs function is limited given its novelty, current evidence has highlighted their roles as modulators of microRNAs, regulators of gene transcription, and biomarkers of disease development and progression. Here, we summarize how circular RNAs may be implicated in the pathogenesis of common retinal diseases including diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, and age-related macular degeneration. Further, we explore the potential of circular RNAs as novel biomarkers and therapeutic targets for the diagnosis and treatment of retinal diseases.

Key Words: age-related macular degeneration; circular RNA; diabetic retinopathy; glaucoma; microRNA; proliferative vitreoretinopathy; retina

Introduction

Retinal disorders encompass a vast array of diseases, many of which can have deleterious effects on vision and even result in blindness. The retina is a vital light-sensitive neural tissue and plays a key role in the central nervous system. Its composition of photoreceptors and other retinal neurons such as bipolar cells and retinal ganglion cells constitutes an extensive neural network responsible for producing vision (D'Orazi et al., 2014). Many processes affecting the structure and function of the central nervous system, such as oxidative stress, inflammation, and disturbance in metabolism, just to name a few, can also have manifestations in the eye. Unfortunately, the initial course of retinal degeneration and dysfunction is sometimes undetectable until irreversible damages occur. In addition, aberrant changes in the retinal vasculature resulting in reduction in retinal blood supply, disruption of the retinal microenvironment, and disturbance in retinal metabolism can also affect retinal structure and function in diseases such as diabetic retinopathy (DR), which is the leading cause of vision loss in working-age adults (Zhang et al., 2015; McLaughlin et al., 2019). The neurodegenerative processes in the retina and optic nerve in glaucoma, also known as glaucomatous neurodegeneration, can persist and progress resulting in complete vision loss (Syc-Mazurek and Libby, 2019). In addition, chronic degenerative changes in the retinal pigment epithelium (RPE), choroidal vasculature, and photoreceptors lead to age-related macular degeneration (AMD), which is the most prevalent cause of central vision deterioration in aging adults (DeAngelis et al., 2017). Current treatment goals are mainly focused on partially delaying disease progression, while effective approaches to prevent and reverse neuronal and vascular degeneration in multiple retinal disorders are yet lacking (Striglia et al., 2020; Fleckenstein et al., 2021). Therefore, it is important to develop new diagnostic measures and treatment options based on the pathogenesis of the diseases.

Circular RNAs (circRNAs) are a novel class of non-coding RNAs originating from covalently closed circular transcripts generated by the back-splicing of a single pre-mRNA. Back-splicing involves the non-colinear linkage between a 5′ terminus of a pre-mRNA upstream exon and a 3′ terminus of a downstream exon, resulting in either a single or multi-exon RNA (Yu and Kuo, 2019). Due to the lack of 5′ and 3′ ends, circRNAs are resistant to exonuclease-mediated degradation and are found abundantly expressed throughout the genome (Enuka et al., 2016). Moreover, circRNAs are non-polyadenylated and therefore undetectable by classical methods of sequencing and traditional molecular techniques used for RNA analysis (Kulcheski et al., 2016). As a consequence, novel bioinformatic approaches and sequencing methodologies, such as deep ribosomal RNA (rRNA)-depleted sequencing, have recently developed, enabling investigators to contribute to current research on circRNA function and biogenesis.

CircRNAs, located in the exon, intron, and/or exon-intron boundary, are abundantly expressed in mammalian cells and demonstrate tissue-specific expression patterns (Yu and Kuo, 2019). Some circRNAs manifest potential as gene regulators and participate in the regulation of multiple biological processes, such as neuronal function, innate immune responses, cell proliferation, and pluripotency (Li et al., 2018; Yang et al., 2020). As a result, an emerging research focus in recent years has been on endogenous circRNAs acting as regulators of microRNAs (miRNAs) and their potential role as biomarkers in human diseases. Mechanically, circRNAs have been shown to act as miRNA sponges by binding to miRNAs and subsequently repressing their function (Hansen et al., 2013; Kristensen et al., 2019).

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Moreover, individual circRNAs can block multiple miRNA binding sites as a "sponge" and inhibit activity of one or more miRNAs (Lin et al., 2019). Accumulating evidence suggests that dysregulated circRNAs may underlie neurological diseases, cardiovascular diseases, cancers, diabetes, and vascular disorders (Han et al., 2018; Lee et al., 2019). There have also been studies highlighting the role of circRNAs in retinal dysfunction and the development of pathological characteristics in DR, glaucoma, and retinal detachment (Charteris et al., 2002; Liu et al., 2017; Wang et al., 2018). In this review, we aim to provide an overview of circRNAs and highlight their importance in the pathogenesis of retinal disorders and their potential use as biomarkers and/or therapeutic targets.

