

# An Eye on Age-Related Macular Degeneration: The Role of MicroRNAs in Disease Pathology

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**Abstract** Age-related macular degeneration (AMD) is the primary cause of blindness in developed countries, and is the third leading cause worldwide. Emerging evidence suggests that beside environmental and genetic factors, epigenetic mechanisms, such as microRNA (miRNA) regulation of gene expression, are relevant to AMD providing an exciting new avenue for research and therapy. MiRNAs are short, non-coding RNAs thought to be imperative for coping with cellular stress. Numerous studies have analyzed miRNA dysregulation in AMD patients, although with varying outcomes. Four studies which profiled dysregulated circulating miRNAs in AMD yielded unique sets, and there is only minimal overlap in ocular miRNA profiling of AMD. Mouse models of AMD, including oxygen-induced retinopathy and laser-induced choroidal neovascularization, showed similarities to some extent with miRNA patterns in AMD. For example, miR-146a is an extensively researched miRNA thought to modulate inflammation, and was found to be upregulated in AMD mice and cellular systems, but also in human AMD retinae and vitreous humor. Similarly, miR-17, miR-125b and miR-155 were dysregulated in multiple AMD mouse models as well as in human AMD plasma or retinae. These miRNAs are thought to regulate angiogenesis, apoptosis, phagocytosis, and inflammation. A promising avenue of research is the modulation of such miRNAs, as the

phenotype of AMD mice could be ameliorated with antagomirs or miRNA-mimic treatment. However, before meaningful strides can be made to develop miRNAs as a diagnostic or therapeutic tool, reproducible miRNA profiles need to be established for the various clinical outcomes of AMD.

## Key Points

Several studies of microRNA, small non-protein coding RNA molecules, were conducted in blood and vitreous humor of AMD patients, but revealed few microRNAs consistently dysregulated in multiple studies.

Similar profiling studies were reported in mouse models of distinct AMD features and demonstrated some similarities with the human AMD findings, including miR-146a, miR-17, miR-125b, and miR-155.

Alteration of the levels of these miRNA in mice revealed an amelioration of the AMD-related damage, providing a promise for novel therapeutic approaches for this devastating blinding disease.

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## 1 Introduction

### 1.1 MicroRNAs: An Historical Perspective

MicroRNAs (miRNAs) are 19–24 nucleotide non-coding RNAs, which negatively regulate gene expression. Although miRNAs were not discovered until 1993, the idea

that non-coding RNA molecules could interfere with gene expression, was first suggested in the late 1970s and early 1980s, when multiple groups found that exogenous oligonucleotides complementary to ribosomal RNA interfere with ribosome function [1–3].

Ironically, the motivation behind the project which ultimately lead to the discovery of miRNAs had only little to do with the field of noncoding RNAs or antisense gene regulation, but instead started with the study of neural development in microscopic worms [4, 5]. In the 1970s, Brenner [6] discovered a line of mutant *lin-4(e912)* *Caenorhabditis elegans* with remarkable developmental timing defects [7]. The “adult” larvae contained many cell types ordinarily only present during an early larval stage, and were devoid of many of the cell types and morphological structures typical of wild-type adults [4, 8].

In 1984, Ferguson et al. [9] discovered a *C. elegans* worm with nearly normal morphology in a culture of the *lin-4* strain, and determined that a previously unknown gene, *lin-14* was the responsible suppressor mutation. Animals with null alleles of *lin-14* were shown to have developmental timing defects opposite to those of *lin-4* [10]. These findings suggested an epistatic interaction between *lin-4* and *lin-14*, and it was theorized that *lin-4* encodes a protein which negatively regulates *lin-14* [5]. In 1993, almost four years after the initial findings, two articles were published in the same issue of the *Cell* journal which elucidated the molecular relationship between *lin-14* and *lin-4*. Lee et al. [4] showed that *lin-4* is not a protein-encoding gene, but instead a 22nt non-coding RNA transcript and a 60nt hairpin structure. The second study reported that seven nucleotides in the 3′ untranslated region (UTR) of *lin-14* are complementary to the *lin-4* small RNAs, and suggested that *lin-4* downregulates *lin-14* translation through RNA-mediated gene expression [11].

## 1.2 MiRNAs and Ocular Pathogenesis

It is difficult to understate the importance of miRNAs, as they regulate proper cell growth, development, and differentiation [12]. Much of the early work on miRNAs focused on their roles in tissue and organ development [4, 13]. In this context, loss of miRNA function produced gross phenotypic defects, and their impact was unmistakable [13]. As technology advanced, researchers began uncovering the function of miRNAs in the mature tissue of higher eukaryotes. In recent years, the theory emerged that miRNA in mature organisms and tissues tend not to affect the primary function of cells, but are imperative for coping with various forms of cellular and organismal stress [13–16]. To date, miRNAs are thought to buffer against fluctuations in gene expression due to either stochastic modulation or environmental stress, in an effort to maintain

cellular homeostasis [13, 17–19]. To facilitate their function, miRNAs are specifically incorporated into the RNA-induced silencing complex (RISC) and bind to transcripts in the cytosol. This binding either results in the degradation of the transcript by nucleases associated with the RISC complex or lead to reduced translation speed by influencing the ribosomal machinery.

As photoreceptors and retinal pigment epithelium (RPE) cells of the retina must maintain their function under exceptionally high rates of metabolism and protein synthesis, there is arguably no system under more stress [13]. Specifically, in pathological conditions of the eye, the intense stress overwhelms the cells’ coping mechanisms resulting in photoreceptor death ultimately causing blindness. This makes the retina a highly interesting organ to elucidate the pathological repercussions of miRNA dysregulation in mature tissue.

