Review

The role of miRNA in retinal ganglion cell health and disease

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Abstract

miRNA are short non-coding RNA responsible for the knockdown of proteins through their targeting and silencing of complimentary mRNA sequences. The miRNA landscape of a cell thus affects the levels of its proteins and has significant consequences to its health. Deviations in this miRNA landscape have been implicated in a variety of neurodegenerative diseases and have also garnered interest as targets for treatment. Retinal ganglion cells are the sole projection neuron of the retina with their axons making up the optic nerve. They are a focus of study not only for their importance in vision and the myriad of blinding diseases characterized by their dysfunction and loss, but also as a model of other central nervous system diseases such as spinal cord injury and traumatic brain injury. This review summarizes current knowledge on the role of miRNA in retinal ganglion cell function, highlighting how perturbations can result in disease, and how modulating their abundance may provide a novel avenue of therapeutic research.

Key Words: exosome; extracellular vesicle; glaucoma; miRNA; neurodegenerative disease; optic neuropathy; retina; retinal ganglion cell

Introduction

Neuronal cell death is a common feature in a variety of diseases, including those of the retina. The functional deficits are typically a manifestation of these lost cells alongside a loss in their connectivity. Great interest has been given to protecting neuronal cells and their connections as well as promoting regeneration to restore connectivity. Considerable progress has been made, typically under the theme of: (A) understanding the signaling pathways involved in degeneration and/or regeneration; and (B), modulating these signaling pathways to push cells towards a desired outcome. This description is inevitably overly simplistic given the great complexity of these signaling pathways, yet even blocking a single protein can have a significant effect, if the correct targets are chosen such as Phosphatase and Tensin Homolog (PTEN) or BAX (Libby et al., 2005; Park et al., 2010; Maes et al., 2017; Syc-Mazurek and Libby, 2019). Given the complexity of neurological diseases, more novel approaches have been developed to modulate multiple pathways. miRNA (discussed below) are interesting in that individual miRNA may exert its effects through hundreds and possibly even thousands of targets, making miRNA potential candidates as treatment (Zuzic et al., 2019). Indeed many articles have been recently published regarding the role of miRNA in neuronal tissue as well as in a variety of neurological diseases (Juzwik et al., 2019; Nuzziello et al., 2019; Prodromidou and Matsas, 2019).

The retina, being an easily accessible part of the central nervous system (CNS), makes it an attractive model to study neuronal death and axon regeneration. The functional role of miRNA in various retinal cells including photoreceptors, Muller cells and retinal pigment epithelium (RPE) has already been explored in other reviews (Sundermeier and Palczewski, 2016; Quintero and Lamas, 2018; Zuzic et al., 2019), however as of yet, limited information exists with regards to retinal ganglion cells (RGC). This review attempts to summarize available data on the role miRNA play in RGC health and disease while also identifying future avenues for research.

Search Strategy and Selection Criteria

Studies cited in this review published from 2000 to 2020 were searched on the PubMed database using the following keywords “miRNA”, “retina”, and “retinal ganglion cell”. Only studies investigating miRNA expression in total retina or RGC were included. miRNA expression in other retinal cells were excluded.

Retinal Ganglion Cells

RGC are located on the innermost cellular layer of the retina, termed the ganglion cell layer, and function as the final stage of the phototransduction pathway. In mice, there are at least 46 different RGC types differing by their morphology, gene expression pattern, susceptibility to different insults, and physiological properties (Sanes and Masland, 2015; Tran et al., 2019). RGC have numerous roles in the processing of visual information, highlighted by heterogeneity of their receptive fields. Most notably however, their axons make up the optic nerve, which projects from the posterior retina to both ipsilateral and contralateral brain hemispheres, and in particular, the lateral geniculate nucleus.