Search Strategy and Selection Criteria

Studies cited in this review published from 2001 to 2021 were searched on PubMed and Google Scholar using the following keywords: circRNA, miRNA, retina, diabetic retinopathy, glaucoma, proliferative vitreoretinopathy, and age-related macular degeneration.

Biogenesis and Biofunction of CircRNAs

CircRNAs are generated through the process of back-splicing and the ligation of a 3′ terminus of a downstream exon to the 5′ terminus of an upstream exon (Zhang et al., 2014). This results in the formation a covalently closed circRNA transcript, as well as an alternatively spliced linear RNA with skipped exons. This process is different from the canonical eukaryotic pre-mRNA splicing, which uses the spliceosomal machinery to remove introns and join an upstream (5′) splice donor site with a downstream (3′) splice acceptor site in exons. However, in some circRNAs canonical splice signals were found to flank the junction site, implicating that both canonical splice signals and canonical spliceosomal machinery are required for back-spliced circularization (Ebbesen et al., 2016; Li et al., 2018). Similar to canonical splicing, back-splicing of circRNAs is tightly regulated by cis-elements and trans-factors (Yu and Kuo, 2019). Although some elements of the biogenesis of circRNAs remain unclear, there are three proposed models on how circularization can be achieved based on the elements identified to act in enhancing the circularization of RNA transcripts [reviewed in (Ebbesen et al., 2016) and (Chen, 2020)].

The first model focuses on the regulation of biogenesis by cis-elements. In this model, the introns flanking the exons, which will become circularized, are thought to contain complimentary sequence motifs. Base-pairing between these motifs is what promotes positioning of the appropriate splice sites necessary for circularization within close proximity (Ebbesen et al., 2016). There has been recent focus on the role of complimentary flanking intronic Alu elements in circularization. Alu elements are copiously expressed repetitive sequences, which may contain CpG islands, and constitute 11% of the reference human genome, with almost half of the Alu elements located in human introns (Lander et al., 2001; Konkel et al., 2015). Jeck et al. (2013) first demonstrate that intronic flanks adjacent to circularized exons are enriched in Alu repeat elements as well as being evolutionarily conserved along with Alus compared to non-circularized exons. A recent analysis has also shown that repetitive elements are responsible for most circRNA formation in humans (Dong et al., 2017).

The second proposed model highlights a role of RNA binding proteins (RBPs) in circRNA formation. RBPs are proteins that bind to single-stranded (ss) or double-stranded (ds) RNA. In this model, RBPs bind to motifs in the upstream and downstream introns that flank the circRNA-forming exon(s), and then form dimers to bring closer the splice sites to facilitate back-splicing (Ebbesen et al., 2016). In addition, RBPs that contain double-stranded RNA-binding domains can bind to the intronic complementary sequence to stabilize the transiently formed intronic RNA pairs thereby promoting circRNA formation. This has been observed with the immune factors, nuclear factor 90 and NF110 protein, each of which contains two double-stranded RNA-binding domains, directly binding to intronic inverted-repeat Alu elements and promoting circRNA formation (Li et al., 2017). Another example is that during epithelial-mesenchymal transition, a large number of circRNAs are produced through *de novo* circRNA formation regulated by a RBP named Quaking, which itself is regulated during epithelial-mesenchymal transition (Conn et al., 2015). However, it was also observed that double-stranded RNA-binding proteins can act as inhibitors of circRNA formation as seen in the enzyme adenosine deaminase acting on RNA 1, which decreases

complementarity and destabilizes RNA pairing (Ivanov et al., 2015). RBPs without a double-stranded RNA-binding domain can also participate in the regulation of circRNA levels by directly binding to specific RNA motifs (Li et al., 2018).

Exon skipping is another potential model in which RBPs can cause circularization of RNA transcripts. In this pathway, one or more exons are spliced out creating an exon-containing lariat. The spliceosome can then recognize the skipped exons within the lariat due to their closer proximity and join them (Jeck et al., 2013). These findings suggest that circRNA production is tightly regulated and highly dependent on biological settings. In addition, expression of circRNAs and their different isoforms is often cell type-, tissue-, and developmental stage-specific. Some circRNAs also show conservation across species, supporting the idea that circRNAs are functional molecules.

