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View on microRNAs as a potential tool to fight blindness: focus on Müller glia and gliosis

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The neural retina is a part of the central nervous system. As it lacks regenerative capacity, in an event of injury or disease, neuronal loss leads to visual impairment and often to blindness. Moreover, Müller glia (MG), the predominant glia in the retina, undergo a variety of molecular and cellular changes and discontinue to carry out their important regular functions in the tissue (e.g., maintaining tissue homeostasis and nurturing neurons). They form the glial scar, a barrier that prevents any form of regeneration and represents a big obstacle for regenerative medicine, in particular for transplantation approaches (Bringmann et al., 2006). For decades, researchers have studied the processes of neurodegeneration and gliosis in the retina, which are very similar to the degenerative events in other brain areas. To this end, urgent questions are: What is happening in MG during gliosis and can the glial scar be prevented or reversed, and if so, how. MG have been monitored and described in different injury/disease models including acute and chronic phases. Various cell markers, cell signaling molecules and pathways have been identified. However, in order to understand which factors are regulating the expression of these molecules, transcriptional and translational regulators need also to be included. A molecule group that gains more and more importance, not only in understanding the regulatory network of particular cellular processes, but also in manipulating them, are microRNAs (miRNAs). miRNAs are inhibitors of protein translation. They bind to mRNA molecules and induce either their destabilization or their decay. Since miRNAs regulate cell cycle events as well as cell death, they play a significant role in cell degeneration and predominantly in cancer (64,345 PubMed entries from 2002–2021). In cancer research, miRNAs did not only gain substantial importance as biomarkers for diagnostics, there is also an increasing interest in the pharmaceutical industry of this "young and relatively immature field of utilizing miRNAs as a therapeutic tool", with first clinical trials on their way (Bonneau et al., 2019).

We believe that miRNAs represent a very promising tool for treating retinal diseases for the following reasons: (1) miRNA levels change rapidly in effected cells/tissue before the onset of cellular (pathophysiological) changes which makes them attractive biomarkers/early indicators. (2) miRNAs target several mRNAs and can be more potent in altering cellular processes than a single protein or monogenetic compound. Since human diseases are very complex, manipulation of gene networks simultaneously might be required. (3) miRNAs can be used to rescue cell/tissue state. (4) They can easily be delivered into a cell, at desired concentrations as well as in various combinations. (5) Some miRNAs are cell type-specific and therefore probably responsible for MG-specific functions including gliosis. (6) Most miRNAs are conserved across all species and will allow knowledge transition from experimental mouse studies to human clinical applications. Although miRNAs are assigned key players in gene regulation, not much is known about their role in physiological glia cell state and gliosis. Here I highlight MG miRNAs and their potential impact on MG function including gliosis, methods to study MG miRNAs, how they could be used as a therapeutic tool by attenuating gliosis or as a reprogramming factor, and lastly, the challenges and downsides of miRNAs as a tool.

miRNAs and MG function: We already know that MG miRNAs are required for MG function and overall retinal health. These insights were obtained from an MG-specific Dicer deletion study in mice. Dicer is the enzyme that produces the mature miRNA from its precursor form. Therefore, deleting Dicer leads to a decline of mature miRNAs in the cell of interest, in our case MG. After the loss of miRNAs, the glia formed aggregations and a glial seal, a characteristic of glia during retinal remodeling (**Figure 1**; Wohl et al., 2017). Retinal remodeling is a process that occurs during retinal degenerative diseases such as age-related macular degeneration or retinitis pigmentosa (RP), two well-known eye diseases with no available cure. The fact that the loss of miRNAs in MG (without injuring a neuron) led to this pathological phenotype, implied fundamental importance of MG miRNAs for a healthy retina and provided new perspectives about retinal disease origination. One form of RP is believed to originate from MG degeneration. An injury model for drastic retinal degeneration, as it occurs in age-related macular degeneration or RP, is light damage, also called photo-oxidative damage. In mice, only a few days after bright white light exposure, the vast majority of photoreceptors die and MG become reactive (glial fibrillary acidic protein upregulation) and form the glial scar. A comparison of the miRNA profile one week after light damage with that obtained one month after Dicer deletion, revealed surprising similarities. Four genes, namely *Atf3*, *Egr2*, *Gadd45b*, and *Maff* were found upregulated in both reactive MG and Dicer-deleted MG and were identified as MG-miRNA targets (Kang et al., 2020). These four genes have been reported to be involved in stress responses and were found in reactive glia. Therefore, the regulating miRNAs of these genes could represent key regulators of a generic stress response that is independent from the nature of the insult or disease. Although more supporting data is required to prove the hypothesis that these miRNAs indeed regulate gliosis, attenuating a generic glia stress response and manipulating gliosis would have a substantial impact on clinical applications and regenerative medicine.