The term “optic nerve” can however be considered a misnomer, as unlike the other cranial nerves, is myelinated by oligodendrocytes and is thus part of the central, not peripheral nervous system, qualifying it as a tract, not a nerve. Being part of the CNS, RGC suffer from several unfortunate

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characteristics: (1) cells that die cannot be replaced; (2) axons that are damaged do not regenerate; (3) specialized vascular barriers significantly limit entry of small molecules and candidate drugs; and (4) small deviations from homeostasis can result in significant cellular and behavioral/visual dysfunction. CNS tissues appear to behave similarly to the optic nerve and therefore it has become the model of choice for much of CNS research (Berry et al., 2019; Williams et al., 2020).

Death and dysfunction of RGC is a characteristic of many blinding diseases including Leber's hereditary optic neuropathy, autosomal dominant optic atrophy, traumatic optic neuropathy, and most notable, glaucoma. Glaucoma affects 80 million people with an estimated 120 million by 2040 (Tham et al., 2014). The principle (but not exclusive) risk factor is an elevation in intraocular pressure, compressing the optic nerve at the lamina cribrosa and leading to the degeneration of RGC. While neuroprotection is an attractive approach for the treatment of glaucoma, it yet remains elusive and the management of intraocular pressure is the only proven treatment for use in the clinic (Adams et al., 2018). Experimental neuroprotective therapies for the above RGC degenerative diseases include neurotrophic factors such as brain-derived neurotrophic factor (Osborne et al., 2018), ciliary neurotrophic factor (Muller et al., 2007; Parrilla-Reverter et al., 2009), platelet-derived growth factor (Johnson et al., 2014), pigment epithelium-derived factor (Vigneswara and Ahmed, 2019), and vascular endothelial growth factor (Froger et al., 2020), transcription factors such as ATF3 (Kole et al., 2020) and NRF2 (Fujita et al., 2017), PTEN/SOCS3 and energy metabolism modulation (Sun et al., 2011; Casson et al., 2020), glutamate receptors antagonist, α-2-adrenergic receptors agonists, calcium channel blockers, nitric oxide synthase inhibitors, enhanced neural activity (Lim et al., 2016), BAX knockout (Donahue et al., 2020), stem cells and neural precursor cells (Johnson et al., 2010; Wu et al., 2018), conversion of Muller cells into RGC (Zhou et al., 2020), and exosomes/extracellular vesicles (EV) (Mead et al., 2018a, b; Pan et al., 2019; Mead and Tomarev, 2020). Interestingly, it was discovered that EV function primarily through the delivery of miRNA, and viral delivery of discrete miRNA can also enhance neuroprotection (Mead et al., 2020). Individual neuroprotective therapies provide different levels of neuroprotection and combination of several factors in some cases produced synergistic neuroprotective effects (Duan et al., 2015; Dulz et al., 2020). However, none of these therapies delivered a long-lasting complete neuroprotective effect that also preserved cellular function. Moreover, different types of RGC demonstrated diverse response to treatments (Norsworthy et al., 2017). The long and still incomplete list of neuroprotective agents/strategies illustrates that there is no magic "single bullet" that can provide complete neuroprotection, and most likely multiple factors or signaling pathways should be modulated to achieve efficient neuroprotection.

