The role of miRNAs in retinal physiology and in Inherited Retinal Disorders

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level. Several studies have highlighted their role as key regulators of different physiological processes that underly retinal homeostasis. Recent evidence suggests that they play a role not only at the intracellular level but also extracellularly, participating in cell-cell crosstalk by being transported via extracellular vesicles (EVs). Moreover, changes in miRNA expression levels have been associated with different forms of retinal diseases, such as Inherited Retinal Diseases (IRDs). IRDs are a group of genetic disorders characterized by photoreceptor cell death and retinal degeneration. Notably, miRNAs can simultaneously regulate multiple molecular pathways associated with disease initiation and progression. Finally, modulation of miRNAs through upregulation or downregulation has shown beneficial effects in different IRD mouse models. Here, we provide a comprehensive overview of retinal miRNA expression profiles in both healthy and IRD conditions and explore their potential as therapeutic targets for clinical applications.

Introduction

The retina is part of the mammalian central nervous system and exhibits a high level of organization and an evolutionary conserved structure(1). It consists of distinct specialized retinal cell types, including photoreceptors, interneurons, and ganglion cells, which, by transducing light into neural impulses, are responsible for vision(2). Photoreceptor cells, including rods and cones, are the main players in light sensing and initiate the cascade of chemical and electrical events that participate in the phototransduction process(3).

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Other non-neuronal cell types, such as Müller cells, astrocytes, and microglia, populate the mammalian retina serving as support for neuronal cells(4). Due to the complex physiology of the retina, a large number of protein-coding genes participate in orchestrating the molecular processes underlying its development and homeostasis(5). However, recent studies have highlighted the emerging role of the non-coding genome in visual function and its involvement in pathological conditions. Thanks to advances in sequencing technologies, the "central dogma" of molecular biology that RNA acts principally as an intermediate molecule between DNA and proteins has been challenged. Around 80% of the mammalian genome is actively transcribed into RNA that is not translated into functional proteins. A significant fraction of the "untranslated RNA" codes for regulatory non-coding RNAs (ncRNAs) whose role in gene expression and modulation of fundamental biological processes is being increasingly appreciated. NcRNAs are a heterogeneous group of molecules that are divided into two different classes based on their length: long ncRNAs and small ncRNAs. The best characterized small ncRNAs are microRNAs (miRNAs), endogenous, regulatory RNA molecules around 25 nucleotides in length that exhibit extensive evolutionary conservation(6). Their biogenesis (Figure 1) is a complex multistep process(7) that begins with the transcription of a primary transcript (pri-miRNA), which is cleaved in the nucleus by Drosha and DGCR8 to generate a ~60–70 nt stem loop termed the miRNA precursor (pre-miRNA)(7). The pre-miRNA is actively exported to the cytoplasm where it is further cleaved by the RNase III endonuclease Dicer and the double-stranded (ds) RNA binding protein TRBP, forming a miRNA duplex of approximately 22 nt (miRNA/miRNA*). One of the two strands forming the duplex, defined as the "guide" miRNA, is incorporated into the RNA-Induced Silencing Complex (RISC), while the complementary strand (miRNA*), termed "passenger", is usually, although not always, degraded. MicroRNAs can direct RISC to downregulate gene expression via two different mechanisms: mRNA cleavage/degradation or translational repression. If there is perfect complementarity between the miRNA and its target mRNA, the latter will be cleaved. In the case of imperfect pairing, the miRNA will cause degradation and/or decrease the translation efficacy of its target mRNA. The recognition of these target sequences, preferentially located in the 3′-untranslated regions (3'-UTR), relies on a 7–8 nt region called the "seed region" in the mature RNA sequence, usually found at positions 2–7 from the miRNA 5'-end(8). By regulating gene expression at the post-transcriptional level, miRNAs control different biological processes in both physiological and pathological conditions(9). It has been demonstrated that each miRNA can simultaneously target a wide number of genes(8,10). The reference repository for miRNAs, i.e., miRbase (https://mirbase.org/), catalogues all published miRNA sequences along with their related annotations(11). Currently, it contains information on 1,917 human miRNAs, and recent studies suggest that many more remain to be identified, particularly those that display tissue-, cell-, and time-specific expression. In this context, miRNAs are important for the development and homeostasis of the retina, and deleterious variants in miRNAs or in their target genes contribute to a range of retinal disease (12,13). In this review, we will describe how miRNAs control retinal health and how they are involved in disease, with a primary focus on Inherited Retinal Disorders (IRDs). In addition, we will highlight the potential role of miRNAs as gene agnostic therapeutic tools for the treatment of IRDs.

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The miRNome in the homeostatic retina

a) Intracellular miRNA expression: Several studies have reported the important role of miRNAs in both developmental processes and in controlling homeostatic functions in the retina (9,14). Indications that miRNAs are important mediators of retinal homeostasis came from studies showing that *Dicer* inactivation within the retina results in progressive loss of photoreceptor-mediated responses to light and subsequent degeneration(15). Early loss of *Dicer* was also associated with severe alterations of RGC axon pathfinding along with a microphthalmia phenotype, highlighting the role of miRNAs in controlling correct axon guidance decisions at the midline(16). Later inactivation of *Dicer* in specific retinal cell types was also linked to significant dysfunction. Indeed, conditional KO (cKO) of *Dicer* in mouse mature rod photoreceptors was accompanied by progressive retinal degeneration(17). A cone photoreceptor-specific *Dgcr8* cKO mouse model showed a progressive loss both of cone outer segments and photopic ERG responses(18). These latter data were subsequently confirmed in another study in which the deletion of *Dicer* in cones using a Chrnb4-cre; $Dicer^{flox/flox}$ conditional knockout mouse caused the degeneration and death of this cell population(19). The observation that the absence of *Dicer* is associated with progressive retinal cell type degeneration clearly indicates that the miRNA machinery is required to ensure the proper function of this tissue, raising the possibility that alterations in specific miRNAs might be associated with pathological processes. To unravel the role of individual miRNAs and the regulatory pathways they govern in both healthy and diseased retina, it was essential to define the retinal miRNA repertoire (miRNome), i.e., identify the miRNAs that are expressed within this tissue. The first studies of the mammalian eye miRNome were conducted in 2006, analyzing the corneal epithelium, lens, ciliary body, and retinal tissues from adult mice using different techniques, including miRNA expression arrays, Northern blot analysis, and RNA *in situ* hybridization(20). This study found that miR-184 expression is higher in both corneal and lens epithelia compared to other retinal tissues and remains stable regardless of the proliferative status of the corneal epithelium(20). The tissue-restricted expression of miRNAs was confirmed by another study, which compared the miRNA expression profiles in adult mouse retina, brain, and heart(21). Transcriptomic analysis revealed the presence of 78 miRNAs that were significantly expressed in the retina as compared to the other examined tissues. Moreover, by RNA *in situ* hybridization, the authors found a polycistronic miRNA cluster that includes miR-96, miR-182, and miR-183 to be preferentially expressed in photoreceptors, retinal bipolar cells, and amacrine cells. Finally, they highlighted a subgroup of 12 miRNAs that are involved in circadian rhythm regulation(21). Subsequently, Krol et al. demonstrated that the expression levels of a subset of microRNAs, including the miR-183/96/182 cluster, miR-204, and miR-211, are regulated by different light exposure levels in the mouse retina. In particular, these miRNAs are downregulated during dark adaptation and upregulated in light-adapted retinas, independently of the circadian rhythm(22). Recently, the role of miRNAs in responding to photo-stimulation has been investigated also in the human retina, using retinal organoids as a model. In this regard, Celiker et al. found that miRNAs are differentially expressed in response to light, show a rapid turnover, and also respond differently depending on light wavelengths(23).