## 1.3 Age-Related Macular Degeneration (AMD)

AMD is a complex disease of the central retina and the primary cause of blindness in developed countries, as well as the third leading cause worldwide [20]. Estimates put the number of people suffering from the late stage of the disease worldwide at 11 million by 2020 [21]. In a healthy eye, light is transduced to a chemical signal by the highly specialized photoreceptors, which have a multifaceted support system. In AMD, the dysfunction of this support system causes progressive damage to the photoreceptors, and ultimately results in vision loss in one or both eyes [22].

Although the pathogenesis of AMD is currently not completely understood, the complex etiology has been linked to cellular, biochemical, and molecular events and is influenced by multiple components involving both environmental factors and genetic predisposition [22–24]. Genome-wide association studies and large scale re-sequencing projects have identified a number of single nucleotide variants (SNVs) enriched in complement and complement-related genes that confer a strong risk for AMD [25–28]. Recent efforts have increased the number of independent genetic signals associated with AMD risk to 52 at 34 different loci [27], explaining around half of the genomic heritability of the disease. Six out of those 34 loci harbor one or more complement or complement-related genes. Together, the known genetic risk factors point to the involvement of oxidative stress, lipid metabolism, extracellular matrix biology, inflammation and dysregulation of the complement cascade and other immune responses in the pathogenesis of the disease.

Early stages of AMD are characterized by an abnormal RPE pigment distribution in the macula, and the formation of drusen-containing lipids and an array of proteins in the

space between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane [22, 29–31]. Early AMD is usually asymptomatic, but may cause a modest decline in visual acuity and function resulting in delayed dark adaptation [22].

Early AMD can progress to late-stage AMD, characterized by geographic atrophy (GA AMD) and choroidal or, less common, retinal neovascularization (both forms subsumed as NV AMD). GA AMD is defined by well-demarcated areas of RPE atrophy, which is followed by degeneration of the corresponding photoreceptors and choroidal capillaries [32]. Disease progression in GA AMD is usually slow, although it can result in significant visual deficits in reading, night vision, and dark adaptation [33, 34]. There is currently no approved or effective treatment to prevent the onset or progression of GA [24, 35].

The hallmark of NV AMD is the growth of new blood vessels generally sprouting from the choroid, penetrating the overlying Bruch's membrane, and growing within the subretinal pigment epithelium space or the subretinal space [36, 37]. In NV AMD the growing neovascular membrane is initially capillary-like [36], and these fenestrated and friable vessels frequently leak serous fluid, lipid, and blood into the retina and surrounding spaces. The neovascularization is accompanied by the infiltration of macrophages and other inflammatory, matrix metalloproteinases producing cells, enabling the growing membrane to digest through tissue planes [38]. Macrophages release a range of other mediators, including pro-inflammatory and pro-angiogenic factors. At some point, the balance between growth factors and inflammatory mediators shifts toward an antiangiogenic, anti-proteolytic, and anti-migratory state, and the CNV lesion becomes fibrosed [36, 37]. This process is known as disciform scar formation, and is associated with irreversible visual loss [36]. Untreated patients with NV AMD experience a consistent, steady deterioration in visual acuity over the first few years following onset of neovascularization, with 75 % of patients reaching legal blindness within 3 years [39].

Choroidal neovascularization can be treated but not cured with inhibitors of vascular endothelial growth factor (VEGF) [40]. In the pathogenesis of NV AMD, increased VEGF production is a defensive response by the tissue to oxidative stress or hypoxia, triggering the proliferation of newly formed blood vessels and damaging the retinal tissues, especially the photoreceptors [41]. In the past several years, the inhibition of VEGF has become the gold standard in the treatment of NV AMD, but is far from an ideal therapy. Monthly injections of VEGF inhibitors are burdensome to patients and a major challenge to healthcare systems worldwide [42]. Additionally, AMD patients with NV exhibit a great deal of variability in response to anti-

VEGF treatments, some patients even lose visual acuity during treatment [40, 43].

Despite advances in the prevention and treatment of the disease, the number of afflicted patients is expected to increase due to a rapidly growing elderly population [20]. In many cases, the damage is not contained to the photoreceptors and RPE cells, the mental health of these patients may also suffer. Depression affects 30 % of older adults with visual impairment, and those with AMD seem to be at particular risk with 39 % affected [44]. The mental deterioration is thought to stem from the frustration and hopelessness elderly patients can feel when the loss of functional capacity becomes apparent [44]. In a national opinion poll of US adults, when asked which disease is the worst that could happen to them, African Americans and Non-Hispanic Whites said that blindness was worse than cancer and HIV/AIDS [45]. This underscores the urgent need for an effective AMD treatment.

The purpose of this review is to inventory the work that has been done to profile miRNA dysregulation in AMD, and further summarize the miRNA work done in well-established mouse models of AMD (Fig. 1). The question arises whether miRNA-based approaches to treatment could be feasible to ameliorate AMD-related tissue damage in the retina and consequently to prevent vision impairment or even blindness.