miRNA

miRNA are short (22 nucleotide) non-coding RNA that function to silence mRNA translation into proteins. Through a highly regulated sequence of cleavages and annealings performed by the proteins Dicer and Drosha, pri-miRNA are converted to pre-miRNA and finally mature miRNA (Zuzic et al., 2019). They act by guiding argonaute proteins, which form part of the RNA-induced silencing complex, to mRNA sequences partially complementary to the miRNA sequence. The targeted mRNA's translational efficiently is subsequently reduced and may even be degraded completely (**Figure 1**). There are approximately 2500 miRNA that have been identified in humans and each miRNA can have hundreds of experimentally observed targets (and thousands of predicted targets), making their role in health and disease incredibly complicated and largely unknown (http://www.miRBase.org; http://mirdb.org; http://mirwalk.umm.uni-heidelberg.de). A subset of microRNA (about 400) is localized to mitochondria (John et al., 2020). An important consideration is the miRNA-mediated down-regulation of mRNA and subsequent reduction in translated protein does not inherently mean their role is to inhibit signaling pathways. Many signaling pathways are activated through miRNA-mediated inhibition of negative regulators. For example, the mTOR/PI3K/Akt pathway leads to the activation of discrete protein synthesis, cell growth, and regeneration. Activation of this pathway leads to axon regeneration. PTEN suppresses this pathway whereas PTEN knockdown activates this pathway and leads to significant regeneration and neuroprotection in models of retinal and spinal cord injury (Park et al., 2008). PTEN is interestingly the target of multiple miRNA including miR-214 (Bera et al., 2017), miR-1908 (Xia et al., 2015), miR-494 (Wang et al., 2010) and miR-21 (Sayed et al., 2010), and thus these miRNA would lead to the activation of the mTOR/PI3K/Akt pathway.

While miRNA have the potential for significant effects on cellular function, studies in various mature organs and tissues suggest that typically this is not the case and their role appears to be that of a buffering system. In this way, miRNA act to help cells cope with stress and environmental changes, protecting them from fluctuations in gene expression (Leung and Sharp, 2010; Emde and Hornstein, 2014). Although most of mature miRNA are relatively stable in the majority of mammalian cell types with half-lives in the hours timescale (Guo et al., 2015; Kingston and Bartel, 2019), they can be quickly degraded outside of the cell by nucleases that are widespread in the extracellular fluids. One notable exception is miRNA within EV. EV, which include microvesicles and exosomes or small extracellular vesicles (sEV), contain DNA, proteins, lipids, mRNA, and miRNA. These are packaged into the EV by the host cell before being released into the extracellular space. The molecular mechanisms and regulation of miRNA sorting into EV remain mostly unknown. A growing number of publications demonstrate that miRNA profiles in exosomes and parental cells are different suggesting selective sorting for some miRNA. RNA-binding proteins whose expression likely

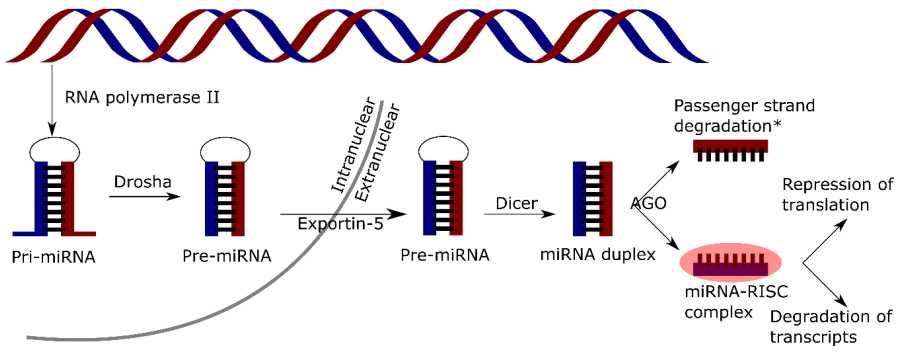

Figure 1 | **A schematic representation of miRNA synthesis.**
Following transcription, Pri-miRNA is converted to Pre-miRNA and then to a mature miRNA duplex. The strands are unwound, and a single strand is packaged into a complex known as RNA-induced silencing complex (RISC) with the help of Argonaute proteins (AGO). While the passenger strand is typically degraded, for some mature miRNA, both strands will form a RISC.

varies between different cell types play an important role in selective sorting of miRNA (Shurtleff et al., 2016; Temoche-Diaz et al., 2019). The sEV can then deliver miRNA into nearby cells, or via the bloodstream, into distant cells. sEV are a rapidly developing area of research and new discoveries relating to miRNA are being made as sEV are studied and the ramifications of delivering or blocking miRNA are unveiled (Zuzic et al., 2019).