In 2010, our group generated a comprehensive survey of miRNA expression in ocular tissues, including retina, lens, cornea, and retinal pigment epithelium (RPE), of the adult mouse eye using both microarray analysis and RNA *in situ* hybridization (24). These analyses were performed at embryonic stage (E)14.5, at postnatal day (P)0 (when retinal organization is not yet complete), at P8 (when the majority of retinal cells are present), and, finally at an adult timepoint, namely P60. Based on this study, 122 miRNAs were shown to display specific expression patterns in different eye tissues and at different stages of development(24). Subsequently, Simpson's group analyzed microRNAs in the retina and RPE/Choroid through deep sequencing analysis, showing the presence of miRNA isoforms called isomiRs that differ from the canonical sequence in terms of length and/or nucleotide substitution, in both the analyzed tissues(25). In 2016, our group used Next Generation Sequencing (NGS) (small RNAseq) to generate the most extensive catalog of miRNAs and isomiRs in the human retina(26). In addition, we studied the miRNome of the RPE/choroid and highlighted distinct miRNA enrichment patterns compared to the retina(26).

Although the overall miRNome in the eye compartment is well characterized, knowledge about miRNA expression in specific retinal cell types is still limited. It is well established that some miRNAs with restricted expression play important roles in specific cell types (Figure 2). For instance, conditional deletion in the RPE of miR-204 and miR-211, which are highly expressed in this tissue, results in retinal degeneration characterized by loss and dysfunction of photoreceptors and retinal inflammation(27). Along the same lines, it was demonstrated that the miR-183/96/182 cluster is highly expressed in photoreceptor cells and plays an important role in light adaptation, likely via modulation of some relevant target genes, such as *Arrdc3, Neurod4* and *Casp2*(28). However, only a few studies have focused on the analysis of miRNA expression profiles in specific retinal cell types under homeostatic conditions. One such study is that of Wohl and Reh, who isolated Müller cells from Rlbp-CreER: $Stop^{f/f}$-tdTomato mice and analyzed their miRNA content (Figure 2)(29). They identified seven miRNAs, including miR-204, miR-9, and miR-125–5p, with high expression levels in Müller cells, while other miRNAs, such as miR-124, were enriched in neurons and expressed at lower levels in Müller glia cells(29).

Therefore, the next challenge is to generate comprehensive retinal cell type-specific miRNome atlases, starting from homeostatic conditions. These atlases would be instrumental for dissecting the role that miRNAs play in retinal function and dysfunction. However, several technical issues need to be addressed to achieve this goal, beginning with the consideration that single cell NGS approaches are not yet suitable for miRNA detection. In this regard, although single cell RNA sequencing technique enables us to explore the transcriptome of individual cells, there are still some limitations in the detection of miRNAs, particularly those not displaying high expression levels. To overcome these issues, different complementary strategies have been devised, such as sorting different cell types from the tissue of interest by flow cytometry to better dissect their RNA content, including miRNAs(30) In the recent past, the Probe-Seq technique, which uses fluorescent gene-specific probe set to label specific RNA markers for subsequent flow cytometry isolation and RNA analysis was developed(31,32). Using the latter technique, the Cepko's group was able to isolate different subtypes of bypolar cells from adult mouse retina(31). However, more

efficient approaches and efforts are still needed for a more effective detection of miRNAs at the single cell level in the retina.

b) Extracellular miRNA component in the retina: In addition to the intracellular distribution of miRNAs, an important aspect of the retinal miRNome that is often overlooked but requires careful consideration is its extracellular component. miRNAs can circulate in a highly stable, cell-free form in various body fluids, through their association with RNA-binding proteins or their inclusion within vesicles. Packaging of miRNAs in vesicles occurs non-randomly, with some specific miRNA populations preferentially sorted into microvesicles(33). Secreted miRNAs play a role in regulating both physiological and pathological processes. Recent evidence has begun to shed light on the role of the exogenous transfer of miRNAs within extracellular vesicles (EVs) in the retina(34).

EVs are lipid-bound structures, ranging in size from 30 nm to 1mm that are being increasingly recognized as crucial mediators of intercellular communication. Depending on their size and biogenesis, they can be classified as exosomes, microvesicles, or apoptotic bodies(35). Exosomes are the smallest vesicles (30–100 nm) and are produced from late endosomes called multivesicular bodies (MVBs)(35). In particular, inward budding of the late endosomal membrane results in the generation of intraluminal vesicles (ILVs) that are released in the extracellular space through the fusion of MVBs with the plasma membrane(36). Microvesicles are more heterogeneous in size (0.1–1.0 μm) and shed by outward blebbing of the plasma membrane(37). Finally, apoptotic bodies range from 50 nm to 5 mm in diameter and are formed by the budding of plasma membrane in cells undergoing programmed cell death(38).