CircRNAs function as miRNA sponges

The most recent focus has been on the role of circRNAs as gene regulators by acting as "sponges" that bind to miRNAs and inhibit their activity. miRNAs are a class of short and common non-coding RNAs that regulate gene expression after transcription via the direct base pairing of miRNA target sites. One circRNA can target one or multiple miRNAs through multiple binding sites in the circular sequence (Li et al., 2018). This property has been first observed in cerebellar degeneration-related protein 1 (CDR1) antisense RNA (*CDR1as*) and *Sry* (Sex-determining region Y) circRNAs, which function by binding to specific miRNAs and acting as target decoys. *CDR1as*, derived from the antisense transcript of the *CDR1* gene, is one of the first discovered functional circRNAs. It is most copiously expressed in the mammalian brain, particularly the cerebellum. *CDR1as* targets miR-7 and is found to have 74 miR-7-specific binding sites (Uhr et al., 2018). Knockdown of *CDR1as* results in dysregulation of miR-7 expression and subsequently affects insulin secretion, cell proliferation, and the pathobiology of myocardial infarction (Li et al., 2018). Like *CDR1as*, *Sry* circRNA contains 16 target sites for miR-138 in the mouse, suggesting its role as a miR-138 sponge (Yu and Kuo, 2019). In addition, several other circRNAs in mammals have been suggested to act as miRNA sponges with potential implications in retinal disorders (Kulcheski et al., 2016; Liu et al., 2017, 2019; Zhang et al., 2017; Wang et al., 2018), which will be discussed in detail in the next section.

CircRNAs function as transcriptional regulators

CircRNAs located in the nucleus are also found to be involved in the regulation of transcription and splicing. EIciRNAs are a novel class of circRNAs composed of exon-intron circRNA. EIciRNAs could interact with U1 small nuclear ribonucleoproteins (U1 snRNPs) and the EIciRNA-U1 snRNP complexes may associate with Pol II at promoter sites to boost gene expression. Blocking such RNA-RNA interactions showed decreased contact of EIciRNAs with Pol II and, subsequently, reduced transcription of their parental genes (Ebbesen et al., 2016).

Role of circRNAs as biomarkers

In recent studies, circRNAs have been found abundantly in saliva and blood samples, suggesting that circRNAs can be utilized as potential clinical biomarkers for human embryonic development, diseases progression, and prognosis (Lee et al., 2019). circRNAs have already been established as biomarkers for different types of cancers such as gastrointestinal caner (Li et al., 2015) and hepatocellular carcinoma (Qin et al., 2016). Emerging research also suggests that circRNAs can be possibly used as biomarkers in DR, vascular dysfunctions, AMD, and glaucoma (Zhou et al., 2015; Liu et al., 2017; Zhang et al., 2017; Wang et al., 2018; Chen et al., 2020a). However, more studies are required to further justify the sensitivity and reliability of circRNAs as a biomarker in retinal disorders and other chronic neurodegenerative diseases in the central nervous system.

Approaches for Analysis and Validation of CircRNAs

CircRNAs lack 3′ and 5′ free ends and do not have a polyadenylated free end, thus posing a challenge when it comes to detection by employing traditional RNA analysis techniques. Circular RNAs can only be identified by ribosomal RNA depleted RNA libraries (rRNA depletion library) or by combining rRNA depleted and RNase R exonuclease treated samples (RNase R) (Jeck and Sharpless, 2014). Algorithms focused on the use of distinct alignment methods and splice signals to detect back-spliced reads in RNA sequencing data

have been created in order to detect true circRNAs. However, their effectiveness is variable and dependent on multiple factors such as the applied statistical methodology and match of mapped circRNAs to the correct genome locations (Li et al., 2019). Examples of tools created to detect back-splice reads by using distinct alignment methods and splice signals from RNAseq data include MapSplice (Wang et al., 2010), find_circ (Memczak et al., 2013), circRNA_finder (Westholm et al., 2014), CIRCexplorer (Zhang et al., 2014), segemehl (Hoffmann et al., 2014), CIRI (Gao et al., 2015), DCC (Cheng et al., 2016), UROBORUS (Song et al., 2016), and acfs (You and Conrad, 2016).