Methods to study miRNAs in MG: To profile miRNAs in MG, the Nanostring platform can be used, a well-accepted, highly sensitive barcode technique that allows quantification of mature miRNAs without an amplification bias (Wohl et al., 2017, 2019; Kang et al., 2020). Although limited to about 800 defined miRNAs, this method is reliable and robust, and permits profiling miRNAs from degenerated samples, which makes it an attractive tool for the analysis of human (postmortem) samples. Another method for miRNA profiling (including primary and precursor miRNAs) is miRNA-Seq, which is similar to conventional bulk RNA-Seq, and allows identification of all miRNAs present in a sample. This technique is therefore broader and might even lead to the

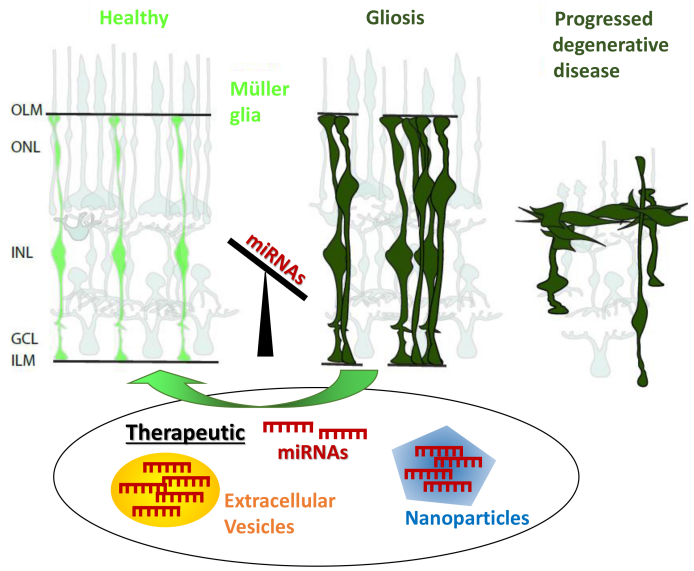

Figure 1 | **Suggested role of MG miRNAs therapeutic tools for eye diseases.**
MG are the predominant glia in the retina, span the entire tissue from ILM to OLM, and are required for overall retinal health. Injury/disease leads to changes in MG function. They undergo gliosis and form a glial seal in progressed retinal degenerative diseases (during retinal remodeling). In mice, reactive MG have reduced levels of their normally expressed set of miRNAs resulting in changes of gene expression. Supplementation with miRNAs can restore imbalances of miRNAs levels in MG and rescue the tissue. Extracellular vesicles or nanoparticles might restore MG function *in vivo*, could counteract further degenerative processes and represent a promising therapeutic tool. GCL: Ganglion cell layer; ILM: inner limiting membrane; INL: inner nuclear layer; MG: Müller glia; OLM: outer limiting membrane; ONL: outer nuclear layer. Adapted from Wohl et al., 2017.

identification of new miRNA candidates but suffers from amplification bias, which can lead to misinterpretations of gene expression. The combination of miRNA-Seq and Nanostring however, would be the best strategy for in-depth profiling, but this is very costly. Since MG display cell heterogeneity, single cell (sc) miRNA-profiling would be necessary for in-depth analyses. As of now, however, and to my knowledge, there is no sc-miRNA-Seq technique commercially available yet. Obtaining miRNA-Seq data from 10x Genomics scRNA-Seq is not possible since this method is based on mRNA poly-A tail capture, and mature miRNAs have no poly-A tail. Nevertheless, an astonishing recent publication reported the establishment of a technique to analyze both miRNA and mRNA levels of the same cell (Wang et al., 2019). The commercialization of this technique will be a break-through in miRNA research and would allow miRNA and mRNA analysis at single cell level in the exact same cell. By then, the only way to find the potential miRNA targets is by measuring mRNA levels of either predicted or known miRNA targets or to analyze whole mRNA profiles (e.g. via RNA-Seq, microarrays or qPCR) and validate miRNA:mRNA interaction via reporter assays, miRNA overexpression/inhibition assays and cross-linking or RNA immunoprecipitation assays (CLIP-Seq/RIP-Seq).