Importantly, EVs are heterogeneous populations with distinct cargoes including lipids, proteins, and nucleic acids, most notably miRNAs. Several studies have proposed that the transfer of exosomal miRNAs may modulate the biological functions of acceptor cells(39,40). Retinal Progenitor cells (RPCs) in culture were found to release EVs containing mRNA of transcription factors responsible for cell specification and multipotency, such as Pax6, Hes1, Ki-67, Sox2, and Nestin, as well as miRNA molecules with known roles in retinal development, such as Let7d, miR-9, miR-182, and miR-204(41). The first study describing a role for exosomes and their miRNA cargo as mediators of homeostasis within the retina was published in 2020 by Natoli's group(42). In this work, the authors demonstrated that EVs contain a set of uniquely enriched miRNAs compared to those found in the whole retina. Using a photo-oxidative damaged mouse retina, they showed that EV secretion is inversely correlated with photoreceptor survival(42). The same group used a multiomics integration approach to investigate the role of EVs in the communication pathways from photoreceptor cells to glial cells within the retina (Figure 3). Specifically, by integrating EV proteomics, miRNA Open Array, and small RNA sequencing in Muller glia and microglia, and retinal single cell RNA sequencing, they demonstrated that neuronal miRNAs are transferred to glial cells by EVs. During degeneration, this process occurs as a targeted response to modulate gliotic inflammation (34).

In 2021, the release of EVs from human retinal organoids was demonstrated(43). Using in-depth transcriptomic analysis, the RNA content within EVs was analyzed at three different

developmental timepoints: Day (D)42, D63 and D90. These timepoints corresponded to: (a) D42, the beginning of retinal neurogenesis, with the first ganglion cell precursors within the developing ganglion cell layer; (b) D63, a stage of active cell differentiation and migration involving ganglion, amacrine, horizontal, and photoreceptor cells; and (c) D90, a stage in which most precursor cells have reached their corresponding laminae. Transcriptomic analysis revealed the presence of miRNAs within EVs, with the highest number of miRNA species found at D42. The miRNA profile of EVs largely corresponded to the one found in the whole organoid, although a small number of miRNAs were detected exclusively in the EVs. Moreover, miRNA cargo-predicted targetome analysis showed the involvement of these EV miRNAs in mechanisms of retinal development. To confirm the ability of EVs released from retinal organoids to regulate gene expression in target retinal cells, the authors co-cultured EVs isolated from retinal organoids at D42 with multipotent human retinal progenitor cells (hRPCs). Differential expression analysis of hRPCs treated with retinal organoid EVs showed changes in gene expression and signaling pathways associated with the cell cycle, differentiation, retinal homeostasis, and neuroprotection, and showed significant overlap with the predicted targetome analysis. Altogether these results highlight the role of EV miRNAs in controlling post-translational modification and regulation of human retinal development(43).

Taken together, the above studies highlight the important role of miRNAs, both intracellular and extracellular, in maintaining homeostatic conditions. Detailed and comprehensive intracellular and extracellular miRNAs profiling will be fundamental for a better understanding of the molecular pathways that ensure retinal health and, consequently, their involvement in retinal dysfunction, either as primary or secondary factors during disease progression. Indeed, miRNAs have been already suggested to play a pathogenic role in multifactorial diseases affecting the retina, such as diabetic retinopathy and Age-related Macular Dystrophy(44–48). Here, however, we focus on the current knowledge on the involvement of miRNAs in the pathogenesis and progression of a specific group of retinal mendelian disorders, namely Inherited Retinal Diseases, as detailed below.

The miRNome in Inherited Retinal Diseases (IRD)

IRDs are a group of genetic disorders that cause visual loss due to photoreceptor and retinal degeneration. They are among the most frequent causes of blindness in the working age population, with a prevalence of approximately 1:3,000 individuals(49), representing a significant cost to the healthcare system. IRDs display a significant range of different phenotypes, varying in terms of cell-type/tissue involvement, disease onset, severity, and progression. Retinitis Pigmentosa (RP) is the most frequent form. Other forms include cone/cone–rod degeneration, Leber congenital amaurosis, and inherited macular dystrophies(50). IRDs are among the most genetically heterogeneous group of disorders in humans, with over 300 causative genes discovered so far. They can be transmitted through various inheritance patterns: autosomal recessive, autosomal dominant, X-linked, and mitochondrial(51,52)

Another important challenge in the IRD field is represented by the poor characterization of the underlying pathogenic events occurring downstream of the causative variant. Retinal degeneration due to photoreceptor (PR) cell death is a common outcome of IRDs. In most

IRDs, rod PRs are the first cells to be affected and die in the early stages of the disease. However, rod loss is almost invariably followed by secondary cone loss, which has a remarkable clinical impact and accounts for the visual disability observed in patients in late disease stages(53). The molecular mechanisms that underlie PR death remain poorly defined(54). This knowledge gap is an obstacle to the development of neuroprotective disease-gene independent approaches to slow down PR death processes that can be applied to a significant proportion of patients.

Although the most abundant cells that constitute the retina are neurons, other cell types, such as microglia and Müller cells, play an important role both in homeostatic conditions, for the maintenance of proper neural signaling, and during degeneration.

In this respect, at early disease stages, Müller glia are activated and release antioxidants and neurotrophic factors to preserve retinal function(55). However, prolonged gliosis is known to be detrimental as it exacerbates neurodegeneration and prevents regenerative processes(55). Additionally, one of the main causes of progressive retinal degeneration is represented by immune reactivity and subsequent inflammation(56,57). Active microglia cells can release pro-inflammatory factors such as reactive oxygen species (ROS), TNF-α, CCL2, and complement activators(58). Although this inflammatory response is required to promote tissue repair and to re-establish homeostasis, sustained microglial inflammatory responses have been implicated in severe alterations in retinal integrity and enhancing neuronal degeneration(59). MicroRNAs are able to simultaneously modulate multiple molecular pathways, regulating metabolism, cell death, angiogenesis, and inflammatory responses(60–62). As previously discussed, the importance of miRNAs in retinal physiology is evident from the impact of global impairment of miRNA biogenesis in specific retinal cell types and developmental stages. Hence, understanding their role in pathological contexts is expected to be instrumental for a better dissection of the molecular mechanisms underlying IRDs and for the identification of potential therapeutic targets.