The function of miRNA in health and disease is extremely complicated. While the role of an individual mRNA can be ascertained with relative ease by following the functions of its protein, miRNA affect many mRNA (and subsequently, proteins) in a highly heterogeneous manner. The importance is however confirmed by the lethality of deletion of Dicer and Drosha, the critical miRNA biogenesis factors (Bernstein et al., 2003; Chong et al., 2010). The level of individual miRNA or total spectrum of miRNA in sEV in a particular cell type very often is modified in development and ageing as well as in different pathologies and physiological or pharmacological treatments *in vivo* (Xia et al., 2019; Du et al., 2020). The study of particular miRNA and their role is typically done via two approaches, overexpression/delivery or downregulation/silencing of discrete miRNA. To deliver miRNA or their mimics into cells, vectors such a viruses (Geisler and Fechner, 2016; He et al., 2018; Wei et al., 2019; Mead et al., 2020), transfection (Zhang et al., 2019c), or liposomes/exosomes (Fu et al., 2019b; Lee et al., 2019) can be used, whereas to silence miRNA, AntagomiRs (Krützfeldt et al., 2005; Murdaca et al., 2019) or miRNA sponges (Ebert et al., 2007; Ebert and Sharp, 2010) are used. These function by binding to the miRNA, inhibiting or competing for the mRNA binding sites, significantly reducing the silencing capacity of discrete miRNA. The global spectrum of miRNA in sEV can be changed by the overexpression or knockdown of different proteins in the host cells (Huang et al., 2020) or by the treatment of host cells with a variety of factors or reagents (Zhu et al., 2020). The level of individual miRNA in sEV can be also changed by the modification of their level in the host cells as described above or by transfection or electroporation of isolated sEV with miRNA mimics or AntagomiRs. The methods of engineered sEV loading, targeting, and functional testing are the subject of intensive current investigations (Donoso-Quezada et al., 2020; Jafari et al., 2020).

miRNA Connection to Neurodegenerative Diseases

A variety of neurodegenerative diseases have been associated with changes in miRNA abundance, and include Parkinson's disease, Huntington's disease, spinocerebellar ataxia type 1, multiple sclerosis and Alzheimer's disease, although their causative role is still an intense area of investigation. Many experimental articles and recent reviews described involvement of miRNA in neuronal pathologies and possible targets and signaling mechanisms involved (Rajgor, 2018). While we will not cover these data here extensively, since information about involvement of individual miRNA in brain pathologies may provide useful hints for their role in retinal diseases, we will summarize some data on miRNA that are associated with several neurological diseases, axonal growth and regeneration, trans differentiation of different types of cells into neuronal types, and are expressed in the retina (**Table 1**).

The miRNA Landscape of the Retina and Retinal Ganglion Cells

Available data indicate that although many miRNA are ubiquitously expressed, there is a tissue-specific set of miRNA for each organ that has been analyzed so far (Isakova et al., 2020). The retina is a highly metabolically active tissue, placing retinal cells under constant stress and thus, as mentioned above, reliant on miRNA to resist this stress and maintain their health. Focus has been given to photoreceptors and RPE, as there high energetic demands are infamous, and the basis for which age-related macular degeneration occurs (Brown et al., 2018). It should be also mentioned that miRNA degradation kinetics in neuronal cells including retinal neurons appears to be more rapid compared with most other cell types and this is linked to neuronal activity (Krol et al., 2010; Kim et al., 2020).