## 2 MiRNA and AMD

### 2.1 Circulating miRNA Profiles of AMD Patients Show Inconsistencies

The function and mode of action of miRNAs in the cell are reasonably well understood and recent advances have yielded promising results for future miRNA-based therapies. MiRNAs have been detected in bodily fluids such as blood, saliva or urine [46]. Although the exact function and origin of those extracellular miRNA are still debated, these molecules seem to function as novel messengers allowing cells in the body to communicate with other cells [47]. A special class of extracellular miRNAs are called circulating miRNAs. They are found in plasma or serum and are theorized to be involved in cell to cell signaling [48]. Since circulating miRNAs can be easily obtained through a blood draw, they are promising targets to investigate as potential liquid biopsy markers. For example, if a marker profile could be linked to an increased risk of developing AMD, this could be used as a minimally invasive screening tool to monitor treatment success. Furthermore, dysregulated miRNAs could potentially be a treatment option themselves by using miRNA mimics or antagomirs to modulate miRNA levels in the cell [49, 50].



**Fig. 1** Word-cloud representation of words found in abstracts related to the search term “miRNAs and AMD”. All words with an occurrence greater than 4 are plotted and their relative abundance is indicated by the size of the respective word. Figure was plotted with

Tagxedo (<http://www.tagxedo.com/app.html>) using words from the abstracts of 21 publications related to miRNAs in AMD [51, 52, 56, 57, 71, 73–75, 77, 82, 89, 90, 99, 103–110]

Four studies published between 2014 and 2016 analyzed the circulating miRNA expression profiles of AMD patients (Table 1; Fig. 2). Of note, there is little overlap in the findings between the four studies. Although some miRNAs were found in several studies, they do not necessarily agree on the orientation of the effect. For example, miR-424-5p was found to be dysregulated in two studies, one study found the miR-424-5p to be downregulated in patients with late-stage NV AMD ( $p$  corrected =  $9.6 \times 10^{-3}$ ) [51] and no significant differences between the expression of miR-424-5p in GA AMD patients and controls. The authors noted, however, that the miR-424-5p expression was significantly higher in GA AMD patients compared to NV AMD patients ( $p$  corrected <0.005). A second study, published almost one year later, showed the opposite results. They found that miR-424-5p was upregulated in NV and GA AMD patients versus controls ( $p < 0.0001$ ) [52]. Both studies agreed on the finding that miR-424-5p expression is higher in GA than NV AMD. The studies used a similar approach, a discovery study to find potential candidates and a validation study relying on quantitative (q)RT-PCR to confirm the candidates' association with AMD, and recruited similarly sized cohorts. Their approach differed in the method used for the discovery study [next-generation sequencing (NGS) vs. qRT-PCR], but this can hardly explain the different direction of dysregulation which each study confirmed by using qRT-PCR. A partial explanation for these

discrepancies may lie in the patient inclusion criteria, as the first study included late-stage NV AMD patients, some of whom had received anti-VEGF therapy, while the second report used newly diagnosed patients, who had not received any therapy prior to their recruitment for the study. Further, some controls in the first study suffered from glaucoma, whereas in the second study, only samples from people without ocular abnormalities were included.

These inconsistencies are not unique to those two studies, in fact all four studies varied in the methods applied to quantify miRNAs, normalizing for qRT-PCR expression and profiling of patients included. Since the reported results differ substantially, the possibility that the different results could be due to methodological characteristics needs to be considered. Additionally, some studies had small sample sizes (13–33 patients, and as many controls), which draws into question the statistical power issue and consequently the validity of the conclusions. Conversely, two of the studies had large sample sizes (188–300 patients, and 147–200 controls). This, and the fact that AMD profiling studies of AMD mouse models revealed some overlapping results, appears reasonable to not entirely disregard the patient cmiRNA profiling studies, but instead to consider these results as preliminary, and to enjoy them with caution.

A more promising candidate may be miR-301. One study found the miR-301-3p arm to be downregulated in NV AMD [51], whereas a second study found the miR-301-5p arm to be upregulated in NV and GA AMD [52]. In miRNA biogenesis,