Several mathematical models have been used to numerically describe the construction of transcripts, abundance, and their differential expression. Examples of such tools are CIRI-AS, FUCHS, and RAISE, which use splice and paired-end reads information to create a complete coverage of circular transcripts (Metge et al., 2017). CIRCexplorer2 uses cufflinks and poly(A) linear alignment for circRNA transcript reconstruction (Zhang et al., 2016). Recently, Li et al. (2019) developed a comprehensively automated circRNA analysis pipeline called circRNAwrap to help further research with circRNAs. The circRNAwrap pipeline includes RNA-Seq read alignment, circRNA identification, circRNA transcript prediction, and circRNA abundance estimation. All of the involved steps combine the use of different circRNA identification tools and Sailfish, which is used for the estimation of circular and linear transcripts.

CircRNAs in Retinal Development

There are limited studies on the characterization of circRNAs during retinal development, but such information would elucidate how and in what ways circRNAs may function in healthy and diseased states. Interestingly, one study by Chen et al. (2020b) discovered that different developmental stages of the mouse retina could be distinguished by unique circRNA expression patterns. CircRNA expression patterns were extrapolated using deep RNA sequencing of rRNA-depleted total RNA samples from five developmental stages, namely the embryonic stage (E18.5), early postnatal stage (P1), outer segment of photoreceptor development stage (P7), eye opening stage (P14), and maturation (P30). Among the 9209 circRNAs detected during development, 438 circRNAs were expressed at all stages while the rest comprised unique signature circRNA profiles per developmental stage, much like a "fingerprint." The importance of proper circRNA expression during developmental stages was demonstrated in rd8 retinal degeneration mouse models, which displayed significantly upregulated circRNAs at P30 prior to onset of disease at P90. This implied that circRNAs play a highly responsive role in retinal degeneration compared to their corresponding linear RNA transcripts, which showed minimal changes prior to disease onset.

Studies of circular RNomes in development can also contribute to the identification of potential therapeutic targets in retinal disease. Among eight of the circRNAs selected for further study by Chen et al. (2020), circHipk2 and circTulp4 was found to act as sponges of miR-124-3p and miR-204-5p/miR-26a-5p, respectively. Moreover, interruption of circTulp4 levels in mouse eyes treated with AAV-circTulp4-shRNA led to compromised retinal function, demonstrated by decreased scotopic and photopic responses on electroretinography, and a thin outer nuclear layer due to upregulation of apoptotic genes. While conservation analysis revealed that more than 13.48% of the mouse circRNAs at each developmental stage could be identified in humans, further investigation is required to confirm and assess the exact implications of circHipk2 and circTulp4 sponging and their translatability from mice to humans.

Implications of CircRNAs in Retinal Disease

Diabetic retinopathy

CircRNAs in the mammalian brain have taken the research spotlight over the past few years. However, the role of circRNAs in retinas remains undetermined. DR is the most frequently occurring complication of diabetes mellitus and the most prominent disease affecting vision globally. It results in retinal microvascular dysfunction and, consequently, neurodegeneration. The precise mechanisms underlying DR pathogenesis are still not fully understood, therefore developing novel diagnostic and treatment techniques is imperative (Zhang et al., 2017). Recent studies have identified specific circRNAs which are highly expressed in the retina and can play a role in the diagnosis and treatment of vascular disorders, such as processes that

arise as a result of diabetes mellitus (**Table 1**).

Biomarkers of diabetic retinopathy

In one study, Zhang et al. used high-throughput circRNA microarray to assess differences of circRNA expression between diabetic retinas and non-diabetic human retinas (Zhang et al., 2017). This data pointed to one circRNA derived from HAS2 gene locus (circ_0005015) which was significantly upregulated in diabetic retinas. In addition, higher levels of circ_0005015 were found in the vitreous sample, plasma fraction of whole blood, and pre-retinal fibrovascular membranes (FVMs) of DR patients (Zhang et al., 2017). Functional studies demonstrated that silencing circ_0005015 decreased retinal endothelial cell proliferation, migration, tube formation, and spheroid sprouting, suggesting an important role of this circRNA in regulation of the angiogenic potential of endothelial cells that may contribute to retinal neovascularization in DR. Circ_0005015 was also found to act as a miRNA sponge to regulate gene expression. Specifically, circ_0005015 can bind to miR-519d-3p and inhibit its activity, resulting in increased MMP-2, XIAP, and STAT3 expression (Zhang et al., 2017). Based on these findings, circ_0005015 might be considered a potential biomarker for DR.