There is limited information on the miRNA landscape of RGC, owing to the challenge in purifying RGC prior to RNA isolation and sequencing. Purification can be done through laser microdissection (Huang et al., 2006) of the ganglion cell layer, although RGC only occupy 50% of the total cell density with displaced amacrine cells occupying the other half (Perry, 1981). Another approach is utilizing surface markers, such as THY-1 and FAX sorting or beads coated with the appropriate antibody. Unfortunately, RGC are extremely heterogeneous with many subtypes all expressing different phenotypic markers. With the example of THY-1 which is considered the best pan-RGC surface marker, only 82% of RBPMS+ RGC express this marker (Rodriguez et al., 2014). Partially for this reason, current knowledge of miRNA is restricted to total retina. There are several examples of such studies which isolated RNA from total rodent retina and sequenced miRNA or used array hybridization (Hackler et al., 2010; Karali et al., 2011; Zhang et al., 2019b; Wang et al., 2020). One such study that has been conducted on human retina used 16 healthy donors, identifying approximately 500 miRNA. Interestingly, this abundance of varying miRNA is deceiving, as only 20 miRNA account for 90% of the total miRNA abundance (Karali et al., 2016) As above, since this study was done on total retina, no inferences can be made regarding RGC expression however differences between neurons and RPE was found. miR-182 and miR-183 were the most abundant in retinal neurons whereas for RPE, miR-204 was found to be the most abundant. It remains to be seen what the differences are between distinct retinal neurons such as photoreceptors, bipolar cells, and RGC. It is very important to characterize the spectrum of miRNA in RGC and in different RGC types in normal retina and after different insults as well as to find how conserved are these spectra in different species. Finally, it is now well established by single cell sequencing that RGC, amacrine cells and bipolar cells contain multiple cell types showing different gene expression patterns. It is reasonable to suggest that differences exist in miRNA expression patterns between different types of cells within individual classes of retinal cells. Such differences may be discovered and analyzed when methods of single cell sequencing for miRNA are developed.

Changes in miRNA – Correlations with Dysfunction and Disease

miRNAseq studies have identified deviations in a variety of retinal diseases including: retinitis pigmentosa (Loscher et al., 2007), diabetic retinopathy (Kovacs et al., 2011; Martins et al., 2020), retinal neovascularization (Shen et al., 2008) and ager-related macular degeneration (Martinez and Peplow, 2021) These studies were able to identify discrete miRNA that were up- or down-regulated in the above disease states, however, it is worth reiterating that these changes are within total retina and as of yet, it is unknown which miRNA changes are associated with which specific retinal cell type.

A miRNA identified as being downregulated in glaucoma is miR-200a, based on RT-qPCR of retinal samples from wildtype and microbead-induced glaucomatous mice (Peng et al., 2019). Intravenous injection of miR-200a mimics into a microbead mouse model of glaucoma provides significant neuroprotection of RGC as well as preservation of RNFL thickness, likely through the knockdown of FGF7. A separate

