Published in final edited form as:

Cell Mol Life Sci. 2012 August ; 69(16): 2739–2750. doi:10.1007/s00018-012-0976-7.

The physiological impact of microRNA gene regulation in the retina

Thomas R. Sundermeier and Krzysztof Palczewski

Department of Pharmacology, School of Medicine, Case Western Reserve University, 10900 Euclid Ave, Cleveland, OH 44106-4965, USA

Abstract

microRNAs (miRNAs) are small, stable RNA molecules that post-transcriptionally regulate gene expression in plants and animals by base pairing to partially complementary sequences on target mRNAs to inhibit protein synthesis. More than 250 miRNAs are reportedly expressed in the retina, and miRNA gene regulation has been shown to affect retinal development, function, and disease. Here we highlight recent advances in understanding the functional roles of vertebrate retinal miRNAs. Details are emerging about the physiological impact of specific miRNAs in the developing and mature retina, and we discuss a group of emerging technologies for studying miRNAs, which can be employed to yield a deeper understanding of retinal miRNA gene regulation.

Keywords

microRNA; Retina; Dicer; Photoreceptor; Argonaute

Introduction

The retina is a multi-layered sensory organ responsible for transducing light into a pattern of electrophysiological signals ultimately interpreted as 'vision' by the brain. The sensitivity of the phototransduction cascade is tightly regulated to adjust to a wide variety of stimuli, including levels of ambient light and circadian rhythm [1–3]. In addition, the retina must rapidly cope with toxic byproducts of phototransduction, maintain cell viability under highly oxidizing conditions, and rapidly regenerate isomerized visual chromophore [1–3]. A complex gene regulation network simultaneously implements the many layers of regulation required to achieve normal vision, and miRNAs are an indispensable component of that regulation.

miRNAs are small, stable RNA molecules that post-transcriptionally regulate gene expression by binding to imperfectly complementary sites on target mRNAs, to effect transcript-specific translational repression and mRNA destabilization [4–6]. First discovered in *C. elegans*, [7–9], miRNAs are ubiquitously expressed in plants and animals and constitute an essential component of gene regulation [10–13]. As summarized in Fig. 1, miRNAs are transcribed in the nucleus, either independently or as part of introns of protein-coding genes [14, 15]. Genes of functionally related miRNAs are often clustered on the same chromosome, and expressed as a single primary transcript (pri-miRNA). Short hairpin

structures within these pri-miRNAs are recognized in the nucleus by a protein complex known as the microprocessor, resulting in endonucleolytic cleavage by the RNase III-type enzyme Drosha, and release of ~70 nt precursor miRNAs (pre-miRNAs). These precursors are exported to the cytosol by exportin5 where they undergo a final endonucleolytic cleavage catalyzed by another RNase III-type enzyme called Dicer.

The ~22-nt mature miRNA is then assembled into a ribonucleoprotein effector complex known as the miRNA-induced silencing complex (miRISC). Core components of the miRISC include proteins of the argonaute (AGO) family that directly bind the miRNA and GW182 family proteins which mediate translational repression and mRNA decay [16–19]. The miRISC is recruited to specific transcripts through base pairing between the mature miRNA and imperfectly complementary target sites (usually in the 3' UTR) on cellular mRNAs. Though a consensus has yet to be reached regarding the primary mechanism of miRNA action, most recent reports favor a model whereby miRNAs repress initiation of translation and facilitate target mRNA decay (Fig. 1).

This review will focus on the substantial progress made recently toward assessing the role of miRNAs in the vertebrate visual system, with particular emphasis on work in mammalian model species. Detailed reviews of advances in understanding miRNA-mediated regulation of insect eye development are available elsewhere [20–22]. Consistent with the complexity of visual system physiology, miRNA transcriptome analyses have revealed a remarkable diversity of miRNAs expressed in the vertebrate retina [23–30]. Moreover, miRNA dysregulation has been demonstrated in models of blinding diseases. Conditional Dicer knockout has revealed roles for miRNAs both in retinal development and in the physiology and survival of mature retinal neurons [31–34]. Finally, genetic loss-of-function studies are beginning to disclose the physiological roles of specific miRNAs or miRNA clusters in the mammalian retina. The recent dramatic increase in interest in retinal miRNAs comes as no surprise considering both our greater awareness of their roles in cellular physiology and disease, and the inherent advantages of the visual system as a model for genetic research. Indeed the number of published reports dealing with miRNA gene regulation has increased by greater than tenfold over the past 6 years, bolstered by discovery of miRNAs involved in a myriad of human diseases together with substantial progress toward targeting miRNAs for therapeutic intervention [35–39]. Additionally, the visual system provides an advantageous model for genetic investigation for several reasons. The laminar architecture and cell-type functionalities of retinal neurons have been clearly defined through decades of investigation [1]. Genes essential for fundamental processes such as phototransduction and the retinoid cycle are generally non-redundant. Furthermore, visual system-inactivating mutations are non-lethal under laboratory conditions, permitting detailed investigation of their corresponding phenotypes. Finally, the retina is readily amenable to genetic manipulation by well-established techniques including virus-mediated gene transfer, injection of nanoparticle-packaged DNA, and retinal lipofection or electroporation of transgene vectors [40–44].

The aims of this review are threefold, to: (a) summarize recent progress toward understanding the physiological impact of miRNAs in the vertebrate retina, (b) highlight recent insights regarding the nature of miRNA gene regulation gained by studying retinal miRNAs, and (c) suggest a set of emerging technologies for studying miRNA gene regulation that can be readily adapted to fill gaps in our current understanding of retinal miRNA function.

miRNAs in the retina

Expression profiling

miRNA transcriptome analyses provided the first clues about the importance of miRNA gene regulation in the retina. About 80 different miRNAs were initially identified in adult mouse retina by microarray, 23 of which were preferentially expressed in retina as compared to other tissues [28, 30, 45]. Recently, the increased sensitivity afforded by next-generation sequencing (NGS) has dramatically expanded the known number of retinal miRNAs to more than 250 [26]. Expression of retinal miRNAs is also subject to precise regulation, varying dramatically by cell type, developmental stage, environmental conditions, and disease pathology. Laser capture microdissection techniques have established significant retina layer-dependent variations in the expression of a limited set of miRNAs [24, 26], results confirmed in some cases by small RNA in situ hybridization approaches [26]. Hackler and colleagues monitored miRNA expression throughout retinal development in mouse eye using locked nucleic acid microarrays and found that of the 138 miRNAs detected in the retina at some point between embryonic day 5 and adult, 50 and 23 varied developmentally by at least 4- or 8-fold, respectively [24]. In a remarkably ambitious study, investigators in the Banfi laboratory performed in situ hybridization analysis to yield a high-resolution map of the expression of over 200 miRNAs in the developing and adult mouse eye [23]. Of miRNAs found in the retina, most displayed variability of expression across retinal layers that generally arose during development [23]. Diurnal variation in expression also was reported for a set of retina-specific miRNAs [28], results later confirmed in a larger study. The latter investigation reported by the Filipowicz laboratory revealed that in contrast to circadian rhythm, these miRNAs were regulated directly by light [26]. The same elegant work combined miRNA transcriptome data from three independent platforms (454 sequencing, Illumina sequencing and microarray analysis) to identify a high-confidence group of five retinal miRNAs (miRs -96, -182, -183, -204, and -211) exhibiting this property [26].

miRNA expression levels also varied in rodent models of human retinal pathologies. Importantly, four different mouse models of retinitis pigmentosa (including both autosomal dominant and autosomal recessive forms) evidenced changes in the expression of six different retinal miRNAs [45, 46]. In these models, miRs -96, -182, and -183 were consistently downregulated and miRs -1, -133, and -142 were consistently upregulated, as compared to wild-type mice. This result suggests involvement of some or all of these miRNAs in the pathology of retinitis pigmentosa. Consistent with this notion, miRs -96, -182, and -183 were later shown to promote survival of rods in the light-damage mouse model of photoreceptor degeneration (see below) [47]. In addition, altered miRNA expression has been demonstrated in experimentally induced uveoretinitis, retinoblastoma, and in rodent models of diabetic retinopathy [48–55] (Table 1).