The primary role of miRNAs in IRDs

A limited number of inherited ocular diseases have been described as being caused by dysregulation or sequence alterations of miRNAs. The heterozygous C-to-T transition (r.57C>U) within the seed region of miR-184 is responsible for familial severe keratoconus combined with early-onset anterior polar cataract(63). The authors showed that the mutant form of miR-184 fails to compete with miR-205 for overlapping target sites on the 3′ UTRs of the *INPPL1* and *ITGB4* genes. Furthermore, the same variant was reported to cause EDICT syndrome, characterized by endothelial dystrophy, iris hypoplasia, congenital cataract, and stromal thinning(64). More recently, genetic variants in the sequence of miR-184 have also been associated with Fuchs endothelial corneal dystrophy (FECD)(65).

However, the only example of a causative role of a miRNA in IRD in humans is a point variant in the seed region of miR-204, which is responsible for an autosomal dominant syndromic form(66). Through a combined linkage and whole exome sequencing approach, it was demonstrated that the n.37C>T variant in miR-204 segregated with RP and iris coloboma in a five-generation family. *In vitro* assays showed that the n.37C>T variant did not affect either the secondary structure or the biogenesis of the miRNA. The variant

acted by disrupting the targeting capabilities of miR-204, both by impairing the recognition of some natural targets and, above all, by generating many novel and aberrant targets. Further confirmation of the causative role of this variant came from subsequent reports that described the association of the same miR-204 variant with RP and iris coloboma, early-onset cataracts, and congenital glaucoma in two additional families(67). In conclusion, miR-204 represents the first example of a miRNA with a primary pathogenic role in IRD.

Several factors may explain the still limited number of miRNAs with a causative role in human mendelian diseases even when using comprehensive genomic NGS-based diagnostic approaches, such as Whole Exome Sequencing (WES) and Whole Genome Sequencing (WGS). First, it is expected that, given their short size, the number of potential pathogenic variants affecting miRNAs will be significantly lower as compared to those found in protein coding genes. Second, since each miRNA can target hundreds of different genes, it is likely that the phenotypic effect of sequence variants, even in the same miRNA, may be linked to different phenotypes, depending upon the target genes specifically affected by each sequence alteration. The latter phenotypic heterogeneity is expected to make more complex pathogenicity interpretation processes. Third, variants altering miRNA function can be localized not only within their mature sequences but also in their target gene binding sites, particularly in their 3′-UTR, as already reported(68).The identification of pathogenic variants altering miRNA binding sites still represents a complex task when performing WES or WGS analyses due to the uncertainty in miRNA target recognition processes when relying exclusively on *in silico* tools. A more precise evaluation of the putative pathogenic role of sequence variants located in 3′-UTRs of mRNAs will benefit from the availability of more accurate information on miRNA target recognition also involving systematic experimental validation efforts. Overall, the recognition of the pathogenic role of miRNAs in IRDs, and in human genetic disorders in general, requires a more careful attention to non-coding parts of the human genome in WES and WGS data interpretation pipelines.

The results of the studies described in the next section add further support to the fact that the current knowledge on the pathogenic contribution, either primary or secondary, of miRNAs to IRDs is still limited.

Alterations of miRNA expression profiles in IRDs

Several studies have highlighted the aberrant expression profiles of miRNAs in IRD models (Figure 4). The first assessment of global miRNA expression profiles in a mouse model of RP was performed in 2007, using microarray and quantitative RT-PCR approaches to compare transgenic mice carrying the Pro347Ser variant in the *RHO* gene (RHO-P347S) with WT animals(69). The authors found an alteration of miRNA expression profiles in the retina, with miR-96 and miR-183 significantly downregulated while miR-1 and miR-133 were upregulated in mutant retinas(69). These data were confirmed in another study from the same group, showing a common signature in four models of RP with decreased levels of miR-96, -182 and -183 and upregulation of miR-1, -133 and -142 in both whole retina and isolated rod photoreceptor cells(70). The potential functions of the above miRNAs were explored in a different study where Palfi et al. combined proteomics data with *in silico* target predictions to identify candidate miRNA-mRNA pairs and validated

their interactions *in vivo*(71). miRNA-mRNA pairs were then associated with cellular regulatory circuits, some of which included signal transduction, synaptic transmission, cell-adhesion, and transmembrane transport(71). In the rd10 mice, a well-established model of autosomal recessive RP due to a spontaneous point variant in the β subunit of the rod phosphodiesterase gene (*Pde6β*), variations in the expression profiles of miRNAs have been reported(72). Here, the overall expression of miRNAs and mRNAs in different stages of the disease were evaluated, with analysis of the tissue immediately before and after the onset of photoreceptor death, namely P15 and P17, respectively. Based on this analysis, 17 miRNAs were found to be upregulated, with miR-6240 and miR-6970 displaying the highest changes, whereas only miR-20b-5p and miR-19b-3p were downregulated. miRNA–mRNA interaction networks were constructed with both differentially expressed miRNAs and their target mRNAs. This analysis predicted a possible involvement of the above miRNAs in the regulation of basic retinal processes, such as apoptosis and inflammation, both of which take place in the rd10 retina(72). Although there is a growing list of miRNAs that are known to be differentially expressed in models of retinal disorders, a deeper analysis of miRNA expression profiles in other models and at different stages of RP is required. Understanding the role of miRNAs in controlling pathways that are commonly dysregulated during retinal degeneration could help to better dissect the molecular mechanisms underlying the disease and also to design effective gene-independent therapeutic approaches. Moreover, a deeper analysis of miRNA expression at the single cell level will be essential to better understand the role of each cell type in the initiation and progression of the disease. This knowledge can be exploited for the design of specific miRNA-based therapies targeting molecular pathways in specific cell types in pathological conditions. In the same direction, the analysis of miRNA profiles at the extracellular level would shed light on the intercellular crosstalk within the retina, uncovering the involvement of specific cell-cell communication axes in the control of both homeostatic and disease conditions, and providing other potential therapeutic targets for the design of gene- and mutation-independent strategies (see next section). Finally, it would be of the upmost importance to perform miRNA expression analyses in human models of IRDs by exploiting induced pluripotent stem cells (iPSC) either patient-derived or appropriately engineered.