miRNAs as a potential therapeutic tool to attenuate gliosis: It has been shown that imbalances such as the loss of all miRNAs in MG, which is causing a pathological phenotype, can be adjusted by replenishing the lost miRNAs. Several miRNAs can be administered in combination and at various desired concentrations. This does not only apply for the actual artificial miRNAs (called mimics) but also for miRNA inhibitors (i.e., antimiRs or antagomiRs) and/or combinations of both (Wohl et al., 2019). These molecules can be delivered via transfection into MG in primary cultures as well as into MG in retinal explants (Wohl et al., 2017, 2019). First successful *in vivo* deliveries have also been reported (Chu-Tan et al., 2020). This is promising for attempts to attenuate gliosis *in vivo*. Other methods proposed for *in vivo* miRNA delivery into MG are extracellular vesicles (Wooff et al., 2020) and nanoparticles (Pi et al., 2018) that can harbor combinations of miRNAs or miRNA inhibitors and are already tested in cancer research. Extracellular vesicles are not cell type specific but the MG are probably the easiest cells to target in the retina based on their morphology and location in the tissue. MG span the entire retina with their processes, form the inner and outer limiting membrane and therefore, offer an excellent target surface for molecule delivery (**Figure 1**). Furthermore, even if an engineered MG-specific carrier would be required for targeted cell-therapy, specific cell surface proteins would allow the implementation of an MG-specific (or any other cell type) specific nanoparticle. Extracellular vesicles/nanoparticles can be delivered to the eye via intravitreal or subretinal injections (Wooff et al., 2020). A non-invasive application such as eye drops would be more ideal and desired, but is probably unrealistic. Since several layers of the eye need to be penetrated to reach the retina, most of the cargo would be lost *en route*. Nevertheless, although it is still a very long and stony way off to develop an miRNA-based therapeutic agents, first steps have been taken. Attenuating MG gliosis would not only slow down secondary neuronal loss and inflammation but also create a more permissive environment for regenerative events.

miRNAs for MG reprogramming in regenerative medicine: Another comprehensive hot topic, that cannot be discussed in detail in this perspective but should be briefly mentioned, is, MG reprogramming. MG represent a natural, endogenous source for retinal regeneration in the fish eye. Involved in this regeneration process are miRNAs, regulating the distinct phases of MG de-differentiation into retinal progenitor cells that can give rise to all six neuron types found in the retina (Konar et al., 2020). Although mammalian MG lack this capacity, they can be reprogrammed by overexpressing proteins such as transcription factors, miRNAs, or a combination of both. miRNAs highly expressed in retinal progenitor cells or neurons can reprogram MG into retinal progenitor cells that adopt neuronal morphology *in vitro*. Most reprogrammed cells displayed bipolar cell characteristics similar to the MG reprogrammed with transcription factors (Wohl et al., 2019). However, whether these neuronal-like cells can be functionally integrated in the mature retina has not been reported yet.

Challenges and downsides of miRNAs as tools: Since miRNAs target several genes, they might also affect genes that are not desired to be affected. The miRNA research is still a very young field, most underlying mechanisms are not yet known and the miRNA regulatory network is extremely complex. This complexity is based on the fact that miRNAs have different half-lives, can bind in canonical and non-canonical ways, and can have multiple binding sites on one single mRNA molecule. Moreover, they are also regulating gene expression indirectly since some of their targets are transcription factors as well as molecules that regulate chromatin accessibility such as histone deacetylases or high motility group proteins (Wohl et al., 2019). This can be beneficial but also might be detrimental, especially since MG have many essential functions in the retina (Bringmann et al., 2006). Nevertheless, because of their importance and their complexity, there is a progressive development of new tools. Conventional computational miRNA:mRNA binding site prediction tools have been improved by linking them to CLIP-Seq data to show true interaction and can also reveal associated signaling pathways or general biological processes, across various species. In the past 10 years, a significant number of miRNA products have been invented and launched including visualization, quantification and profiling tools, artificial miRNA molecules/inhibitors, a variety of miRNA:mRNA reporter assays, and miRNA carriers such as nanoparticles. In order to understand why, when, and how an MG miRNA targets a particular mRNA that encodes for stress factors, and they certainly do under normal healthy conditions, more in-depth basic research is imperative. A close collaboration of basic and applied scientists and clinicians with companies specializing in miRNA technologies is the key to a rapid and successful translation of basic knowledge to therapeutic applications to attenuate or prevent gliosis and fight eye diseases and blindness.

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