Relevance to retinal diseases

Studies of retinal pathologies in animal models suggest that miRNAs can be involved in disease progression, and recent findings also suggest that miRNAs will be useful targets for the prevention or treatment of retinal degenerative disorders. For example, miRNAs have been shown to promote the survival of both rod and cone photoreceptors, a crucial finding as photoreceptor cell death is the primary cause of blindness in retinal degenerative diseases such as Stargardt disease, retinitis pigmentosa, and age-related macular degeneration [56–58]. Knockout of one of the genes encoding miR-124a led to premature apoptosis of developing cones, whereas simultaneous disruption of miR -96, -182, and -183 activities resulted in dramatically enhanced sensitivity to light-induced degeneration of rods [47, 59]. These results make these miRNAs and the pathways they control attractive targets for the

rational design of therapeutics to prevent or ameliorate various retinal degenerative disorders. In addition, the let-7 miRNA has recently been shown to play a role in Müller glia dedifferentiation in zebrafish [60]. Although mammals are unable to regenerate retinal neurons in response to injury, teleost fish retinas can restore visual function through dedifferentiation of Müller glia cells, followed by subsequent maturation of these restored retinal precursors into any type of neuroretinal cell [61]. Mammalian Müller glia lacks this dedifferentiation property, making retinal degenerative vision loss permanent. Ramachandran and colleagues [60] recently demonstrated that let-7 miRNA inhibits zebrafish Müller glia dedifferentiation by repressing the expression of many regeneration-associated target genes. Downregulation of let-7 after retinal injury permits expression of these factors, facilitating glial dedifferentiation and retinal repair. These results identify the let-7 pathway as an intriguing therapeutic target to promote mammalian retinal self-renewal.

The physiological roles of retinal miRNAs

Impact of global loss of miRNA regulation in the retina

Disruption of the pre-miRNA processing enzyme *Dicer* in mice leads to death early in embryonic development. However, conditional knockout (CKO) of *Dicer* has become the most common technique used to assess the phenotypic consequences of miRNA gene regulation loss in selected tissues. *Dicer* CKO tends to yield more severe phenotypes than disruption of other miRNA pathway components for several reasons [62, 63]. First, mammalian genomes encode only one *Dicer* protein, in contrast to four argonaute family proteins. Furthermore, many miRNAs do not require microprocessor activity for pri-miRNA maturation. For example, mirtrons are short introns of protein coding genes whose pre-miRNA is directly released by the splicing machinery [64–68]. *Dicer* CKO is the most reliable method for tissue-specific disruption of miRNA responses. However, as there are rare examples of *Dicer*-independent production of mature miRNAs, complete loss of *Dicer* activity in a given tissue results only in loss of most mature miRNAs [62, 63]. In addition, a recent report has implicated *dicer* in the processing of Alu RNAs in the retinal pigmented epithelium [69]. This study concluded that loss of *dicer*-mediated cleavage of toxic Alu RNA transcripts (and not loss of mature miRNAs) in these cells led to the phenotype of retinal pigmented epithelium-specific *Dicer* CKO. Therefore, the possible impact of loss of miRNA-independent *dicer* RNA processing activities must also be considered when evaluating the phenotype of *Dicer* CKO models.

Four different recently developed retinal *Dicer* CKO mouse models suggest that miRNAs play diverse roles in the development and physiology of the mammalian retina. *Cre*-mediated *Dicer* excision in retinal progenitors resulted in phenotypes of variable severity, likely dependent on the extent of *Dicer* deletion across the developing retina (Table 2). More severe phenotypes resulted when *Dicer* excision began earlier in retinal development, or when *Cre* was more uniformly expressed throughout the developing retina. *Dicer* CKO driven by the *Chx10-cre* transgene led to decreased electroretinogram (ERG) responses, morphological anomalies, and progressive retinal degeneration [31]. However, retinal development was essentially normal in these mice. By contrast, α *Pax6cre*-driven *Dicer* CKO resulted in abnormal differentiation of retinal cell types [32], and *Dkk3-cre* or *Rx-cre* driven *Dicer* deletion led to widespread apoptosis of retinal progenitors [33, 34], a phenotype consistent with the effect of *Dicer* disruption in *Xenopus* [70]. Due to the well-defined laminar architecture and cell type organization of the retina, it should be possible to define cell type-dependent roles of miRNA gene regulation through cell type-specific *Dicer* CKO. It will be interesting to discover which cell types require miRNAs for proper function in the adult retina. In summary, a wealth of evidence from *Dicer* disruption studies suggests that miRNA gene regulation is essential for both retinal development and mature retinal

neuronal survival and function. These findings have fueled further studies of the functional impact of specific miRNAs or groups of miRNAs on retinal development and physiology.

Roles of specific miRNAs in the retina

Robust defects in retinal development reported in *Dicer*CKO studies suggest that miRNAs are important both for proper retinal neuronal differentiation and also for survival of retinal precursors during development. Studies performed in frogs, fish, and mice are beginning to identify specific miRNAs and target genes that regulate these global miRNA loss-of-function phenotypes (Table 3). Walker and Harland reported that inhibition of miR-24a in *Xenopus* resulted in increased apoptosis of retinal precursors, leading to reductions in eye size [71]. miR-24a is predicted to target the pro-apoptotic factors *caspase-9* and *apoptosis protease-activating factor 1* (*apaf1*). Both these factors were upregulated by inhibition of miR-24a which also repressed expression of reporter genes bearing either the *caspase-9* or *apaf1* 3'UTR. These results suggest that miR-24a targeting of these cell death genes is important for the survival of neuroretinal progenitors. As the timing of differentiation of retinal precursors is a key determinant of their developmental cell fate, Decembrini et. al. hypothesized that miRNAs exhibiting differential expression during retinal patterning might play a role in regulating the differentiation of retinal cell types [72]. They demonstrated that simultaneous inhibition of a set of miRNAs (miRs -129, -155, -214, and -222) expressed early in *Xenopus* retinal development could, through de-repression of the homeobox genes *otx2*, and *vsx1*, promote differentiation of additional retinal bipolar cells, a late-developing retinal cell type. Thus, in addition to supporting the survival of retinal progenitors, miRNAs also regulate cell fate in the developing visual system. Using the medaka fish as a model system, investigators in the Banfi laboratory recently demonstrated that loss of miR-204 function resulted in gross abnormalities in eye development, an effect mediated in part by dysregulation of the transcription factor *Meis2* [73]. In both mice and humans, there are three genetic sources of mature miR-124a (miR-124a-1, miR-124a-2, and miR-124a-3), a highly abundant, brain-enriched miRNA implicated in regulation of neuronal development [74–78]. Knockout of one of these genes in mice resulted in specific apoptosis of newly differentiated cone photoreceptors (along with pronounced effects elsewhere in the CNS, including microencephaly and defects in hippocampal axogenesis) [59]. This cone cell death was partially rescued by shRNA-mediated reduction of the miR-124a target gene, *Lhx2*, which encodes a homeobox transcription factor required for eye development. This study is the first report of a robust retinal phenotype for knockout of a single miRNA in a mammalian model species. Interestingly, this model represents only partial loss of miR-124a in the retina, as significant retinal expression of pre-miR-124a-2 was observed in the retinas of knockout mice [59]. Considering the paucity of strong reported phenotypes for individual miRNA disruptions in mammals, the severity of the impact of partial loss of miR-124a is surprising and highlights the importance of this miRNA in the neuroretina, and for neurons in general.