MiRNAs as potential therapeutic targets for IRD

Modulation of miRNA expression represents a potential therapeutic strategy in different human diseases. The major strength of such an approach originates from miRNA regulatory activity. Due to the large number of mRNA targets, miRNAs can simultaneously modulate vast regulatory networks involved in the maintenance of essential cellular functions. In this regard, the use of miRNA modulators, such as mimics and inhibitors, in preclinical studies, already showed promising results in cancer, cardiovascular diseases, and metabolic disorders. For example, the small molecule drug Obefazimod, which enhances miR-124 expression, is being considered for the treatment of moderately to severely active ulcerative colitis (UC) and Crohn’s disease (CD) (73,74)while LNA-i-miR-221 inhibits miR-221 and is being evaluated for the treatment of Refractory Multiple Myeloma patients and advanced solid tumors (NCT04811898)(75).

Recently, increasing evidence shows that miRNA modulation can exert a beneficial role in the treatment of IRDs, whose genetic heterogeneity represents a limitation for the application of gene-dependent therapeutic strategies. Despite the clinical success of *Voretigene Neparvovec* (Luxturna) in the treatment of Leber Congenital Amaurosis (LCA) (76), gene-replacement therapies cannot be applied to IRDs caused by a gain-of-function mechanism. Furthermore, such approaches are not readily applicable for large numbers of patients that have different causative sequence variants. Consequently, current alternative efforts are directed to develop strategies that are able to modulate common dysregulated pathways that are downstream the primary genetic defect. Specifically, these mutation-/gene-independent interventions are designed to target pathological mechanisms that govern photoreceptor cell death, oxidative stress, and neuroinflammation. In this scenario, miRNAs emerge as an attractive therapeutic target, which may confer neuroprotection during retinal degeneration to a large fraction of patients (Figure 5). Below, we report some of the published evidence in support of the possible therapeutic use of miRNA modulation (either silencing or overexpression) in IRDs.

It is known that the miR-181a/b family is highly expressed in the retina and has a role in axon growth and mitochondrial function (77,78). An example of a miRNA silencing-based strategy was reported in a study from our group, in which the functional consequences of miR-181a/b downregulation in the retina of IRD models were analyzed. We showed that adeno-associated viral vector (AAV)-mediated subretinal injection of miR-181a/b-silencing “sponges” exerted protective effects in two genetically different mouse models of RP, i.e. the already mentioned autosomal dominant RHO-P347S and autosomal recessive rd10 models(79). Specifically, delivery of the miR-181a/b “sponge” under the control of both the constitutive *CMV* promoter and the rod photoreceptor-specific RHO promoter led to improved retinal morphology and visual function. This neuroprotective effect was partially associated with amelioration of mitochondrial function. It has been demonstrated that mitochondrial dysfunction is one of the main pathological signatures in different forms of IRDs(77).

In the same direction, downregulation of miR-6937 by AAV-mediated subretinal injection of a miRNA inhibitor in the rd10 mouse resulted in a delay of photoreceptor degeneration(80). Specifically, photoreceptor function and structure were improved, as indicated by preserved electrophysiological responses and histological analysis. Microarray analysis identified 101 differentially expressed mRNAs, some of which, involved in pro-survival pathways, being upregulated. Surprisingly, another subset of upregulated genes was reported to play a role in pro-apoptotic signaling. Further studies are needed to understand the protective effects of miR-6937 in IRD mouse models.

Inflammation is known to be a common feature of retinal diseases. miR-155 and miR-146a represent well characterized examples of immunomodulatory miRNAs. Their role has been established in many inflammation-related conditions(81,82), whereas in retinal diseases, their precise role has not been fully elucidated. Previously, it was observed that the expression of miR-155 and miR-146a is increased in different models of retinal degeneration. To evaluate the possible benefits resulting from their inhibition, downregulation of both miR-155 and miR-146a was investigated using miRNA inhibitors in

a rodent photo-oxidative damage model(83). Combinatorial downregulation of miR-155 and miR-146a attenuated retinal function and enhanced inflammatory responses. When analyzed independently, only miR-155 suppression yielded beneficial effects on the degenerating retina, as revealed by increased photoreceptor viability and reduced inflammation. In addition, similar neuroprotective effects were also observed in a miR-155 knock out mouse model.

By contrast, the overexpression of some miRNAs can exert a protective action on photoreceptor death, and hence represents a potential therapeutic approach for IRDs, as in the case of miR-204. This miRNA is involved in the development and maintenance of photoreceptor cells and is required for the proper function of the RPE. As described above, a sequence variant in miR-204 is responsible for autosomal dominant forms of RP associated with coloboma and other eye anomalies(13). To assess the protective effects of miR-204 upregulation in IRDs, we overexpressed it in the retina of the RHO-P347S and *Aipl1*$^{-/-}$ mouse models using AAV-mediated subretinal administration(84). miR-204 overexpression preserved retinal function and increased photoreceptor survival in both models. In addition, comparative transcriptomic analysis revealed that miR-204 delivery shifted transcript profiles toward those of a healthy retina by downregulating degeneration-induced pathways such as innate immunity, inflammation, and cell death. This led to the hypothesis that the protective response mediated by miR-204 could be due to an attenuation of microglia activation(84).

Recently, by exploiting the results of an *in vitro* high-content imaging (HCI) miRNA screen in 661W cells, our group identified miR-429 as an additional potential therapeutic candidate(85). To determine its efficacy *in vivo*, an AAV-miR-429 viral construct was administered to the subretinal space in the RHO-P23H mouse model. Overexpression of this miRNA slowed down retinal degeneration and improved retinal function. In addition, transcriptomic data highlighted downregulation of pathways associated with inflammation and innate immune responses, suggesting a potential immunomodulatory mechanism of miR-429.