**Table 1** Circulating miRNAs dysregulated in AMD

cmiRNA	Family	FC <sup>b</sup>	Tissue	Pathology	Source
hsa-Let-7 <sup>a</sup>	let-7	6.74; 2.7 <sup>c</sup>	Serum	NV and GA AMD	[52]
hsa-Let-7c <sup>a</sup>	let-7	o.p.	Plasma	NV AMD	[103]
hsa-miR-106a-5p	mir-17	3.45	Plasma	NV AMD	[103]
hsa-miR-106b-5p	mir-17	-1.5	Plasma	NV AMD	[57]
hsa-miR-139-3p	mir-139	o.p.	Plasma	NV AMD	[103]
hsa-miR-140-3p	mir-140	-2.81	Plasma	NV AMD	[103]
hsa-miR-146a-5p	mir-146	2.5	Plasma	NV AMD	[57]
hsa-miR-146b-5p	mir-146	-8.68	Plasma	NV AMD	[103]
hsa-miR-152-3p	mir-148	-1.7	Plasma	NV AMD	[57]
hsa-miR-17-5p	mir-17	2.77	Plasma	NV AMD	[103]
hsa-miR-192-5p	mir-192	-3.54	Plasma	NV AMD	[103]
hsa-miR-20a-5p	mir-17	2.02	Plasma	NV AMD	[103]
hsa-miR-212-3p	mir-132	o.p.	Plasma	NV AMD	[103]
hsa-miR-21-5p	mir-21	-3.09	Plasma	NV AMD	[103]
hsa-miR-223-3p	mir-223	1.65	Plasma	NV AMD	[103]
hsa-miR-24-3p	mir-24	1.58	Plasma	NV AMD	[103]
hsa-miR-25-3p	mir-25	-1.84	Plasma	NV AMD	[103]
hsa-miR-26b-5p	mir-26	o.p.	Plasma	NV AMD	[103]
hsa-miR-27b-3p	mir-27	o.p.	Plasma	NV AMD	[103]
hsa-miR-29a-3p	mir-29	o.p.	Plasma	NV AMD	[103]
hsa-miR-301-3p	mir-130	-0.318	Plasma	NV AMD	[51]
hsa-miR-301-5p	mir-130	3.94; 4.47 <sup>c</sup>	Serum	NV and GA AMD	[52]
hsa-miR-3121 <sup>a</sup>	mir-3121	2.85; 8.7 <sup>c</sup>	Serum	NV and GA AMD	[52]
hsa-miR-324-3p	mir-324	o.p.	Plasma	NV AMD	[103]
hsa-miR-324-5p	mir-324	o.p.	Plasma	NV AMD	[103]
hsa-miR-335-5p	mir-335	-7.00	Plasma	NV AMD	[103]
hsa-miR-342-3p	mir-342	-2.65	Plasma	NV AMD	[103]
hsa-miR-361-5p	mir-361	-0.373	Plasma	NV AMD	[51]
hsa-miR-374a-5p	mir-374	-12.92	Plasma	NV AMD	[103]
hsa-miR-410 <sup>a</sup>	mir-154	-9.84	Plasma	NV AMD	[103]
hsa-miR-424-5p	mir-322	-0.338	Plasma	NV AMD	[51]
hsa-miR-424-5p	mir-322	3.28; 3.97 <sup>c</sup>	Serum	NV and GA AMD	[52]
hsa-miR-4258 <sup>a</sup>	unknown	8.55; 2.6 <sup>c</sup>	Serum	NV and GA AMD	[52]
hsa-miR-532-3p	mir-188	o.p.	Plasma	NV AMD	[103]
hsa-miR-574-3p	mir-574	-2.44	Plasma	NV AMD	[103]
hsa-miR-660-5p	mir-188	-3.39	Plasma	NV AMD	[103]
hsa-miR-661 <sup>a</sup>	mir-661	1.96; 9.05 <sup>c</sup>	Serum	NV and GA AMD	[52]
hsa-miR-744-5p	mir-744	o.p.	Plasma	NV AMD	[103]
hsa-miR-889 <sup>a</sup>	mir-889	3; 8.9 <sup>c</sup>	Serum	NV and GA AMD	[52]
miR438 <sup>a</sup>	unknown	3.3; 3.3 <sup>c</sup>	Serum	NV and GA AMD	[52]

FC fold change, AMD age-related macular degeneration, miRNA micro RNA, cmiRNA circulating micro RNA, GA AMD graphic atrophy AMD, NV AMD neovascularization AMD

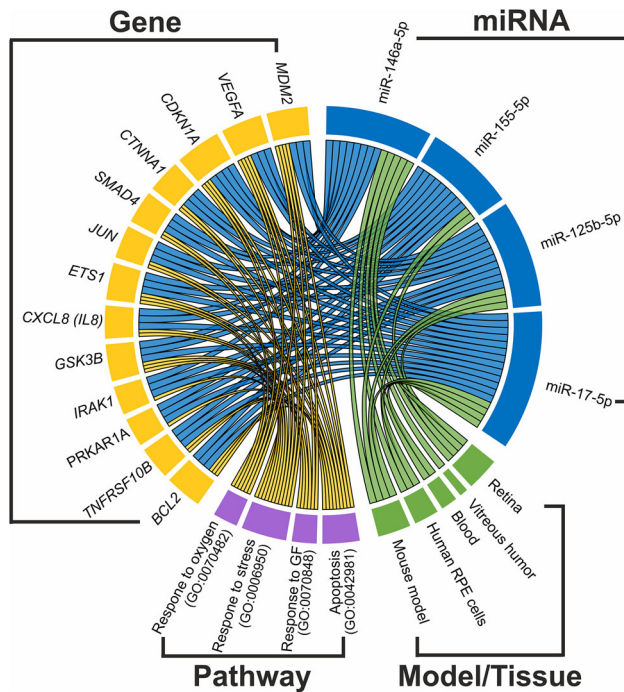
<sup>a</sup> Mature miRNA strand unknown/not mentioned

<sup>b</sup> o.p. miRNA was only expressed in the patient group

<sup>c</sup> Fold change for NV AMD; fold change for GA AMD

the 5p and 3p arms of a mature miRNA are processed from a long precursor miRNA [53]. The 5p and 3p arms are separated, the guide strand is incorporated into the RNA-induced

silencing complex, RISC, and subsequently becomes a functional gene inhibitor. The second strand, termed the complementary strand, usually becomes degraded. These two studies



**Fig. 2** Interaction between AMD related miRNAs and genes, pathways and tissues. We selected four miRNAs (plotted in blue) with the most evidence for an involvement in AMD and used Tarbase v7.0 [111] to extract genes (plotted in yellow) with validated interactions with those miRNAs. Genes that are regulated by at least three out of four miRNAs were used to search for significantly enriched pathways using WebGestalt [112]. Pathways containing at least six of these genes and being significantly enriched (Bonferroni corrected  $p$  value  $<0.01$ ) are plotted in purple. The role of the miRNAs in AMD pathology was evaluated in different tissues and/or mouse models (plotted in green)

may indicate that miR-301-5p is a negative gene regulator in AMD, while miR-301-3p is subsequently degraded, and therefore downregulated in the patient.