To begin assessing the physiological impact of specific groups of miRNAs in photoreceptors, our laboratory recently evaluated the role of the miR-183/96/182 cluster (hereafter referred to as the miR-183 cluster) in rods. The miR-183 cluster is an evolutionarily conserved, paralogous miRNA gene cluster. Cluster miRNA components exhibit similar seed region sequences and are predicted to share common target mRNAs. Enriched in rod and cone photoreceptors, these miRNAs are regulated by light, suggesting they might play a role in phototransduction or the visual cycle [26, 28]. To test this hypothesis, we developed a miR-183 cluster sponge transgenic mouse model (Fig. 2b) [47]. Briefly, we engineered a transgene driving rod-specific expression of a reporter mRNA bearing ten target sites for each cluster miRNA, inactivating these miRNAs by sequestration. The retinas of transgenic mice were morphologically indistinguishable from

wild-type mice when kept under normal laboratory lighting conditions. However, when the visual system of transgenic mice was stressed with intense light, they evidenced a dramatically increased sensitivity to light-induced retinal degeneration. A 30-min light exposure caused the death of ~80 % of rods in the superior retina, whereas similar light exposure led to no discernable changes in wild-type mice. Though there are many examples of miRNA-dependent defects in development, this light-induced retinal degeneration is a rare example of a robust miRNA loss-of-function phenotype in a mature tissue. Moreover, we identified *Casp2* as a direct target of the miR-183 cluster. Expression of both the cluster miRNAs and *Casp2* increased in response to light in wild-type mice, and pharmacological inhibition of caspase-2 partially rescued light-induced retinal degeneration in transgenic mice. Therefore, miR-183 cluster-mediated repression of this apoptotic gene is at least partially responsible for the observed miRNA-driven rod photoreceptor protection. However, miRNAs are generally thought to 'fine tune' gene expression, by effecting only minor changes in the expression of multiple genes. Therefore, it is likely that dysregulation of other direct miRNA target genes contributes to the enhanced light sensitivity of these transgenic mice. Clarification of this point will require systematic, biochemical identification of these miRNAs' physiologically relevant targets (see emerging technologies below).

Other than our 'sponge' transgenic mice, two additional mouse models exhibiting disrupted miR-183/96/182 cluster activity have been characterized. An *N*-ethyl-*N*-nitrosurea (ENU)-induced mutation leading to a dominant auditory system developmental defect was mapped to a seed region substitution mutation in miR-96 [79], and a miR-182 knockout mouse model was recently generated [80]. Consistent with the phenotype of miR-183 cluster sponge transgenic mice, knockout of miR-182 resulted in no discernible change in retinal morphology under normal laboratory lighting conditions and visual system function has yet to be systematically characterized in miR-96 mutant mice. Evaluating these two mouse models for sensitivity to light induced retinal degeneration will provide insight into the relative importance of each cluster miRNA in supporting photoreceptor survival upon light stress. However, it is quite possible that disruption of all three miRNAs will be required to elicit a robust phenotype due to functional redundancy. The seed region sequences of these three miRNAs are quite similar and target prediction algorithms generate overlapping lists of putative target genes for them. Importantly, functional redundancy of the three miRNAs has been directly established in the zebrafish auditory system. Morpholino-induced knockdown of all three cluster miRNAs produced auditory system morphological defects that were more severe than those resulting from knockdown of miR-96 alone, or miR-182 and -183 together [81]. In addition, transgenic expression of miR-182 partially rescued the phenotype of miR-96 knockdown in this system [81].

In addition to the miR-183 cluster, miR-26a has also been shown to be important for the physiology of mature photoreceptors (Table 3). A study demonstrated that miR-26a in chickens directly regulates the expression of the $\alpha 1C$ subunit of the photoreceptor L-type voltage-gated calcium channel (L-VGCC $\alpha 1C$) [82]. Circadian rhythm-dependent fluctuations in the expression of this miRNA led to changes in the rate of L-VGCC $\alpha 1C$ translation, contributing to the circadian regulation of light-dependent neurotransmitter release in these dynamic sensory neurons.

Mammalian retina as a model for studying miRNA gene regulation

Despite the expenditure of much energy and resources over recent years, the precise mechanism of miRNA gene regulation remains unclear. Many possible mechanisms of miRNA action have been proposed, including miRNA-mediated repression of translation initiation, post-initiation translational repression, destabilization of nascent polypeptides,

facilitated decay of target mRNAs, and even miRNA-mediated enhancement of gene expression under certain circumstances [5, 6]. In addition, we are now only beginning to understand the cellular regulation of the miRNA response. There are at least two possible explanations for the disparity among mechanisms advocated for the miRNA response. First, most studies dealing with miRNA gene regulation were performed with cultured cell lines or in vitro extracts derived from immortalized cells. These systems provide invaluable experimental flexibility and have greatly advanced our knowledge of miRNA action. However, miRNA dysregulation is a hallmark of transformed cells [39, 83, 84]. Therefore, it is unclear whether the insights gleaned from these model systems are relevant to miRNA action in animals. Alternatively, all the proposed mechanisms might be relevant to the biology of different tissues under different physiological conditions. Targeting of a particular transcript by a given miRNA, as well as the primary mode of miRNA gene regulation, could depend on multiple factors such as the presence of other miRNAs, the abundance of other target transcripts, proteins associated with the miRISC complex, the presence of other mRNA-binding proteins that bind near the miRNA target site, etc. In either case, it has become increasingly apparent that studying the nature of the miRNA response in animal models promises to yield more physiologically relevant insights.

Thus, many groups have turned to the retina as a model system for studying miRNA gene regulation. The retina has several advantages including: (a) its laminar architecture and cell type functionalities are well characterized, (b) as a central nervous system component, the retina is remarkably amenable to experimental manipulation, and (c) genetic manipulations that disrupt visual system development or physiology do not cause premature death, permitting detailed analysis of phenotypic consequences. Hence, several significant insights regarding the regulation of the miRNA response and its manipulation for therapeutic purposes have recently been gained from studies of the vertebrate retina.