Table 1 | **Examples of miRNA associated with neurodegenerative diseases**

miRNA	Disease	Model	Up/down regulation	Possible targets	Biological functions	References
Let-7	Peripheral nerve injury	SD rat	Down	NGF	Sciatic nerve regeneration	Li et al., 2015
Mir-9a-5p	Cerebral ischemia	Middle cerebral artery occlusion	Down	ATG5	Autophagy	Wang et al., 2018
Mir-17–92	Acute and chronic neurological disorders	Human animal models	Up and down	Multiple targets including PTEN, GP130, Sgk1	Regulation of developmental and adult neurogenesis	Xia et al., 2020
Mir-21	Traumatic brain injury	Mouse model	Up	PDCD4, Tiam1	Blood-brain barrier function	Redell et al., 2011
Mir-29b-3p	Amyotrophic lateral sclerosis	Wobbler mice	Up	BMF, Bax, and cleaved-Caspase 3	Apoptosis	Klatt et al., 2019
Mir-34a	Alzheimer's disease	(TetR-TetO-miR-34a) Transgenic Mice	Up	Sirt1, Ptpa (phosphatase 2A activator)	Memory formation, APP metabolism	Sarkar et al., 2019
Mir-96	Hearing loss	Human mutant	Down		Defects in hair cells of adult cochlea	Mencia et al., 2009; Schluter et al., 2018
		Hindbrain development	Dmdo/Dmdo mice	KV1.6; BK channel protein	Auditory hindbrain circuits	
mir-124	Alzheimer's disease	SD rat Human	Down	BACE1,	APP cleavage	Johnson and Buckley, 2009; Fang et al., 2012; Angelopoulou et al., 2019
	Parkinson's disease	Mouse models	Down	PTBP1, APP		
	Huntington's disease	Bioinformatic analysis	Down	Calpain 1, Bim, Stat3, AMPK	Apoptosis, inflammation	
			Down	REST		
Mir-132-3p	Alzheimer's disease	Human	Down	PTEN, FOXO3a	Neurite outgrowth	Magill et al., 2010
		Mouse				
Mir-137	Schizophrenia	Human	Down	ERBB4, ABRA1, GRIN2A, GRM5, GSK3B, NRG2	Neurogenesis, neurodevelopment, dendritic arborization	Wright et al., 2013
Mir-155-5p	Peripheral nerve injury	Wistar rat	Down	Protein kinase inhibitor peptide (PKI-α)	Sciatic nerve regeneration	Wang et al., 2019
Mir-200 family (Mir200a-c, -141,-429	Several neurodegenerative diseases	Human rodent models	Up and down	OGG1-2a, APP, SIRT1, FUS	Cell apoptosis, APP metabolism, α-synuclein aggregation, DNA repair	Fu et al., 2019a
Mir-495	Amyotrophic lateral sclerosis	Mouse	Up	Gria2	Motor neuron degradation	Capauto et al., 2018

ABRA1: BRCA1-A complex subunit abraxas 1; AMPK: protein kinase AMP-activated catalytic subunit alpha 2; APP: amyloid beta precursor protein; ATG5: autophagy related 5; BACE1: beta-secretase 1; Bax: BCL2 associated X; Bim: BCL2 like 11; BK channel: potassium calcium-activated channel subfamily M apha 1; BMF: Bcl2 modifying factor; Dmdo: diminuendo; ERBB4: Erb-B2 receptor tyrosine kinase 4; FOXO3a: forkhead box O3; FUS: FUS RNA binding protein; GP130: glycoprotein 130; Gria2: glutamate ionotropic receptor AMPA type subunit 2; GRIN2A: glutamate ionotropic receptor NMDA type subunit 2A; GRM5: glutamate metabotropic receptor 5; GSK3B: glycogen synthase kinase 3 beta; KV1.6: potassium voltage-gated channel subfamily A member 6; NGF: nerve growth factor; NRG2: neuregulin 2; OGG1-2a: 8-oxoguanine DNA glycosylase; PDCD4: programmed cell death 4; PTBP1: polypyrimidine tract binding protein 1; PTEN: phosphatase and tensin homolog; REST: RE1 silencing transcription factor; SD: Sprague-Dawley; Sgk1: serum/glucocorticoid regulated kinase 1; Sirt1: sirtuin 1; Stat3: signal transducer and activator of transcription 3; TetR-TetO: tetracycline-controlled transcriptional activation system; Tiam1: T-lymphoma invasion and metastasis-inducing protein 1.

study investigating how 17 different miRNA change in a mouse model of glaucoma identified nine to be differentially expressed from controls. These include the down regulation of miR-27a, which is associated with anti-apoptosis, and the up regulation of miR-29b, -497, and -16, which are associated with pro-apoptosis. These findings suggest RGC undergo miRNA changes following glaucomatous damage, possibly in an attempt to prevent RGC death (Jayaram et al., 2015). In contrast to modelling the chronic aspects of glaucoma, it is also possible to analyse miRNA changes in RGC immediately after ocular hypertension induction. To model these acute effects of ocular hypertension, authors administered saline for 7 days into the anterior chambers of rats prior to retina removal and miRNA array analysis. A total of 31 miRNA were differentially expressed between ocular hypertensive retina and normal retina with many targeting mRNA of the mitogen-activated protein kinase pathway (Wang et al., 2017). Importantly however, the above studies only tested retinal miRNA, not purified RGC miRNA. We compared changes in miRNA levels in isolated RGC after optic nerve crush and after elevation of intraocular pressure in rats (Ben and Tomarev, unpublished). Multiple changes in miRNA spectrum were detected in both cases when compared to undamaged RGC. Moreover, optic nerve crush and glaucoma model showed some common and unique changes when compared with control samples or with each other similar to the picture observed when changes in mRNA levels were analyzed. Some miRNA with the modified abundance level that we detected were also observed in in the brain tissues from different neurological pathologies (**Table 1**).