In some cases the 5p and 3p arms can be directly correlated with another [54, 55]. Unfortunately, most publications do not specify which miRNA arm was studied, making it difficult to interpret their results. Furthermore, multiple members of the same miRNA families have been found in the various studies, i.e. let-7 and let-7c of the let-7 family, and miR-106a and b of the miR-17 family, among others. This is not as helpful as it might appear. MiRNAs are grouped into families based on similar sequence or their location in the genome, not based on their function. So, even though two members of the mir-188 family are indicated to be dysregulated in AMD, it still is difficult to draw conclusions from this.

## 2.2 MiRNA Dysregulation in AMD Ocular Tissue

To date, three studies profiled miRNA from samples of AMD retinæ and RPE cells, and one study reported

miRNA measurements from vitreous humor (VH) of AMD patients (Table 2; Fig. 2). MiR-146a was found to be upregulated in both forms of AMD, and in different tissue. One study showed the miRNA to be upregulated in the retina of GA AMD patients [56], whereas a second study found a 3.0-fold increase in the VH of NV AMD patients [57]. Both studies used similar sample sizes (12–13 patients and 9–13 controls), and methods to assess miRNA levels. The second study further discovered an upregulation of the miRNA in the plasma of NV AMD patients. Interestingly, a third group confirmed the upregulation of miR-146a in the serum of NV and GA AMD patients, although these findings were not validated using qRT-PCR [52]. Unfortunately, two of these studies did not specify whether they analyzed the 5p or 3p arm [52, 56].

MiR-146a is a well characterized small RNA and has been linked to progressive, age-related, inflammatory neurodegenerative disorders [58]. MiR-146a is theorized to modulate innate immune responses, inflammation, and the microglial activation state, although it is not yet clear whether the induction of miR-146a is protective or detrimental to the cell. It is under transcriptional control by nuclear factor-kappaB (NF- $\kappa$ B), and has been found to be upregulated by reactive oxygen species, pro-inflammatory cytokines, and amyloid peptides. Multiple studies have established that upregulation of miR-146a in stressed human neural cells such as astroglia and microglia, results in the downregulation of complement factor H (*CFH*) [59, 60]. *CFH* is a major repressor of the innate-immune and inflammatory response, and one of the key players in AMD pathogenesis [58, 61, 62]. It has been suggested that the upregulation of miR-146a has detrimental effects and promotes the pathogenesis of AMD [61]. There is evidence suggesting the opposite behavior as MiR-146a has been shown to interfere with interleukin-1-receptor-associated kinase-1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) [63, 64] as well as directly down-regulating interleukin-6 (IL-6) [57, 65]. IRAK1 and TRAF6 are known modulators of the NF- $\kappa$ B pathway, and their translational repression inhibits pro-inflammatory cytokine signaling [57, 66–68]. IL-6 acts as a pro-inflammatory cytokine, and is routinely used in the diagnosis of sepsis [69]. Since IL-6 is associated with progression of NV [64, 70] miR-146a may be a protective regulator of inflammation in NV AMD [57]. However, as the effect of miR-146a is not fully protective nor fully detrimental for AMD, the molecule is unlikely a good candidate for a therapeutic intervention.

A closely related small RNA to miR-146a, is miR-146b-5p, which is also theorized to be a negative regulator of inflammation, although it has not been characterized nearly as well as miR-146a [71]. MiR-146b is known to target IRAK1 and TRAF6 thereby inhibiting pro-inflammatory

**Table 2** MiRNA dysregulated in ocular tissue of AMD patients

miRNA	Family	FC <sup>c</sup>	Tissue	Pathology	Source
miR-106b-5p	miR-17	-0.23	Vitreous humor	NV AMD	[57]
miR-125b <sup>a</sup>	miR-10	+	Retina	GA AMD	[56]
miR-146a <sup>a</sup>	miR-146	2.1	Retina	GA AMD	[56]
miR-146a-5p	miR-146	3.02	Vitreous humor	NV AMD	[57]
miR-152-3p	miR-148	-0.33	Vitreous humor	NV AMD	[57]
miR-155 <sup>a</sup>	miR-155	+	Retina	GA AMD	[56]
miR-184 <sup>a</sup>	miR-184	-	RPE cell culture <sup>b</sup>	AMD	[89]
miR-23a <sup>a</sup>	miR-23	-	Macular RPE cell culture <sup>b</sup>	AMD	[104]
miR-34a <sup>a</sup>	miR-34	6.3	Retina and macula	GA AMD	[56]
miR-9 <sup>a</sup>	miR-9	+	Retina	GA AMD	[56]

FC fold change, AMD age-related macular degeneration, miRNA micro RNA, cmiRNA circulating micro RNA, GA AMD graphic atrophy AMD, NV AMD neovascularization AMD, RPE retinal pigment epithelium

<sup>a</sup> Mature miRNA strand unknown/not mentioned

<sup>b</sup> Primary culture of human RPE isolated from eyes of AMD donors

<sup>c</sup> A “+” indicates miRNA upregulated/higher levels and a “-” indicates miRNA downregulated/lower levels

cytokine signaling [63, 65, 71, 72]. One study showed that the expression of miR-146a and miR-146b is highly induced by a combination of pro-inflammatory cytokines (IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$ ) in human RPE cells [71]. Conversely, miR-146b was shown to be downregulated in NV AMD plasma. Since both miRNAs bind similar targets, this could be a method of harnessing an immune reaction.