In a study of broad scope and implications, investigators in the Filipowicz laboratory set out to determine whether retinal miRNAs are important for adaptation to variable levels of illumination [26]. While examining the dynamic expression levels of a set of light-regulated miRNAs, they noticed an unexpectedly sharp decline in the levels of these miRNAs upon dark adaptation. Based on this initial observation, they went on to demonstrate that dynamic activity-dependent enhancement in the rate of miRNA decay is a common feature of regulation of the miRNA response, especially in neurons. Such rapid, activity-dependent miRNA turnover could enable more dynamic modification of cellular miRNA composition, facilitating miRNA regulation of a rapidly changing transcript pool in active neurons. In addition, knockout of *Dicer* in the mouse retinal pigmented epithelium revealed that this pre-miRNA processing enzyme might also be involved in cleavage of other cellular RNAs [69]. Finally, Karali and colleagues [85] recently showed that engineering miRNA target sites into the 3' UTR of virally delivered transgenes could effectively modify the pattern of transgene expression in the mammalian retina. The last result has interesting implications for the design of gene delivery vectors, not only for the visual system, but other organs and tissues as well. Hence, studies in retina are beginning to yield useful knowledge about regulation of the miRNA response, as well as ways to hijack this gene regulatory mechanism to facilitate eventual gene therapy.

Targeting miRNA gene regulation for treatment of ocular diseases

Retinal pathologies are attractive candidates for gene therapy approaches based on the relative accessibility of this tissue for targeted gene delivery. Indeed, gene therapy approaches for the treatment of ocular pathologies have met with much success in recent years. Adeno-associated virus-mediated gene replacement has proven both safe and effective for treating Leber congenital amaurosis in clinical trials with patients carrying mutations in

the Rpe65 gene [86–92]. In addition, gene therapy for neovascular age-related macular degeneration, as well as retinoblastoma is undergoing clinical trials [93, 94]. miRNA-based gene therapeutic approaches can offer enhanced efficacy based on targeting multiple genes in the same pathway, and substantial progress has been made recently in developing efficient delivery systems for targeting miRNA pathways therapeutically [35–39]. Multiple strategies both for augmenting and repressing the activity of specific miRNAs have been reported, including delivery of synthetic miRNA mimics, miRNA-encoding transgenes, competitive miRNA inhibitory antisense oligonucleotides (known as antagomirs) and miRNA sponges (Fig. 2b) [39]. The relative ease of gene delivery to the retina also promises to facilitate the introduction of small RNAs for therapeutic purposes, as evidenced by recent clinical trials involving introduction of siRNAs to the retina for treatment of neovascular age-related macular degeneration [95–97]. Thus miRNA gene therapy for retinal degenerative disorders melds two successful, recently developed approaches (i.e., gene therapy in the eye and gene therapy targeting miRNA genes). In light of recent results which clearly demonstrate that specific miRNAs play a role in photoreceptor survival [47, 59], manipulation of miRNA activity constitutes an attractive strategy to treat retinal degenerative disorders such as retinitis pigmentosa, and age-related macular degeneration.

Future directions

Recent findings have been discussed that have dramatically expanded our understanding of the physiological impact of miRNA gene regulation in the retina. *Dicer* CKO studies have clearly demonstrated that global loss of retinal miRNAs has markedly detrimental effects on retinal development and physiology. Loss-of-function studies with lower vertebrate model species have identified fundamental roles for miRNAs in regulating the differentiation and promoting the survival of retinal neuronal progenitors. Finally, we are beginning to discern roles for specific miRNAs in adult retina. Some remaining challenges toward understanding the roles of retinal miRNAs are to: (a) assess the specific physiological roles of each retinal miRNA, (b) systematically identify genes directly targeted by miRNAs in the retina, and (c) determine specific target gene(s) whose dysregulation in the absence of miRNAs results in abnormal retinal phenotypes. Though this list seems daunting, emerging technologies developed for studying miRNAs in other systems have the potential to dramatically accelerate progress. It is possible to envision an experimental scheme wherein the direct targets of a retinal miRNA are identified biochemically by using AGO crosslinking immunoprecipitation followed by next generation sequencing (CLIPseq), the physiological impact of that miRNA determined using a miRNA sponge, and the targets whose de-repression is responsible for the phenotype identified utilizing target protector oligonucleotides (Fig. 2). In this way, a complete, detailed picture of the physiological impact of each retinal miRNA could be revealed.

AGO CLIPseq

Reliable identification of genes targeted by miRNAs is of paramount importance to understanding the physiological impact of miRNA gene regulation. Until recently, targets of a given miRNA were identified by using one of several bioinformatic miRNA target prediction algorithms (e.g., TargetScan [98], PicTar [99], Microcosm [100], etc.). Selected target genes were then validated by demonstrating that their 3'UTR could be directly targeted in a reporter-based, cell culture transfection assay. However, this approach suffers from several inherent limitations. First, target prediction algorithms suffer from a high rate of false positives, requiring potentially biased selection of putative targets for further analyses. Second, constraints inherent in each of the different target prediction algorithms render them unlikely to identify certain classes of physiologically relevant target genes. For example, reliance on perfect seed region complementarity causes certain algorithms to ignore targets exhibiting non-canonical (i.e., G-U wobble) base pairing or seed region

mismatches. Similarly, analyses limited to the mRNA 3'UTR miss a well-characterized class of target sites residing within the coding sequence [101–103]. Finally, cell type and tissue-specific factors such as expression of other miRNA target transcripts, mRNA binding proteins or target site-bearing non-coding RNAs can influence miRISC interaction with a given putative target transcript [84, 104–108]. Therefore, reliable, non-biased miRNA target identification requires a systematic, biochemical assay of miRISC-target mRNA interactions directly in the cell-type or tissue of interest.

The recently developed technology of CLIP-seq offers an elegant solution to this problem (Fig. 2a) [109, 110]. Conveniently, miRNA-interacting proteins of the AGO family exist in sufficiently close proximity to both the miRNA guide strand and its bound target mRNA to permit in vivo UV-crosslinking to both RNAs [109, 111]. The resulting covalently linked protein-RNA complexes then can be purified through high-stringency immunoprecipitation, and the RNA sequences identified by NGS. First reported in 2009, the utility of AGO CLIP-seq is now well established in a variety of systems including HeLa cells, mouse embryonic stem cells, *C. elegans*, and mouse neocortex [109, 112–114]. However, no systematic, biochemical analysis of miRNA targets in the retina has yet been reported. CLIPseq analysis represents a dramatic improvement over earlier AGO immunoprecipitation-based miRNA target analyses which lack crosslinking, because in vivo crosslinking stabilizes the AGO-RNA complexes such that the resulting genome-wide map of retinal miRNA regulation represents a snapshot of RNAs bound by AGO in living cells. In addition, covalent attachment of AGO to bound RNAs allows more stringent immunoprecipitation, reducing background, and identification of RNAs whose interaction with the miRISC might be too transient to survive immunoprecipitation and washing.