Implications on retinal pericyte biology

An early hallmark of DR is the loss of pericytes in retinal blood vessels (Hammes et al., 2002). Pericytes are critical regulators of endothelial cell (EC) proliferation and play a vital role in vascular stabilization, maturation and remodeling. Loss of pericyte coverage exacerbates EC dysfunction contributing to increased vascular permeability, macular edema, and angiogenesis in DR (Hammes et al., 2002). An investigation led by Liu et al. (2019) studied a role of circRNA in communication between vascular pericytes and ECs in DR. They discovered 844 circRNAs that were differentially expressed in diabetic and non-diabetic mouse retinas, and the most upregulated circRNA was cPWWP2A in pericytes. Functional studies demonstrated that silencing cPWWP2A in the retina aggravated vascular pathology in DR including increased vascular permeability and acellular capillary formation associated with decreased pericyte coverage in retinal vasculature. In addition, silencing cPWWP2A also exacerbated retinal inflammation (Liu et al., 2019). Mechanistically, cPWWP2A acting as a miRNA sponge sequesters and inhibits the activity of miR-579, thereby upregulating the expression of target genes of miR-579 including angiopoietin 1, occludin, and SIRT1 in pericytes (Liu et al., 2019). Protective effects of overexpression of cPWWP2A on promoting cell survival through regulation of miR-579/Sirt1 were also observed in human osteoblasts (Hong et al., 2019) and macrophages (Ma et al., 2019). Whether targeting the cPWWP2A /miR-579/ Sirt1 signaling can provide benefits to other retinal cell types, such as retinal neurons, thus improving retinal function and preventing neurodegeneration in DR and other retinal diseases remain to be investigated.

Adding to the evidence of circRNA-mediated pericyte function is the identification of cZNF532 as a protective regulator in DR-induced pericyte degeneration (Jiang et al., 2020). In cultured human retinal pericytes, silencing cZNF532 reduced expression of pericyte markers, cell viability and proliferation and increased apoptosis. Moreover, silencing cZNF532 in pericytes suppressed pericyte recruitment to endothelial cells, suggesting that cZNF532 plays an important role in the regulation of pericyte health and function and in maintaining retinal vascular integrity. *In vivo* studies using streptozotocin-induced murine DR models showed that, similar to cPWWP2A, cZNF532 silencing led to increased formation of microaneurysms, acellular capillaries, and pericyte ghosts, or degenerated intramural pericytes. Similar results were also seen in conditional knockdowns of cZNF532 in pericytes, further suggesting its pericyte-specific actions. As with many discovered circRNAs, the mechanism by which cZNF532 imposes protective effects may be through the sponging of miR-29a-3p, whose overexpression was shown to result in the same insults to retinal pericytes. The downstream targets of miR-29a-3p included NG2, LOXL2, and CDK2, suggesting that a cZNF532-miR-29a-3p/NG2/ LOXL2/CDK2 network is at the basis of retinal vascular dysfunction. From a clinical standpoint, vitreous samples from patients with diabetic macular edema, proliferative DR, or neovascularization of the iris exhibited higher levels of cZNF532 that also correlated with disease severity as well as unchanged miR-29a-3p levels. While the investigators interpreted this finding to imply that cZNF532 acted as a protector in the etiology of DR in humans, it is still curious that its upregulation was detected alongside severe disease given the disease-attenuating roles that were elucidated with cZNF532 upregulation in *in vitro* and murine *in vivo* models. Future studies