### 2.3 Dysregulated miRNA in AMD Models Provide Clues to Understanding the Function of miRNA Dysregulated in AMD

Multiple studies have profiled miRNAs in RPE cells of mice and rats in experimental models meant to mimic characteristic pathological changes seen in AMD patients (Supplementary Table 1; Fig. 2). These studies have shown a combined total of 176 miRNAs to be dysregulated. Fourteen of these miRNAs were also dysregulated in the same direction in AMD patients, which makes them exciting contenders for further research (Table 3). Importantly, the miRNA with the highest number of studies supporting its involvement in AMD is miR-146a. Two studies performed in patients, and three studies performed in human RPE cells or in mouse retinae revealed a miR-146a upregulation, although one study indicated this miRNA species to be downregulated after oxygen-induced retinopathy (OIR) [56, 57, 71, 73–75]. Although technically not an AMD model, OIR is a well-established protocol used to study hyperoxia-induced retinal neovascularization and inflammation in mice, two well-known characteristics of AMD [76, 77]. One upregulated miRNA in an OIR model, and oxidative stress model in RPE cells, as well as in NV AMD plasma was miR-17, a

regulator of angiogenesis [78]. MiR-17 targets several pro-angiogenic genes, and the inhibition of miR-17 along with miR-20 increased the number of perfused vessels in Matrigel plugs [78]. Besides its antiangiogenic activity, miR-17 was recently shown to be anti-apoptotic as well [79]. Pretreatment with miR-17 significantly prevented the norepinephrine-induced apoptosis of cardiomyocytes, by inhibiting apoptotic protease activating factor 1 (Apaf-1), a facilitator of apoptosome formation. miR-17 is part of the miR-17-92 cluster, which is a negative regulator of angiogenesis, and encodes miR-17, -18a, -19a/b, -20a, and miR-92a [50]. Of the other members of this cluster, only miR-92a was upregulated in RPE cells after oxidative stress, and in mouse retinae after OIR [80]. miR-92a was not dysregulated in AMD patients. MiR-92a is a well-studied miRNA which also regulates angiogenesis. First, Bonauer et al. [50] showed the miRNA was upregulated in a hind limb mouse ischemia model in response to ischemic injury. The authors then systemically injected mice with a miR-92 antagonist before inducing ischemia, and showed that these mice had improved functional recovery of ischemic limbs, due to improved neovascularization and perfusion [50]. Similarly, treating mice with the miR-92 antagonist after inducing an acute myocardial infarction, resulted in reduced infarct size and improved left ventricular systolic and diastolic function [50]. miR-92a was significantly reduced in patients with coronary artery disease, a major risk factor for acute myocardial infarction [81].

Another miRNA which may regulate neovascularization and apoptosis is miR-106b, which was downregulated in NV AMD plasma and VH, and in RPE cells after oxidative stress [57, 82]. MiR-106b-25 cluster knockout mice

**Table 3** Fourteen miRNAs dysregulated in experimental animal models of AMD, and in AMD patient tissue

miRNA	Experimental AMD models				Patient				
	Strand	FC <sup>d</sup>	Tissue	Manipulation <sup>c</sup>	Source	Strand	FC <sup>d</sup>	Tissue	Source
miR-106a	mmu-miR-106a <sup>a</sup>	+	Mouse retina	OIR	[90]	hsa-miR-106a-5p	3.45	Plasma	[103]
miR-106b	hsa-miR-106b-5p	0.11	ARPE-19 cells	Oxidative stress	[82]	hsa-miR-106b-5p	-1.5	Plasma	[57]
miR-125b	hsa-miR-125b-5p	+	ARPE-19 cells	Oxidative stress	[82]	hsa-miR-106b-5p	-0.23	VH	[57]
	mmu-miR-125b-5p	2.07	Mouse retina	OIR	[75]	hsa-miR-125b <sup>a</sup>	+	Retina	[56]
miR-146a	hsa-miR-146a <sup>a</sup>	+	Human RPE	Chronic oxidative stress	[73]	hsa-miR-146a-5p	2.5	Plasma	[57]
	mmu-miR-146a-5p	3.02	Mouse retina	Selective Müller cell ablation	[74]	hsa-miR-146a <sup>a</sup>	2.1	Retina	[56]
	hsa-miR-146a <sup>a</sup>	+	Human RPE	treatment with pro-inf cytokines	[71]	hsa-miR-146a-5p	3.02	VH	[57]
	mmu-miR-146a-5p	0.3	Mouse retina	OIR	[75]				
miR-155	mmu-miR-155-5p	55.92	Rat retinas	Light induced retinal degeneration	[99]	hsa-miR-155 <sup>a</sup>	+	Retina, macula	[56]
	mmu-miR-155-5p	5.87	Mouse retina	OIR	[75]				
	hsa-miR-155 <sup>a</sup>	+	Human RPE	Treatment with pro-inf cytokines	[113]				
miR-17	hsa-miR-17-5p	+	ARPE-19 cells	Oxidative stress	[82]	hsa-miR-17-5p	2.77	Plasma	[103]
	mmu-miR-17-5p	3.16	Mouse retina	OIR	[75]				
miR-184	mmu-miR-184 <sup>a</sup>	-	Mouse retina	OIR	[90]	hsa-miR-184 <sup>a</sup>	-	RPE culture	[89]
miR-21	hsa-miR-21 <sup>a</sup>	-	Human RPE	Chronic oxidative stress	[73]	hsa-miR-21-5p	-3.09	Plasma	[103]
	hsa-miR-21-5p	+	ARPE-19 cells	Oxidative stress	[82]				
miR-223	hsa-miR-223-3p	+	ARPE-19 cells	Oxidative stress	[82]	hsa-miR-223-3p	1.65	Plasma	[103]
	mmu-miR-223-3p	0.32	Mouse retina	OIR	[75]				
miR-23a	hsa-miR-23a-3p	+	ARPE-19 cells	Oxidative stress	[82]	hsa-miR-23a <sup>a</sup>	-	Macular RPE	[104]
	mmu-miR-23a-3p	0.16	Mouse retina	OIR	[75]				
miR-29a	hsa-miR-29a <sup>a</sup>	+	Human RPE	Chronic oxidative stress	[73]	hsa-miR-29a-3p	o.p. <sup>b</sup>	Plasma	[103]
	hsa-miR-29b-3p	0.05	ARPE-19 cells	Oxidative stress	[82]				
miR-342-3p	mmu-miR-342-3p	0.47	Mouse retina	OIR	[75]	hsa-miR-342-3p	o.p. <sup>b</sup>	Plasma	[103]
miR-374a	hsa-miR-374a-5p	0.1	ARPE-19 cells	Oxidative stress	[82]	hsa-miR-374a-5p	-12.92	Plasma	[103]
miR-424	mmu-miR-424 <sup>a</sup>	+	Mouse retina	OIR	[90]	hsa-miR-424-5p	-0.338	Plasma	[51]
						hsa-miR-424-5p	3.97; 3.28	Serum	[52]