The miRNA sponge

With reported mammalian retinal miRNAs numbering in the hundreds, it is impractical to knockout these miRNAs individually in mice to evaluate their physiological function (even though a resource of mouse embryonic stem cells bearing deletion of most individual miRNAs has recently been reported) [115]. Inhibition of miRNA activity with synthetic antagomirs is another option, but though the retina is more amenable to this type of manipulation than other tissues, the extent and duration of this effect is limited. A useful alternative is to express a sponge transgene to inactivate one or a group of miRNAs by sequestration (Fig. 2b). This approach has the advantages of decreased model development time along with control over cell-type specificity of miRNA inactivation through selection of an appropriate promoter to drive sponge transgene expression.

miRNA target protectors

Once the impact of loss of a specific miRNA has been established and its direct targets biochemically validated, the next challenge is to establish which of the identified target genes mediates the observed phenotype. The ideal way to address this question would be to knock-in mutation to the target site(s) to see if this disruption has an effect similar to miRNA inactivation. However, as miRNAs tend to target many genes, this approach is likely to be prohibitively laborious. An alternative has recently been developed involving the use of antisense oligonucleotides termed 'target protectors' (Fig. 2c). First developed in zebrafish [116], this strategy has since been used to inhibit miRNA-target interactions in *Drosophila* [117], and more recently in mice [118]. Briefly, a target protector is an antisense oligonucleotide that binds to a region of a given mRNA overlapping the miRNA target site, acting as a competitive inhibitor of miRISC-target interaction that is specific for the selected target gene. Zovoilis and colleagues [118] used hippocampal injection of target protector oligonucleotides to demonstrate that miR-34c mimic-dependent learning impairment is mediated through targeting of the SIRT1 mRNA in mice. One can easily envision

application of this approach to study retinal miRNA-target interactions by subretinal injection of precisely designed target protectors, or through cell type-specific expression of target protector transgenes.

Conclusions

A wealth of new information has evolved recently regarding the functional significance of miRNAs in the vertebrate retina. Global as well as specific miRNA loss-of-function studies have revealed important roles for miRNAs in retinal development, physiology and disease. Furthermore, the vertebrate retina has served as an attractive model system for studying both the regulation and experimental manipulation of the miRNA response. New technologies for studying miRNA gene regulation now make it possible to evaluate precisely the impact of loss of specific miRNAs or miRNA-target mRNA interactions in selected tissues or cell types. Moreover, technology permitting tissue-specific, biochemical identification of miRNA targets will allow systematic analysis of downstream factors that mediate loss-of-function phenotypes. Though much progress has recently been made in this field, the pace of discovery is certain to increase greatly in the near future.

Acknowledgments

We thank Dr. Leslie T. Webster, Jr. for critical comments on the manuscript. This work was supported, in whole or in part, by National Institutes of Health Grants EY009339, and P30 EY11373. KP is John H. Hord Professor of Pharmacology.

Abbreviations

AGO	Argonaute
CKO	Conditional knockout
ERG	Electroretinogram
CLIPseq	Crosslinking immunoprecipitation followed by next-generation sequencing
L-VGCCα1C	α 1C subunit of the photoreceptor L-type voltage-gated calcium channel
NGS	Next-generation sequencing
miRISC	microRNA-induced silencing complex
miRNA	microRNA
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA

References

1. Rodieck, RW. The first steps in seeing. Sinauer Associates; Sunderland: 1998.
2. Palczewski K. Focus on vision: 3 decades of remarkable contributions to biology and medicine. *FASEB J*. 2011; 25(2):439–443. [PubMed: 21282210]
3. Ridge KD, Palczewski K. Visual rhodopsin sees the light: structure and mechanism of G protein signaling. *J Biol Chem*. 2007; 282(13):9297–9301. [PubMed: 17289671]
4. Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet*. 2011; 12(2):99–110. [PubMed: 21245828]
5. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem*. 2010; 79:351–379. [PubMed: 20533884]

6. Fabian MR, Sundermeier TR, Sonenberg N. Understanding how miRNAs post-transcriptionally regulate gene expression. *Prog Mol Subcell Biol.* 2010; 50:1–20. [PubMed: 19841878]
7. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell.* 1993; 75(5):843–854. [PubMed: 8252621]
8. Wightman B, Burglin TR, Gatto J, Arasu P, Ruvkun G. Negative regulatory sequences in the *lin-14* 3'-untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development. *Genes Dev.* 1991; 5(10):1813–1824. [PubMed: 1916264]
9. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell.* 1993; 75(5):855–862. [PubMed: 8252622]
10. Lee RC, Ambros V. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science.* 2001; 294(5543):862–864. [PubMed: 11679672]
11. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science.* 2001; 294(5543):858–862. [PubMed: 11679671]
12. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science.* 2001; 294(5543):853–858. [PubMed: 11679670]
13. Pasquinelli AE, et al. Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA. *Nature.* 2000; 408(6808):86–89. [PubMed: 11081512]
14. Newman MA, Hammond SM. Emerging paradigms of regulated microRNA processing. *Genes Dev.* 2010; 24(11):1086–1092. [PubMed: 20516194]
15. Siomi H, Siomi MC. Posttranscriptional regulation of microRNA biogenesis in animals. *Mol Cell.* 2010; 38(3):323–332. [PubMed: 20471939]
16. Fabian MR, et al. miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4-NOT. *Nat Struct Mol Biol.* 2011; 18(11):1211–1217. [PubMed: 21984185]
17. Chekulaeva M, et al. miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. *Nat Struct Mol Biol.* 2011; 18(11):1218–1226. [PubMed: 21984184]
18. Braun JE, Huntzinger E, Fauser M, Izaurralde E. GW182 proteins directly recruit cytoplasmic deadenylase complexes to miRNA targets. *Mol Cell.* 2011; 44(1):120–133. [PubMed: 21981923]
19. Tritschler F, Huntzinger E, Izaurralde E. Role of GW182 proteins and PABPC1 in the miRNA pathway: a sense of déjà vu. *Nat Rev Mol Cell Biol.* 2010; 11(5):379–384. [PubMed: 20379206]
20. Rapicavoli NA, Blackshaw S. New meaning in the message: noncoding RNAs and their role in retinal development. *Dev Dyn.* 2009; 238(9):2103–2114. [PubMed: 19191220]
21. Xu S. microRNA expression in the eyes and their significance in relation to functions. *Prog Retin Eye Res.* 2009; 28(2):87–116. [PubMed: 19071227]
22. Li X, Jin P. Roles of small regulatory RNAs in determining neuronal identity. *Nat Rev Neurosci.* 2010; 11(5):329–338. [PubMed: 20354535]
23. Karali M, et al. miRNeYE: a microRNA expression atlas of the mouse eye. *BMC Genomics.* 2010; 11:715. [PubMed: 21171988]
24. Hackler L Jr, Wan J, Swaroop A, Qian J, Zack DJ. MicroRNA profile of the developing mouse retina. *Invest Ophthalmol Vis Sci.* 2010; 51(4):1823–1831. [PubMed: 19933188]
25. Karali M, Peluso I, Marigo V, Banfi S. Identification and characterization of microRNAs expressed in the mouse eye. *Invest Ophthalmol Vis Sci.* 2007; 48(2):509–515. [PubMed: 17251443]
26. Krol J, et al. Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal microRNAs. *Cell.* 2010; 141(4):618–631. [PubMed: 20478254]
27. Makarev E, Spence JR, Del Rio-Tsonis K, Tsonis PA. Identification of microRNAs and other small RNAs from the adult newt eye. *Mol Vis.* 2006; 12:1386–1391. [PubMed: 17149364]
28. Xu S, Witmer PD, Lumayag S, Kovacs B, Valle D. MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. *J Biol Chem.* 2007; 282(34):25053–25066. [PubMed: 17597072]