Despite current progress in miRNA target screening technologies and promising results obtained in preclinical studies, miRNA-based interventions still face a number of limitations. Specifically, off-target effects as well as adverse immune response activation represent major safety concerns associated with the use of miRNA modulators in the clinic. Until now, this issue has been addressed by targeted delivery and chemical modification of miRNA modulators, which help to improve miRNA specificity and decrease its immunogenic potential. AAV2 vectors, which can transduce various retinal cell types and induce low immunogenicity, are most frequently used to obtain retina-specific delivery. In addition, targeting the retina with these vectors has several other advantages. Their small size, compartmentalized structure, and the presence of the blood-retinal barrier allows for low therapeutic doses and ensure tissue specificity. In addition, it is important to provide optimal administration routes. While, as shown in the above preclinical studies, subretinal injection has proven to be effective, it may be necessary to provide less invasive techniques in the future, such as intra-vitreal or, even better, eye drop-based delivery.

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Undoubtedly, miRNA-based strategies hold great promise for IRD therapy: however, their development is still at an early stage. Studies on their safety and optimization of delivery systems are necessary to successfully exploit the clinical potential of miRNA modulators.

Concluding remarks and future perspectives

MiRNAs play an important role in controlling developmental processes and participate in the maintenance of homeostasis within the retina, thanks to their ability to simultaneously modulate multiple pathways underlying different physiological processes. Several recent studies aimed at characterizing the miRNA expression profile in the whole retina, both in homeostatic and in pathological conditions, and the primary pathogenic role of sequence variants in these noncoding RNAs in IRD is also emerging. Finally, the expression modulation of miRNAs holds great promise for the development of gene agnostic therapeutic approaches that could be applied to a relevant fraction of IRD patients either alone (to slow down disease progression) or in combination with gene-specific procedures (to enhance the efficacy).

However, several challenges need to be tackled both for a better comprehension of the overall contribution of miRNAs to retinal physiology and for their effective exploitation as neuroprotective therapeutic agents. Firstly, despite the extensive characterization of miRNA expression in the whole retina, there is still a significant lack of information regarding the distribution of miRNAs at the single cell level. Obtaining this information will contribute to a better understanding of the molecular pathways in which retinal miRNAs are involved and pinpoint the best cellular targets, as well as the optimal therapeutic strategies, to slow down retinal degeneration. Secondly, there is still an important knowledge gap concerning the contribution of extracellular miRNAs to retinal physiology and disease. In particular, there is little or no information about the role of EV miRNAs in IRDs. One approach to address this issue would be to carry out further expression analysis and modulations in appropriate IRD mouse models and/or in human retinal organoids. This analysis would help to define the intercellular crosstalk within the retina during degeneration. Thirdly, concerning the potential therapeutic exploitation of miRNAs, a fundamental goal would be that of devising effective and “user-friendly” delivery strategies to the retina. So far, convincing proof-of-principle results have been obtained using subretinal injection of AAV constructs. However, alternative strategies based on the use of oligonucleotides, either naked or enclosed in nanoparticles or in EVs, which could be injected by a less invasive route (intravitreally or as eye drops) are predicted to significantly enhance the translational potential of such therapeutic avenues. Finally, although the available data are quite reassuring, additional evidence in support of safety and lack of serious side effects from the expression modulation of miRNAs in IRDs is definitely needed. Altogether, we can conclude that a deeper analysis of miRNAs within the retina is essential to better understand the mechanisms underlying IRDs as well as to identify the cellular and molecular changes that lead to degeneration and, finally, to design the best therapeutic strategies to tackle the disease.

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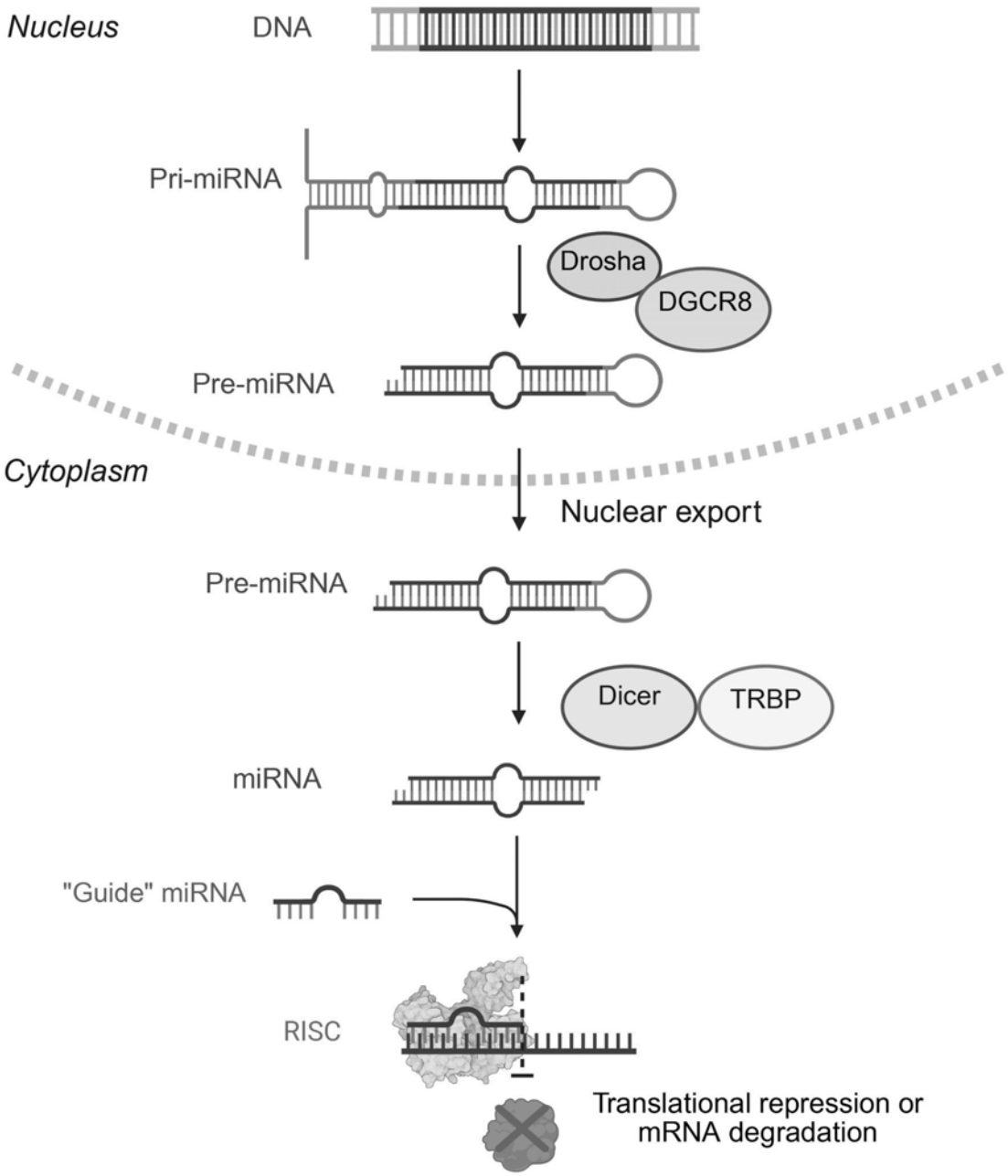