Optic nerve injury is characterized by the acute loss of RGC. Muller glia, a supportive cell type in the retina initially acts in a preventive, pro-survival manner but switches to pro-inflammatory, further exacerbating the RGC death. It is suggested that the expression of miR-21 plays a role in this muller glia activation as its inhibition modulates it, subsequently leading to a neuroprotective effect on RGC (Li et al., 2019). The following study however only utilized 4 animals per group once consideration is given to all of the time points and further studies would be necessary to corroborate these conclusions. Equally, the study only tested photoreceptor/bipolar cell function using ERG, whereas RGC function was not tested.

miRNA as Targets for Therapies

miRNA and their mimics or inhibitors can be delivered into RGC to act as a potential therapeutic through a variety of approaches including viral and non-viral vectors (Fu et al., 2019b; Wang, 2020). While a single candidate may be delivered, it is also possible to deliver a cocktail of candidate miRNA (Hu et al., 2011; Mead et al., 2020). These are typically tested *in vitro* initially, such as retinal culture systems, prior to their testing in *in vivo* animal models of retinal disease. One of the promising approaches is to deliver miRNA through application of nanoparticles or artificial vesicles loaded with miRNA as well as natural or modified sEV. The main advantages of artificial vesicles including liposomes are that it is easier to prepare a characterized population of such vesicles in large amounts and modify them to target a particular type of cells or organs. sEV as a rule are well compatible with cellular membranes of the host cells and they show good biostability. One of the disadvantages of sEV is that there are problems with their large-scale preparation from some cells.