Table 1 │ **Targets and roles of circRNAs in retinal diseases**

CircRNA	Targets	Tissues/Cell types of expression	Disease association	Biological function	References
cZNF609	miR-615-5p	Retinal endothelial cells Retinal ganglion cells Müller cells (rMC-1)	Diabetic retinopathy Glaucoma	Regulation of endothelial cell survival, proliferation, migration, and tube formation Retinal neurodegeneration and gliosis Increases expression of MEF2A and METRN	Liu et al., 2017; Wang et al., 2018
circHIPK3	miR-30s-3p	Retinal endothelial cells	Diabetic retinopathy	Regulation of endothelial cell proliferation, migration, and tube formation Increases expression of VEGF-C, FZD4, and WNT2	Shan et al., 2017
Circ0005015	miR-519d-3p	Plasma Vitreous Fibrovascular membranes Retinal endothelial cells	Diabetic retinopathy	Regulation of endothelial cell proliferation, migration, and tube formation Increases expression of MMP-2, XIAP, and STAT3	Zhang et al., 2017
cPWWP2A	miR-579	Retinal pericytes	Diabetic retinopathy	Regulation of pericyte-endothelial cell crosstalk, pericyte coverage, and vascular integrity Increases expression of angiopoietin 1, occludin, and SIRT1	Liu et al., 2019
circ0043144	–	Vitreous ARPE-19 cells	Proliferative Vitreoretinopathy	Proliferation, migration and secretion ability of ARPE-19 cells Increases production of CCL2, CXCL8, IL-6, and VEGF-A	Yao et al., 2019
circDMNT3B	miR-20b-5p	Retinal endothelial cells	Diabetic retinopathy	Decreases endothelial cell proliferation, migration, and tube formation Increases expression of BAMBI	Zhu et al., 2019
circNR3C1	miR-382-5p	ARPE-19 cells	Age-related macular degeneration	RPE marker expression, phagocytosis of photoreceptor outer segments, and accelerated anti-oxidation Regulates the PTEN/AKT/mTOR pathway	Chen et al., 2020
cZNF532	miR-29a-3p	Retinal pericytes	Diabetic retinopathy	Regulation of pericyte marker expression, recruitment towards retinal endothelial cells, cell viability, and proliferation	Jiang et al., 2020

ARPE-19: Adult retinal pigment epithelial cell line-19; BAMBI: bone morphogenetic protein (BMP) and activin membrane bound inhibitor; CCL2: C-C motif chemokine ligand 2; CXCL8: C-X-C motif chemokine ligand 8; FZD4: frizzled class receptor 4; IL-6: interleukin 6; MEF2A: myocyte enhancer factor 2A; METRN: meteorin; MMP-2: matrix metalloproteinase-2; SIRT1: sirtuin 1; STAT3: signal transducer and activator of transcription 3; VEGF-A: vascular endothelial growth factor A; VEGF-C: vascular endothelial growth factor C; WNT2: wnt family member 2; XIAP: X-linked inhibitor of apoptosis.

are warranted to evaluate the protective effects of targeting the cZNF532/miR-29a-3p network on retinal vasculature in large animal models and clinical trials.

Other mechanisms of retinal vascular dysfunction
CircHIPK3 is another circRNA found to be expressed at higher levels in retinal endothelial cells. It is thought to play a role in the regulation of diabetes mellitus-induced retinal vascular dysfunction by acting as a miRNA sponge (Shan et al., 2017). CircHIPK3 expression is significantly upregulated as a response to high glucose stress *in vivo* and *in vitro*. Silencing of CircHIPK3 shows a decrease in abnormal proliferation, mobility, and tube formation of retinal endothelial cells *in vitro*, implying that it could have a role as a potential therapeutic target. CircHIPK3 is mainly expressed in the cytoplasm of human retinal vascular ECs and can also act as an endogenous miR-30a-3p sponge, thus upregulating VEGFC, FZD4, and WNT2 expression (Shan et al., 2017). This data suggests that altering circHIPK3 expression could prevent or reduce vascular complications.

cZNF609 is a circRNA that is significantly up-regulated by high glucose and hypoxia stress *in vivo* and *in vitro* and is an important mediator of vascular dysfunction (Liu et al., 2017). cZNF609 is found at high levels in ECs and is dysregulated when there is vascular dysfunction. In a study to explore the biological role of cZNF609 in retinal ECs, Liu et al. (2017) discovered that silencing cZNF609 protects cultured retinal ECs from oxidative stress and hypoxia induced apoptosis, while overexpressing cZNF609 exacerbates EC injury. In addition, silencing cZNF609 increases endothelial cell viability and proliferation and accelerates cell migration and tube formation. These protective effects of cZNF609 inhibition were further confirmed by *in vivo* studies, demonstrating that silencing cZNF609 decreases retinal neovascularization, reduces vascular obliteration and degeneration, alleviates inflammatory response, and decreases vascular permeability in animal models of DR and oxygen-induced retinopathy. These findings strongly imply that cZNF609 silencing could provide beneficial effects to retinal ECs under stress conditions, thus minimizing vascular injury and subsequent aberrant vascular growth (pathological angiogenesis) resulting from retinal ischemia (Liu et al., 2017). Mechanistically, cZNF609 was found to interact with miR-615, potentially acting as an endogenous sponge that sequester and inhibit miR-615 activity. This in turn leads to an increase in transcription factor MEF2A (myocyte-specific enhancer

factor 2A) expression, which was believed to mediate the detrimental effects of cZNF609 in retinal ECs. Thus, targeting the cZNF609/miR-615-5p/MEF2A network may be used as a promising approach for protecting retinal ECs against stress at the early stages of DR and preventing advanced vascular pathologies including pathological angiogenesis (Liu et al., 2017).