Figure 1. Biogenesis of miRNAs.
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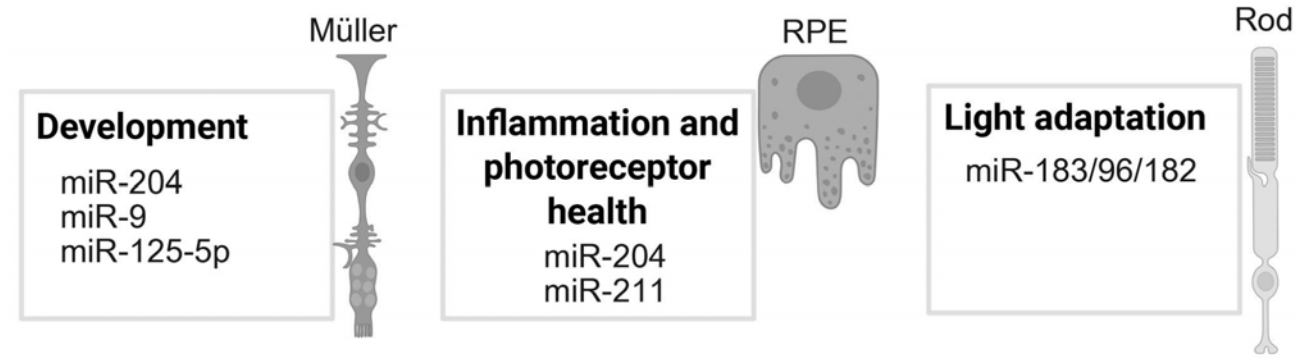

Figure 2. Example of miRNAs with relevant functional roles in specific retinal cell types.
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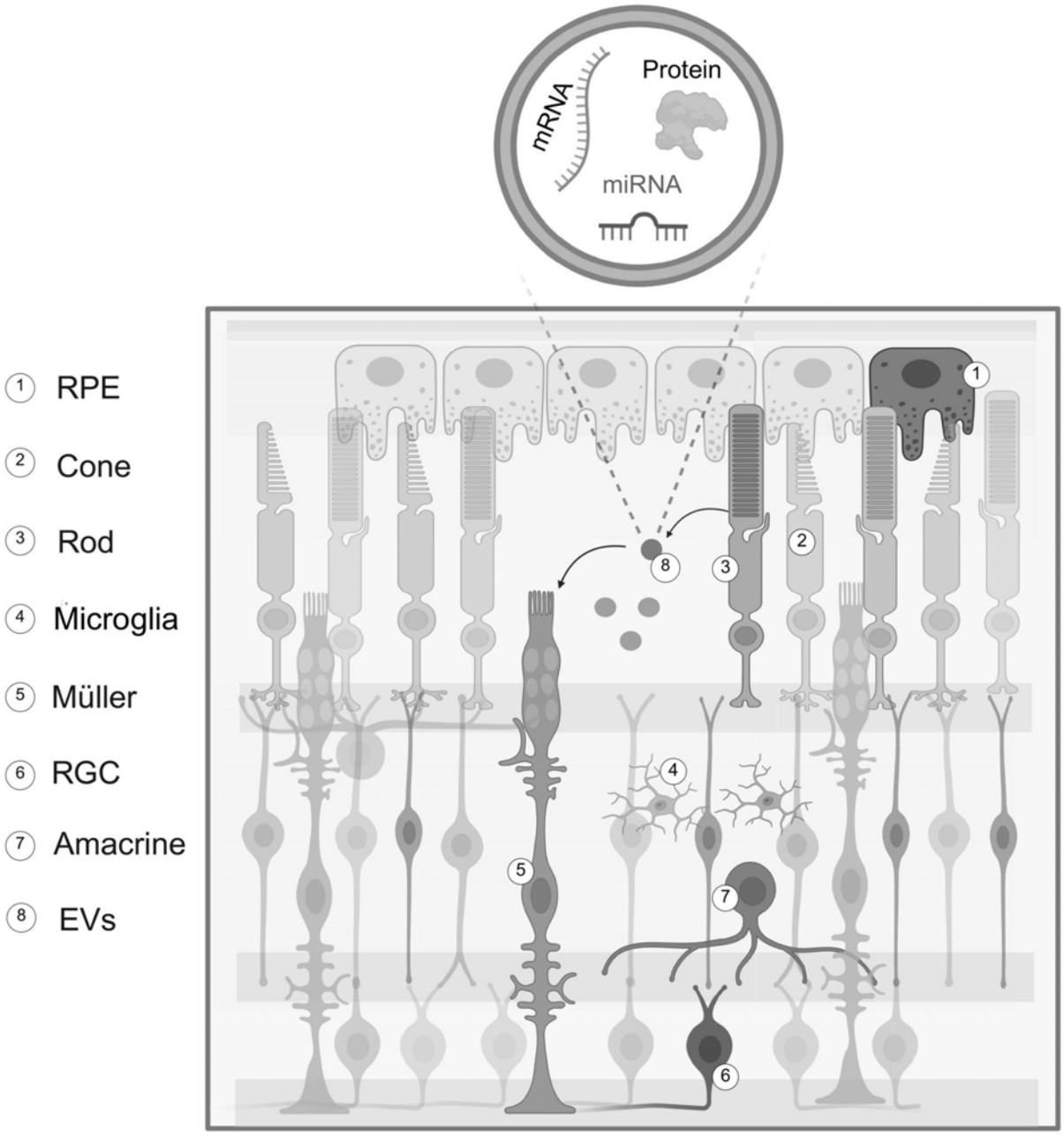