FC fold change, AMD age-related macular degeneration, miRNA micro RNA, GA AMD geographic atrophy AMD, RPE retinal pigment epithelium, OIR oxygen-induced retinopathy, VH vitreous humor, pro-inf pro-inflammatory, ARPE-19 adult retinal pigment epithelium cell line

<sup>a</sup> Mature miRNA strand unknown/not mentioned

<sup>b</sup> o.p.: miRNA was only expressed in the patient group

<sup>c</sup> Oxidative stress was induced using H<sub>2</sub>O<sub>2</sub> [82] or Paraquat [73]

<sup>d</sup> A “+” indicates miRNA upregulated/higher levels and “-” indicates miRNA downregulated/lower levels



showed impaired neovascularization after hind limb ischemia in mice [83]. This effect was confirmed in a study which induced cerebral ischemia in mice which had received a miR-106b antagomir, and observed decreased infarct volume and neuronal injury [84]. The same miR-106b antagomir-protected cells derived from a pheochromocytoma of the rat adrenal medulla (PC12) against glutamate-induced apoptosis and oxidative damage, as evidenced by decreased lactate dehydrogenase release, and enhanced superoxide dismutase activity [84]. MiR-106b showed an age-dependent expression, and was downregulated in serum from patients aged 70 versus 22 years [85].

With neovascularization as the hallmark of NV AMD, it is not surprising that many of the miRNAs associated with the disease have also been linked to angiogenesis. MiR-125b was upregulated in patient retinæ, in mouse retinæ after OIR, and in ARPE-19 cells under oxidative stress [56, 75, 82]. Overexpression of miR-125b was negatively correlated with VEGF mRNA, and inhibited tumor-induced angiogenesis in ovarian cancer cells [86]. In brain endothelial cells (ECs), miR-125b overexpression resulted in a trend towards reduced tubule length [87]. This inverse correlation between miR-125b and in vitro tube formation by ECs was confirmed by Muramatsu et al. [88] who theorized that prolonged overexpression of miR-125b could result in blood vessel regression due to a transient proangiogenic stimulation of miR-125b induction in EC cells.

MiR-184 was downregulated in primary culture of human RPE cells isolated from eyes of AMD donors and in OIR mouse retinæ [77, 89, 90]. One of the studies showed that miR-184 negatively regulates Wnt signaling in OIR retinæ, a conserved intracellular signaling pathway also involved in regulating angiogenesis and inflammation [77]. This interaction was confirmed by other findings that showed corneal neovascularization induced by suture was ameliorated by topical administration of miR-184 in rats [91]. Inhibition of miR-184 significantly reduced phagocytosis efficiency in adult RPE cells [89]. Phagocytosis is crucial for the maintenance of photoreceptors, and an age-related decline in phagocytosis is thought to contribute to AMD [89, 92, 93]. Interestingly, a group studying ocular *Chlamydia trachomatis* infection demonstrated that miR-184 and miR-155 had a direct relationship with the degree of clinical inflammation, where miR-184 was downregulated, and miR-155 was upregulated as the severity increased [94]. This inverse relationship may also be true in AMD, since miR-184 was downregulated in AMD RPE cell culture, while miR-155 was upregulated in AMD retina and macula [56, 89].

MiR-424, which was dysregulated in opposite directions in two independent studies with AMD patients was upregulated in OIR mouse retinæ [90]. This may indicate that the upregulation is a true effect, although the evidence of upregulation in OIR mouse retinæ is far from

conclusive. OIR mimics two key traits of AMD such as inflammation and angiogenesis. In AMD, however, the macula is primarily affected, and mice do not have a macula. Therefore, information gained from mouse models of AMD should be considered with caution.

## 2.4 Manipulation of miRNA in AMD Mouse Models Ameliorate Damage

Multiple studies have manipulated miRNA levels in vivo following a pathologic insult and tested the effect. MiRNA were either over-expressed using a pre-miR or miR-mimic, or under-expressed using an antisense-miR or a knockout mouse. Subsequently, neovascularization was quantified and many miRNAs were shown to ameliorate the inflicted damage (Table 4; Fig. 2). Unfortunately, most of the miRNAs tested were not homologous to those which were dysregulated in the AMD profiling studies (see above), but there are two that overlap, miR-155 and miR-184.