Common to the theme of endogenous circRNA sponges is circDMNT3B, which was shown to act as a sponge of miR-20b-5p (Zhu et al., 2019). CircDMNT3B was found to have a therapeutic effect in rat DR models that received intravitreal injections of AAV-Dj-circDMNT3B. The mechanistic pathway was purported to involve the downregulation of miR-20b-5p via the sponge action of circDMNT3B, which then consequently led to decreased targeting of BMP and activin membrane-bound inhibitor (BAMBI). Given that BAMBI knockdown in human retinal microvascular endothelial cells resulted in enhanced proliferation, migration, and tube formation under high-glucose diabetic conditions, a downregulation of miR-20b-5p via circDMNT3B was hypothesized to upregulate BAMBI levels and maintain vascular homeostasis in human retinal microvascular endothelial cells. Thus circDMNT3B/miR-20b-5p/BAMBI serves as additional compelling evidence that circRNAs represent a realm of non-coding RNAs whose roles may have important effects on the development of DR.

Glaucoma
In addition to regulation of angiogenesis and vascular injury, cZNF609 also plays a role in modulating glaucoma, a neurodegenerative retinal disease characterized by progressive and gradual loss of retinal ganglion cells and their axons (Almasieh et al., 2012). cZNF609 silencing is found to inhibit retinal reactive gliosis and glial cell activation as well as support retinal ganglion cell survival in glaucoma (Wang et al., 2018). As mentioned earlier, cZNF609 acts as a miR-615 sponge that sequesters and inhibits miR-615 activity. This can lead to increased Meteorin (METRN), which is a secreted protein that has been linked to the control of neuritogenesis, angiogenesis, and gliogenesis (Nishino et al., 2004). These studies suggest that cZNF609 or its downstream targets may be a potential therapeutic target for vascular disorders and retinal neurodegeneration.

Proliferative vitreoretinopathy
CircRNAs also have implications in proliferative vitreoretinopathy

(PVR), one of the major complications of rhegmatogenous retinal detachment surgery. PVR is a complex disease that can result in severe vision reduction mainly by the formation of contractile pre-retinal fibrous membranes (Zhou et al., 2015). Even though retinal surgery has undergone major advances over the past few years and has taken measures to reduce the incidence of PVR, there are no agents that have been approved for clinical applications (Pastor et al., 2016). Current methods of PVR treatment mainly include vitrectomy, systematic peeling, and dissecting epiretinal membranes and retinal tamponade with silicone oil or gas (Charteris et al., 2002). circ_0043144 is a circRNA with high levels in vitreous samples and corresponding serum samples of patients with PVR. circ_0043144 expression was significantly down-regulated after PVR operation. *In vitro* studies conducted by Yao et al. (2019) revealed that circ_0043144 was involved in the regulation of the proliferation, migration, and secretion ability of ARPE-19 cells, which is critical for epiretinal membrane formation. This implies that circ_0043144 can be used as a biomarker of PVR and can aid the distinction between PVR and other ocular diseases. Detection of circulating circ_0043144 levels would be used as a non-invasive, rapid, and sensitive tool for molecular diagnosis and monitoring of PVR disease (Yao et al., 2019).

Age-related macular degeneration

Among the aforementioned major retinal diseases, AMD ranks as the most common cause of irreversible vision impairment in adults over the age of 50 worldwide (Friedman et al., 2004). Multiple studies have shown that RPE dedifferentiation is a key pathological event in the early stages of atrophic AMD, or AMD characterized by RPE atrophy (Zhao et al., 2011). A recent study by Chen et al. (2020a) suggests that circNR3C1, derived from the NR3C1 gene, may have protective effects on RPE function and dedifferentiation, thereby preventing AMD progression. CircNR3C1 expression levels were vastly reduced under abnormal RPE conditions *in vitro* and downregulated in the blood serum of AMD patients. ARPE-19 cells transfected with circNR3C1-siRNA exhibited downregulated expression of RPE markers, suppressed phagocytosis of photoreceptor outer segments, and accelerated generation of reactive oxygen species—all of which are considered pathologic events that are contributory to AMD progression. CircNR3C1 was also found to serve as a sponge for miR-382-5p, a miRNA whose overexpression can suppress the protective role of Phosphatase and tensin homolog on chromosome 10 (PTEN) in retinal cells. The circNR3C1/miR-382-5p/PTEN network was then correlated with the proper mediation of RPE phenotypes via the AKT/mTOR pathway, further supporting the idea that aberrant circRNAs may have a wide range of deleterious effects on retinal cell function and physiology.