Figure 3. miRNA exchanges by EVs mediate intercellular communication in the retina.
EVs display a set of uniquely enriched miRNAs compared to the whole tissue. The exchange of miRNAs between photoreceptor and Muller cells, in response to damage, controls the inflammatory response. Created with assistance of BioRender. https://BioRender.com/wxmenvx

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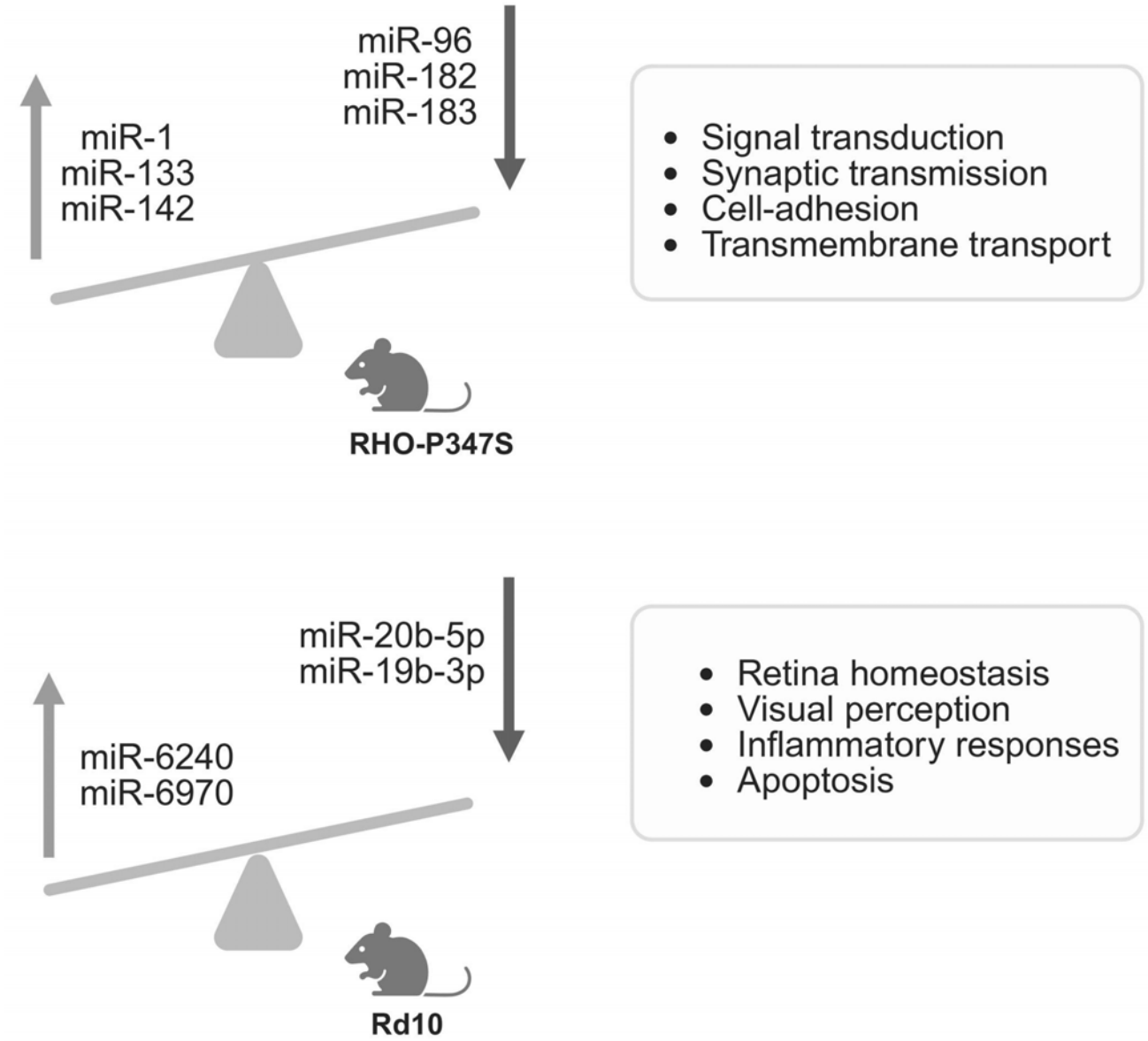

Figure 4. Changes in miRNA expression between healthy and degenerating retina in IRD mouse models and potential cellular pathways affected.

(Top) Analysis of miRNA expression levels comparing WT and RHO-P347S mouse models revealed upregulation of miR-1, miR-133, and miR-142, and downregulation of miR-96, miR-182, and miR-183. (Bottom) Analysis of miRNA expression levels in WT and Rd10 mouse models revealed upregulation of miR-6240 and miR-6970, and downregulation of miR-20b-5p and miR-19b-3p. The right panels show pathways associated with dysregulation of these miRNAs. Created with assistance of BioRender. https://BioRender.com/wxmenvx

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miRNA modulation strategy	Mouse model	Therapeutic effect
miR-181-sponge ↓	RHO-P347S; Rd10	Improved retinal morphology and function, ameliorated mitochondrial function (70)
anti-miR-155 ↓	C57BL/6J, miR-155-KO mice after photo-oxidative damage	Increased photoreceptor cells survival, reduced inflammation (74)
anti-miR-6937 ↓	Rd10	Improved retinal morphology and function (71)
AAV-miR-204 ↑	RHO-P347S; Aipl1 KO	Increased photoreceptor cells survival, reduced inflammation (75)
AAV-miR-429 ↑	RHO-P23H	Improved retinal function, reduced inflammation (76)

Figure 5. miRNA-based therapeutic strategies and their effects in different pre-clinical IRD mouse models.

Therapeutic effects have been observed after miRNA-204 and miRNA-429 overexpression and after downregulation of miRNA-181a/b, miR-155 and miRNA-6937. In the mouse models analyzed, retinal degeneration was induced either by genetic mutations (RHO-P347S, Rd10, miR-155KO, *Aipl1*$^{-/-}$, RHO-P23H) or by external conditions (photo-oxidative damage). Modulation of these miRNAs leads to improvement of specific retinal functions. Created with assistance of BioRender. https://BioRender.com/wxmenvx