In mouse models of photoreceptor degeneration, MSC and MSC-derived exosomes were sufficient in preventing cellular degeneration and functional deterioration. Interestingly, exosomes isolated from MSC of miR-21 knockout mice were no longer therapeutically efficacious (Deng et al., 2020). miR-155 is associated with retinal inflammation following injury and its expression in glial cells is up-regulated in response to stress. miR-155 is translocated to photoreceptors via exosomes/EV and likely contributes directly to the retinal degeneration. Knockdown/depletion of miR-155 attenuates inflammation and protects against retinal degeneration (Aggio-Bruce et al., 2021). In RGC-5 cells, overexpression of miR-211 (Yang et al., 2018) or miR-187 (Zhang et al., 2015) has been shown to be neuroprotective. The RGC-5 cell-line is however no longer considered be a reliable model of RGC and these findings have yet to be corroborated in primary RGC models. Primary RGC cultures have however been used to test the therapeutic efficacy of other miRNA candidates. These *in vitro* models are characterized by a rapid loss of RGC, and delivery of miRNA via Schwann cell-derived exosomes proved to be effective in promoting neuritogenesis and survival, evident by the lack of effect if exosomes were ablated of RNA (Ching et al., 2018). Authors identified several candidate miRNA including miR-21, miR-222, miR-18a, and miR-182. NMDA can be added to these RGC cultures to promote and model autophagy of RGC. In this model, miR-93-5p is down-regulated, leading to the authors testing the delivery of miR-93-5p as a potential therapeutic (Li et al., 2018). miR-93-5p protected RGC from NMDA-induced death and was shown to target PTEN, reducing the NMDA-mediated autophagy of RGC. RGC death can also be modelled *in vivo*, typically in rodent models whereby the optic nerve has been damaged (traumatic optic neuropathy) or pressure in the eye has been artificially increased (glaucoma). In both models we demonstrated that the therapeutic efficacy of exosomes was at least partially due to their miRNA cargo (Mead and Tomarev, 2017; Mead et al., 2018). The neuroprotective effects were shown to be muted when miRNA packaging into exosomes was abolished through Argonaute-2 knockdown. Intravitreal injection of nanoparticles carrying mir-124 protected RGC in a mouse optic nerve crush model although only early stages of RGC death (3 days after the crush) were analyzed (Li et al., 2020). Using episcleral vein ligation to model glaucoma in mice, authors demonstrated that inhibitors of miR-149 were RGC neuroprotective along with an associated upregulation of the PI3K/Akt pathway (Nie et al., 2018). A second study investigated the role of miRNA-mediated autophagy in glaucoma. Using a different mouse model of glaucoma, in which ocular hypertension is induced through laser photocoagulation of the outflow pathways, miR-708 and miR-335-3p were found to be down-regulated in retinal tissues, whereas overexpression was neuroprotective. The mechanism of action appeared to be through the down-regulation of autophagy-related-3 and subsequent inhibition of autophagy in RGC (Zhang et al., 2021). A third glaucoma model known as DBA/2J mice, which spontaneous develop ocular hypertension has also been used to test miRNA therapies. Young DBA/2J mice injected with glutamate to induce RGC excitotoxicity received an intravitreal delivery of a virus expressing miR-141-3p. Significant RGC survival was observed compared to controls, along with down-regulation of apoptotic signaling pathways such as Bax and caspase-3 (Zhang et al., 2019a). Recent data suggest that miR-124 may participate in the conversion of Muller glial cell into RGC *in vivo*, stimulated by down-regulation of a single RNA-binding protein, polypyrimidine tract-binding protein 1 (Zhou et al., 2020). Polypyrimidine tract-binding protein 1 normally inhibits miR-124 in non-neuronal cells and in its absence releases miR-124 activity which suppresses the REST complex responsible for silencing a large number of neuron-specific transcription factors. It would be interesting to test whether overexpression of miR-124 in Muller cells may stimulate their conversion into functional RGC.

Future Perspectives

It became clear in recent years that miRNA play an important role in development and normal functioning of adult tissues as well as in pathology. miRNA-based therapeutics surfaced as a promising approach in the treatment of different diseases including optic neuropathies. Many important general questions should be addressed and clarified before miRNA-based therapeutics becomes a clinical reality.

As in case of other neurological diseases, the selection of the appropriate miRNA providing significant RGC neuroprotective effects is one of the first critical steps. Testing candidate neuroprotective miRNA in larger animals having eyes more similar to human eyes is an important step before clinical testing in human. Such knowledge may help to select better miRNA candidates for neuroprotective studies in human, since most neuroprotective experiments with miRNA *in vivo* were performed using rodent models. There are many publications describing neuroprotective effects of individual miRNA in different systems. However, some publications demonstrate that a cocktail of several miRNA may provide much better neuroprotection. Testing of different miRNA combinations for neuroprotection represent an important step in the developing neuroprotective strategy. Another important step in this process is the development of easy and inexpensive methods of measuring miRNA levels in experimental samples in general and in RGC in particular. Other critical challenges include the development of efficient methods of miRNA delivery and potential off-target effects of delivered miRNA. It is also important to conduct side-by-side comparison of neuroprotective properties of different cell types versus EV versus miRNA, although EV or miRNA appear to be safer than cells for clinical applications.

In summary, RGC neuroprotection is a complex process involving multiple players that may differ after different insults like intraocular pressure elevation of optic nerve crush. There is no single factor or "magic bullet" that may efficiently protect or help to replace damaged RGC in optic neuropathies. miRNA, affecting multiple targets, represent a useful tool in RGC neuroprotection.

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