Perspective and Future Directions

The discovery of circRNAs in humans has been a breakthrough in the scientific field in recent years. The function of circRNAs as miRNA sponges in most human diseases presents a novel potential for therapeutic targets or biomarker use. Multiple circRNAs have implications in retinal disorders and clearly serve as important regulators of retinal neurodegeneration and microvascular dysfunction. As with many studies of biomarkers and genes implicated in disease pathogenesis, the multitudinous availability of potential disease markers and therapeutic targets presents as both an asset and challenge to investigators seeking to develop therapies for retinal dysfunction and degeneration of neuronal and vascular systems.

Though the concept of miRNA sponging by circRNAs is a seemingly simple one, the reality of circRNA research often involves focusing on one miRNA among a handful of other identified miRNAs whose levels were also altered based on circRNA action (Thomson and Dinger, 2016). The choice to investigate a single miRNA is often based on looking at its relative abundance. Moreover, several circRNAs seem to be implicated in many of the same disease processes that lead to eye disease—both cPWWP2A and cZNF532, for example, play highly similar protective roles in pericyte function, and both circRNAs were selected for investigation among a myriad of other circRNAs that also showed differential expression in diabetic versus control retinas (Liu et al., 2019; Jiang et al., 2020). A single study on circRNAs in mice with oxygen-induced retinopathy resulted in the construction of a vast network of 236 mRNAs, 4 circRNAs, and 42 predicted miRNAs, further illustrating the complexity of circRNA-miRNA interactions (Cao et al., 2019). Such abundance of miRNAs and circRNAs implicated in retinal disease is promising in that numerous possible targets may be assessed for therapy. However, it also presents a significant challenge to piecing together the comprehensive picture of the circRNA/miRNA/downstream-target network that governs retinal disease. Such knowledge may be important for understanding whole implications of circRNA targeting. For this reason, more research, perhaps involving multiple circRNA targets, is needed to fulfill this knowledge gap.

Despite the inherent challenges of attaining a comprehensive understanding of circRNA-mediated signaling, it is evident that circRNAs themselves may serve as useful biomarkers of retinal disease. Most of the circRNAs described in this review have the potential to be used as biomarkers of DR, PVR, AMD, and possibly glaucoma. Biomarkers are important in their utility as markers of disease progression, particularly for diseases for which prognoses can be difficult to make, such as DR; the temporal progression from non-proliferative DR—the mildest classification of DR—to severe proliferative DR, for example, can largely vary per individual (Wong et al., 2009). In such cases, levels of the aforementioned cZNF532 may be of use in determining DR prognoses for each patient given its high correlation with DR severity (Jiang et al., 2020). Biomarkers can also be utilized as primary or secondary endpoints in clinical trials; circ_0043144, for instance, could be examined to distinguish between PVR and other ocular diseases in clinical settings (Yao et al., 2019).

There are many avenues through which future research can embark to advance the field of circRNAs in retinal disease. For one, it is important to note that circRNAs underscore the important role of miRNAs in retinal degeneration. It is not necessarily the circRNAs themselves that cause disease, but rather their deregulation in sponging miRNAs that often triggers pathogenesis. This emphasizes the importance of developing therapies that ultimately affect the action of miRNAs, whether this be through engineering anti-miRNAs that target the specific miRNAs implicated in circRNA deregulation, designing artificial circRNA sequences to inhibit specific miRNAs, or possibly exploiting the endogenous degradation mechanism of circRNAs, which itself remains elusive (Lima et al., 2018; Schreiner et al., 2020; He et al., 2021). Research in these areas is scant, particularly in the context of retinal disease. Overall, further studies are required to both support the role of circRNAs as biomarkers of disease development and/or progression as well as establish the therapeutic potential of targeting circRNAs for the treatment of retinal diseases.

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