29. Wang FE, et al. MicroRNA-204/211 alters epithelial physiology. *FASEB J.* 2010; 24(5):1552–1571. [PubMed: 20056717]
30. Ryan DG, Oliveira-Fernandes M, Lavker RM. MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity. *Mol Vis.* 2006; 12:1175–1184. [PubMed: 17102797]
31. Damiani D, et al. Dicer inactivation leads to progressive functional and structural degeneration of the mouse retina. *J Neurosci.* 2008; 28(19):4878–4887. [PubMed: 18463241]
32. Georgi SA, Reh TA. Dicer is required for the transition from early to late progenitor state in the developing mouse retina. *J Neurosci.* 2010; 30(11):4048–4061. [PubMed: 20237275]
33. Iida A, Shinoe T, Baba Y, Mano H, Watanabe S. Dicer plays essential roles for retinal development by regulation of survival and differentiation. *Invest Ophthalmol Vis Sci.* 2011; 52(6):3008–3017. [PubMed: 21273552]
34. Pinter R, Hindges R. Perturbations of microRNA function in mouse dicer mutants produce retinal defects and lead to aberrant axon pathfinding at the optic chiasm. *PLoS One.* 2010; 5(4):e10021. [PubMed: 20386732]
35. Kota J, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell.* 2009; 137(6):1005–1017. [PubMed: 19524505]
36. Trang P, et al. Regression of murine lung tumors by the let-7 microRNA. *Oncogene.* 2010; 29(11):1580–1587. [PubMed: 19966857]
37. Lanford RE, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science.* 2010; 327(5962):198–201. [PubMed: 19965718]
38. Thum T, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature.* 2008; 456(7224):980–984. [PubMed: 19043405]
39. Sayed D, Abdellatif M. MicroRNAs in development and disease. *Physiol Rev.* 2011; 91(3):827–887. [PubMed: 21742789]
40. Liu MM, Tuo J, Chan CC. Gene therapy for ocular diseases. *Br J Ophthalmol.* 2011; 95(5):604–612. [PubMed: 20733027]
41. Conley SM, Cai X, Naash MI. Nonviral ocular gene therapy: assessment and future directions. *Curr Opin Mol Ther.* 2008; 10(5):456–463. [PubMed: 18830921]
42. Chalberg TW, Genise HL, Vollrath D, Calos MP. phiC31 integrase confers genomic integration and long-term transgene expression in rat retina. *Invest Ophthalmol Vis Sci.* 2005; 46(6):2140–2146. [PubMed: 15914635]
43. Farjo R, Skaggs J, Quiambao AB, Cooper MJ, Naash MI. Efficient non-viral ocular gene transfer with compacted DNA nanoparticles. *PLoS One.* 2006; 1:e38. [PubMed: 17183666]
44. Diebold Y, Calonge M. Applications of nanoparticles in ophthalmology. *Prog Retin Eye Res.* 2010; 29(6):596–609. [PubMed: 20826225]
45. Loscher CJ, et al. Altered retinal microRNA expression profile in a mouse model of retinitis pigmentosa. *Genome Biol.* 2007; 8(11):R248. [PubMed: 18034880]
46. Loscher CJ, et al. A common microRNA signature in mouse models of retinal degeneration. *Exp Eye Res.* 2008; 87(6):529–534. [PubMed: 18834879]
47. Zhu Q, et al. Sponge transgenic mouse model reveals important roles for the microRNA-183 (miR-183)/96/182 cluster in postmitotic photoreceptors of the retina. *J Biol Chem.* 2011; 286(36):31749–31760. [PubMed: 21768104]
48. Kovacs B, Lumayag S, Cowan C, Xu S. MicroRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. *Invest Ophthalmol Vis Sci.* 2011; 52(7):4402–4409. [PubMed: 21498619]
49. Ishida W, et al. Dynamic changes of microRNAs in the eye during the development of experimental autoimmune uveoretinitis. *Invest Ophthalmol Vis Sci.* 2011; 52(1):611–617. [PubMed: 20881307]
50. Konkrite K, et al. miR-17 ~92 cooperates with RB pathway mutations to promote retinoblastoma. *Genes Dev.* 2011; 25(16):1734–1745. [PubMed: 21816922]
51. Silva VA, et al. Expression and cellular localization of microRNA-29b and RAX, an activator of the RNA-dependent protein kinase (PKR), in the retina of streptozotocin-induced diabetic rats. *Mol Vis.* 2011; 17:2228–2240. [PubMed: 21897745]

52. McArthur K, Feng B, Wu Y, Chen S, Chakrabarti S. MicroRNA-200b regulates vascular endothelial growth factor-mediated alterations in diabetic retinopathy. *Diabetes*. 2011; 60(4): 1314–1323. [PubMed: 21357793]
53. Feng B, et al. miR-146a-Mediated extracellular matrix protein production in chronic diabetes complications. *Diabetes*. 2011; 60(11):2975–2984. [PubMed: 21885871]
54. Wu JH, et al. Altered microRNA expression profiles in retinas with diabetic retinopathy. *Ophthalmic Res*. 2011; 47(4):195–201. [PubMed: 22156553]
55. Mu G, et al. Correlation of overexpression of HMGA1 and HMGA2 with poor tumor differentiation, invasion, and proliferation associated with let-7 down-regulation in retinoblastomas. *Hum Pathol*. 2010; 41(4):493–502. [PubMed: 20004941]
56. Zack DJ, et al. What can we learn about age-related macular degeneration from other retinal diseases? *Mol Vis*. 1999; 5:30. [PubMed: 10562654]
57. Sun H, Nathans J. The challenge of macular degeneration. *Sci Am*. 2001; 285(4):68–75. [PubMed: 11570044]
58. Organisciak DT, Vaughan DK. Retinal light damage: mechanisms and protection. *Prog Retin Eye Res*. 2010; 29(2):113–134. [PubMed: 19951742]
59. Sanuki R, et al. miR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression. *Nat Neurosci*. 2011; 14(9):1125–1134. [PubMed: 21857657]
60. Ramachandran R, Fausett BV, Goldman D. Ascl1a regulates Muller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. *Nat Cell Biol*. 2010; 12(11):1101–1107. [PubMed: 20935637]
61. Lamba DA, Karl MO, Reh TA. Strategies for retinal repair: cell replacement and regeneration. *Prog Brain Res*. 2009; 175:23–31. [PubMed: 19660646]
62. Yang JS, Lai EC. Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. *Mol Cell*. 2011; 43(6):892–903. [PubMed: 21925378]
63. Yang JS, Lai EC. Dicer-independent, Ago2-mediated microRNA biogenesis in vertebrates. *Cell Cycle*. 2010; 9(22):4455–4460. [PubMed: 21088485]
64. Sibley CR, et al. The biogenesis and characterization of mammalian microRNAs of mirtron origin. *Nucleic Acids Res*. 2011; 40(1):438–448. [PubMed: 21914725]
65. Flynt AS, Greimann JC, Chung WJ, Lima CD, Lai EC. MicroRNA biogenesis via splicing and exosome-mediated trimming in *Drosophila*. *Mol Cell*. 2010; 38(6):900–907. [PubMed: 20620959]
66. Berezikov E, Chung WJ, Willis J, Cuppen E, Lai EC. Mammalian mirtron genes. *Mol Cell*. 2007; 28(2):328–336. [PubMed: 17964270]
67. Okamura K, Hagen JW, Duan H, Tyler DM, Lai EC. The mirtron pathway generates microRNA-class regulatory RNAs in *Drosophila*. *Cell*. 2007; 130(1):89–100. [PubMed: 17599402]
68. Ruby JG, Jan CH, Bartel DP. Intronic microRNA precursors that bypass Drosha processing. *Nature*. 2007; 448(7149):83–86. [PubMed: 17589500]
69. Kaneko H, et al. DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. *Nature*. 2011; 471(7338):325–330. [PubMed: 21297615]
70. Decembrini S, Andreazzoli M, Barsacchi G, Cremisi F. Dicer inactivation causes heterochronic retinogenesis in *Xenopus laevis*. *Int J Dev Biol*. 2008; 52(8):1099–1103. [PubMed: 18956342]
71. Walker JC, Harland RM. microRNA-24a is required to repress apoptosis in the developing neural retina. *Genes Dev*. 2009; 23(9):1046–1051. [PubMed: 19372388]
72. Decembrini S, et al. MicroRNAs couple cell fate and developmental timing in retina. *Proc Natl Acad Sci USA*. 2009; 106(50):21179–21184. [PubMed: 19965369]
73. Conte I, et al. miR-204 is required for lens and retinal development via Meis2 targeting. *Proc Natl Acad Sci USA*. 2010; 107(35):15491–15496. [PubMed: 20713703]
74. Baudet ML, et al. miR-124 acts through CoREST to control onset of Sema3A sensitivity in navigating retinal growth cones. *Nat Neurosci*. 2012; 15(1):29–38. [PubMed: 22138647]
75. Visvanathan J, Lee S, Lee B, Lee JW, Lee SK. The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. *Genes Dev*. 2007; 21(7):744–749. [PubMed: 17403776]