As mentioned above, strong data suggest that miR-184 is involved in angiogenesis. The studies showed that miR-184 is downregulated in OIR mouse retinæ, and one of the groups also showed that intraocular injection with pre-miR-184 reduced retinal neovascularization in OIR mice [90]. The authors also tested the miRNA in the most widely accepted NV AMD model, the laser-induced choroidal neovascularization [95–98]. This model relies on laser injury to perforate Bruch's membrane, resulting in sub-retinal blood vessel recruitment from the choroid, recapitulating the main characteristic of NV AMD [95]. Of note, in this context treatment with pre-miR-184 failed to reduce the area of choroidal NV.

A far more complex candidate is miR-155 as it regulates not only angiogenic but also inflammatory responses in the retina. MiR-155 was upregulated in GA AMD retina and macula, in OIR mouse retina, and in rat retinas after light-induced retinal degeneration [56, 75, 99]. Yan et al. [75] subjected miR-155 knockout mice to OIR, but found no protective effects against OIR provided by miR-155 deficiency. Instead, it induced rapid revascularization and prevented the development of aberrant neovascularization. Next, the authors demonstrated that intraocular injection of a miR-155 mimic in WT mice (postnatal day 3) delayed migration of the regularly and radially expanding superficial capillary plexus and hindered advancement toward the retinal edge. To elucidate the effect of miR-155 on inflammation, they first showed that miR-155 knockout mice had significantly reduced numbers of microglia. miR-155-deficient mice showed a decreased expression of mitochondrial translocator protein, a selective marker of microglia in their highly reactive state [100], but an increased expression of CD200R, which blocks pro-inflammatory activation in microglial cells [75, 101]. The

**Table 4** MiRNAs manipulated prior to insult in AMD animal model

miRNA	Delivery	Compound	Model	Effect <sup>b</sup>	Source
mmu-miR-126 <sup>a</sup>	Intravitreal injection	miR-mimic	OIR	↓ Retinal NV	[114]
mmu-miR-128 <sup>a</sup>	Intravitreal injection	miR-mimic	OIR	↓ Retinal NV	[105]
mmu-miR-132 <sup>a</sup>	Intraocular injection	anti-miR	OIR	↓ NV	[106]
mmu-miR-150 <sup>a</sup>	Intraocular injection	pre-miR	OIR	↓ Retinal NV	[90]
	Intraocular injection	pre-miR	Laser induced CNV	↓ Choroidal NV	[90]
	Intravitreal injection	miR-mimic	OIR	↓ Retinal NV	[108]
mmu-miR-155 <sup>a</sup>	Knockout mice	miR-150-/-	Laser induced CNV	↑ Choroidal NV	[108]
	Knockout mice	miR-155-/-	OIR	↑ Retinal NV	[75]
	Knockout mice	miR-155-/-	Retinal development	↓ Vascular area	[75]
	Intravitreal injection	miR-mimic	Retinal development	↓ Vascular area	[75]
mmu-miR-184 <sup>a</sup>	Intraocular injection	pre-miR	OIR	↓ Retinal NV	[90]
mmu-miR-23/27 <sup>a</sup>	Intravitreal injection	anti-miR	Laser induced CNV	↓ Choroidal NV	[107]
mmu-miR-24 <sup>a</sup>	Subretinal	miR-mimic	Laser induced CNV	↓ Choroidal NV	[109]
mmu-miR-31 <sup>a</sup>	Intraocular injection	pre-miR	OIR	↓ Retinal NV	[90]
	Intraocular injection	pre-miR	Laser induced CNV	↓ Choroidal NV	[90]

OIR oxygen-induced retinopathy, CNV choroidal neovascularization, AMD age-related macular degeneration, miRNA micro RNA

<sup>a</sup> Mature miRNA strand unknown/not mentioned

<sup>b</sup> A “↓” indicates a decrease and a “↑” indicates an increase in the respective phenotype

OIR miR-155 knockout mice showed reduced translocator protein (TPSO) and inflammatory cytokines [75]. It is of note, however, that miR-155 has been shown to have a cell-type specific function. The growth of six breast cancer cell lines showed a wide range of sensitivities when transfected with a miR-155 mimic [102]. MiR-155 may have a similar cell-type specific function in the microglia, endothelial cells, RPE cells, or some of other cell types implicated in AMD pathogenesis.

### 3 Conclusions

Taken together, increasing research efforts on small RNAs suggests that dysregulated miRNAs may regulate key aspects of AMD pathology. While data on circulating miRNA in AMD are still scarce, findings on dysregulated ocular miRNA show more promise. For example, miR-146a and miR-155 are regulators of inflammation and microglial activation state in response to stress, and are both dysregulated in AMD retinae. Furthermore, angiogenesis and response to varying oxygen levels is likely modulated by miR125b, and miR-17, which may also regulate apoptosis. These key candidate miRNAs could be useful as novel therapeutic approaches in AMD, as exemplified by miR-155 which was shown to modulate damage in AMD mouse models in vivo. Still, studies in mouse models are not ideal in the long term, and must be complemented by comprehensive studies establishing miRNA profiles in AMD development and progression.

### Compliance with Ethical Standards

**Conflict of interest** The authors Patricia Berber, Felix Grassmann, Christina Kiel, and Bernhard HF Weber declare no conflict of interest.

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