76. Maiorano NA, Mallamaci A. The pro-differentiating role of miR-124: indicating the road to become a neuron. *RNA Biol.* 2010; 7(5):528–533. [PubMed: 20523124]
77. Makeyev EV, Zhang J, Carrasco MA, Maniatis T. The microRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol Cell.* 2007; 27(3): 435–448. [PubMed: 17679093]
78. Cheng LC, Pastrana E, Tavazoie M, Doetsch F. miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. *Nat Neurosci.* 2009; 12(4):399–408. [PubMed: 19287386]
79. Lewis MA, et al. An ENU-induced mutation of miR-96 associated with progressive hearing loss in mice. *Nat Genet.* 2009; 41(5):614–618. [PubMed: 19363478]
80. Jin ZB, et al. Targeted deletion of miR-182, an abundant retinal microRNA. *Mol Vis.* 2009; 15:523–533. [PubMed: 19279690]
81. Li H, Kloosterman W, Fekete DM. MicroRNA-183 family members regulate sensorineural fates in the inner ear. *J Neurosci.* 2010; 30(9):3254–3263. [PubMed: 20203184]
82. Shi L, Ko ML, Ko GY. Rhythmic expression of microRNA-26a regulates the L-type voltage-gated calcium channel $\alpha 1C$ subunit in chicken cone photoreceptors. *J Biol Chem.* 2009; 284(38): 25791–25803. [PubMed: 19608742]
83. Kasinski AL, Slack FJ. Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. *Nat Rev Cancer.* 2011; 11(12):849–864. [PubMed: 22113163]
84. van Kouwenhove M, Kedde M, Agami R. MicroRNA regulation by RNA-binding proteins and its implications for cancer. *Nat Rev Cancer.* 2011; 11(9):644–656. [PubMed: 21822212]
85. Karali M, et al. MicroRNA-restricted transgene expression in the retina. *PLoS One.* 2011; 6(7):e22166. [PubMed: 21818300]
86. Bainbridge JW, et al. Effect of gene therapy on visual function in Leber's congenital amaurosis. *N Engl J Med.* 2008; 358(21):2231–2239. [PubMed: 18441371]
87. Cideciyan AV, et al. Vision 1 year after gene therapy for Leber's congenital amaurosis. *N Engl J Med.* 2009; 361(7):725–727. [PubMed: 19675341]
88. Cideciyan AV, et al. Human RPE65 gene therapy for Leber congenital amaurosis: persistence of early visual improvements and safety at 1 year. *Hum Gene Ther.* 2009; 20(9):999–1004. [PubMed: 19583479]
89. Cideciyan AV, et al. Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. *Proc Natl Acad Sci USA.* 2008; 105(39):15112–15117. [PubMed: 18809924]
90. Maguire AM, et al. Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. *Lancet.* 2009; 374(9701):1597–1605. [PubMed: 19854499]
91. Maguire AM, et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med.* 2008; 358(21):2240–2248. [PubMed: 18441370]
92. Simonelli F, et al. Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. *Mol Ther.* 2010; 18(3):643–650. [PubMed: 19953081]
93. Campochiaro PA, et al. Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. *Hum Gene Ther.* 2006; 17(2):167–176. [PubMed: 16454650]
94. Ildefonso CJ, et al. Absence of systemic immune response to adenovectors after intraocular administration to children with retinoblastoma. *Mol Ther.* 2010; 18(10):1885–1890. [PubMed: 20606647]
95. Kaiser PK, et al. RNAi-based treatment for neovascular age-related macular degeneration by Sirna-027. *Am J Ophthalmol.* 2010; 150(1):33–39. e32. [PubMed: 20609706]
96. Bumcrot D, Manoharan M, Koteliensky V, Sah DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol.* 2006; 2(12):711–719. [PubMed: 17108989]
97. Campa C, Harding SP. Anti-VEGF compounds in the treatment of neovascular age related macular degeneration. *Curr Drug Targets.* 2011; 12(2):173–181. [PubMed: 20887245]

98. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120(1):15–20. [PubMed: 15652477]
99. Krek A, et al. Combinatorial microRNA target predictions. *Nat Genet*. 2005; 37(5):495–500. [PubMed: 15806104]
100. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. *Nucleic Acids Res*. 2008; 36:D154–D158. [PubMed: 17991681]
101. Kang JG, et al. Kaposi's sarcoma-associated herpesvirus ORF57 promotes escape of viral and human interleukin-6 from microRNA-mediated suppression. *J Virol*. 2011; 85(6):2620–2630. [PubMed: 21209110]
102. Moretti F, Thermann R, Hentze MW. Mechanism of translational regulation by miR-2 from sites in the 5' untranslated region or the open reading frame. *RNA*. 2010; 16(12):2493–2502. [PubMed: 20966199]
103. Schnall-Levin M, Zhao Y, Perrimon N, Berger B. Conserved microRNA targeting in *Drosophila* is as widespread in coding regions as in 3' UTRs. *Proc Natl Acad Sci USA*. 2010; 107(36):15751–15756. [PubMed: 20729470]
104. Karreth FA, et al. In vivo identification of tumor-suppressive PTEN ceRNAs in an oncogenic BRAF-induced mouse model of melanoma. *Cell*. 2011; 147(2):382–395. [PubMed: 22000016]
105. Cesana M, et al. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell*. 2011; 147(2):358–369. [PubMed: 22000014]
106. Tay Y, et al. Coding-independent regulation of the tumor suppressor PTEN by competing endogenous mRNAs. *Cell*. 2011; 147(2):344–357. [PubMed: 22000013]
107. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell*. 2011; 146(3):353–358. [PubMed: 21802130]
108. Mukherji S, et al. MicroRNAs can generate thresholds in target gene expression. *Nat Genet*. 2011; 43(9):854–859. [PubMed: 21857679]
109. Chi SW, Zang JB, Mele A, Darnell RB. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature*. 2009; 460(7254):479–486. [PubMed: 19536157]
110. Licatalosi DD, et al. HITS-CLIP yields genome-wide insights into brain alternative RNA processing. *Nature*. 2008; 456(7221):464–469. [PubMed: 18978773]
111. Wang Y, et al. Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. *Nature*. 2008; 456(7224):921–926. [PubMed: 19092929]
112. Leung AK, et al. Genome-wide identification of Ago2 binding sites from mouse embryonic stem cells with and without mature microRNAs. *Nat Struct Mol Biol*. 2011; 18(2):237–244. [PubMed: 21258322]
113. McKenna LB, et al. MicroRNAs control intestinal epithelial differentiation, architecture, and barrier function. *Gastroenterology*. 2010; 139(5):1654–1664. 1664, e1651. [PubMed: 20659473]
114. Zisoulis DG, et al. Comprehensive discovery of endogenous Argonaute binding sites in *Caenorhabditis elegans*. *Nat Struct Mol Biol*. 2010; 17(2):173–179. [PubMed: 20062054]
115. Prosser HM, Koike-Yusa H, Cooper JD, Law FC, Bradley A. A resource of vectors and ES cells for targeted deletion of microRNAs in mice. *Nat Biotechnol*. 2011; 29(9):840–845. [PubMed: 21822254]
116. Choi WY, Giraldez AJ, Schier AF. Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. *Science*. 2007; 318(5848):271–274. [PubMed: 17761850]
117. Gehrke S, Imai Y, Sokol N, Lu B. Pathogenic LRRK2 negatively regulates microRNA-mediated translational repression. *Nature*. 2010; 466(7306):637–641. [PubMed: 20671708]
118. Zovoilis A, et al. microRNA-34c is a novel target to treat dementias. *EMBO J*. 2011; 30(20):4299–4308. [PubMed: 21946562]
119. Bai Y, et al. MicroRNA-126 inhibits ischemia-induced retinal neovascularization via regulating angiogenic growth factors. *Exp Mol Pathol*. 2011; 91(1):471–477. [PubMed: 21586283]

Fig. 1. miRNA biogenesis and mechanism of action

miRNAs are transcribed in the nucleus, either independently or as introns of protein coding genes, by RNA Polymerase II. Functionally related miRNAs are often clustered on a chromosome and co-transcribed. miRNA primary transcripts (pri-miRNAs) fold into hairpin structures, which are recognized by the nuclear microprocessor complex and cleaved by the RNase III-type enzyme Drosha, generating ~70 nt precursor miRNAs (pre-miRNAs). Pre-miRNAs are exported to the cytoplasm by exportin-5 and further processed into ~22 nt double-stranded RNAs. One strand is selected and bound by AGO family proteins, then assembled into a large ribonucleoprotein effector complex known as the miRISC. Imperfect base pairing between the miRNA and target mRNAs recruits the miRISC to target transcripts where it represses their expression either by repressing translation, or enhancing the rate of mRNA decay [4–6]

Fig. 2.

Emerging technologies for the study of miRNA gene regulation. **a** AGO CLIPseq permits systematic biochemical identification of miRNA targets directly in selected tissues [109]. AGO proteins are crosslinked in vivo to both miRNA and target mRNA. Limited RNase digestions followed by high stringency immunoprecipitation with AGO-specific antibodies results in purification of AGO proteins covalently linked to either miRNAs or short fragments of target mRNAs. These co-purified RNAs are then identified by NGS. **b** A miRNA sponge is a synthetic gene bearing multiple target sites for selected miRNA(s) in its 3' UTR. Sponges sequester miRISCs bound to a miRNA, de-repressing synthesis of that miRNA's target genes. **c** Target protectors are oligonucleotides that interact with target mRNAs by base-pairing, thus inhibiting interaction of the miRISC with overlapping target sites, de-repressing the expression of specific target transcripts

Table 1

Modulation of miRNA expression in models of retinal pathologies

Disease model	miRNAs	References
Diabetic retinopathy: Streptozotocin injected rat model	miR-10a, miR-10b, miR-29b, miR-96, miR-124a, miR-144, miR-146a, miR-182, miR-183, miR-199-3p, miR-200b, miR-204, miR-211, miR-219-2-3p, miR-338	[48, 51–54]
Experimental autoimmune uveoretinitis	miR-21, miR-142-5p, miR-182	[49]
Neovascularization—oxygen-induced retinopathy mouse model	miR-126	[119]
Photoreceptor degeneration: light damage model (mouse)	miR-96, miR-182, miR-183	[47]
Retinitis pigmentosa: <i>opsin</i> ^{−/−} mice <i>P347S opsin</i> transgenic mice, <i>rds</i> ^{−/−} mice <i>rds</i> Δ <i>307</i> transgenic mice,	miR-1, miR-96, miR-133, miR-142, miR-182, and miR-183 (common miRNA signature across RP models)	[45, 46]
Retinoblastoma: Rb/p107DKO mouse model	miR-17, miR-18a, miR-19a, miR-20a, miR-19b1, miR-92-1	[50]
Retinoblastoma: Human patients	let-7	[55]

Table 2Functional consequences of retinal *Dicer*CKO in mice

Cre transgene	Pattern of cre-recombination	Phenotype	Reference
<i>Chx10-cre</i>	Mosaic pattern, beginning before embryonic day 14.5 in precursors of all retinal layers	Decreased ERG responses, retinal disorganization, progressive retinal degeneration	[31]
α . <i>Pax6cre</i>	Expressed in peripheral regions of developing retina, beginning at embryonic day 10.5; also expressed in differentiated amacrine cells, beginning around embryonic day 14.5	Impaired patterning of retinal cells. Overproduction of ganglion cells, failure to generate late cell types such as Müller glia and rod photoreceptors	[32]
<i>Dkk3-cre</i>	Ubiquitously expressed in precursors of all neuroretinal cell types beginning at embryonic day 10.5	Microphthalmia, massive apoptosis of retinal progenitor cells	[33]
<i>Rx-cre</i>	Ubiquitously expressed in the developing neuroretina, optic stalk, and later in the optic chiasm. Some expression also in the lens	Microphthalmia, massive apoptosis of retinal progenitors, additional defects in retinal ganglion cell axon pathfinding	[34]

Table 3

Impact of specific miRNA loss of function on retina

miRNA	Impact of loss in retina	Reference
miR-24a	Morpholino-induced inhibition in <i>Xenopus</i> leads to apoptosis of retinal progenitors	[71]
miR-124a	Knockout of one of the miR-124a genes (<i>miR-124a-1</i>) leads to apoptosis of newly differentiated cone photoreceptors in mice	[59]
miRs -129, -155, -214, and -222	Simultaneous inhibition of these miRNAs in <i>Xenopus</i> leads to aberrant timing of retinal precursor differentiation	[72]
miR-183/96/182 cluster	Simultaneous, sponge-driven inactivation in mouse rods leads to light-induced retinal degeneration	[47]
miR-204	Inhibition in the medaka fish leads to gross defects in eye development	[73]