THE MICRORNA *miR-18a* REGULATES NEURAL REGENERATION IN THE INJURED RETINA

by

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ABSTRACT

THE MICRORNA *miR-18a* REGULATES NEURAL REGENERATION IN THE INJURED RETINA

Evin Tyson Magner

In mammals, retinal injuries and photoreceptor degeneration (PD) can damage photoreceptors causing permanent blindness. However, retinal injury in zebrafish (*Danio rerio*) elicits a rapid stem cell-based response that regenerates photoreceptors and restores vision. Crucial to this are the Müller glia (stem cells) that generate multipotent progenitor cells, which regenerate photoreceptors. Photoreceptor regeneration requires accurate control over the immune response (inflammation), cell cycle exit, and differentiation. Understanding the mechanisms which control photoreceptor regeneration in zebrafish could lead to treatments for blindness that stimulate Müller glia to reestablish vision in humans. Past investigations have demonstrated that microRNAs (miRNAs) are essential regulators of retinal neurogenesis, but lacking is an understanding of the roles of miRNAs in regulating photoreceptor regeneration. Preliminary data with *miR-18a* mutant fish generated using CRISPR/Cas9 gene-editing indicated that this microRNA regulates photoreceptor regeneration. The objective of this study was to determine if and how *miR-18a*, following light-induced photoreceptor injury, functions to regulate the cell cycle and/or differentiation in Müller glia and photoreceptor progenitors. In-situ hybridization (ISH) and immunohistochemical (IHC) labeling showed that *miR-18a* is expressed in microglia, proliferating Müller glia and photoreceptor progenitors, suggesting roles at both early and later stages of the photoreceptor regeneration response. Bromodeoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA) immunolabeling experiments showed that, following photoreceptor injury, there are more proliferating cells in *miR-18a* mutant (CRISPR) retinas compared with

wild-type (WT), demonstrating that *miR-18a* regulates the cell cycle. Expression of *miR-18a* in Müller glia and microglia (macrophages) indicates a role in the early regeneration response during which inflammation plays a critical role, and microRNA databases predict that *miR-18a* might interact with and regulate several molecules involved in inflammation. These results led to the hypothesis that *miR-18a* regulates photoreceptor regeneration by regulating inflammation. As an initial test of this hypothesis, ISH was used to show that, in *miR-18a* mutants compared with WT, the inflammatory mediator NFkB is expressed for a more extended period of time, indicating that *miR-18a* might typically function to suppress inflammation. To test this, dexamethasone (corticosteroid) was administered at critical time points to reduce inflammation levels in the *miR-18a* mutants. By blocking inflammation from days 2 to 6 post-injury, the treatment rescued the phenotype in the mutants, reducing the numbers of proliferating cells to the levels observed in WT fish. Finally, to determine if *miR-18a* also regulates photoreceptor differentiation, ISH for rods and cones showed that *miR-18a* mutant fish generate photoreceptors slower than wild-type fish but that by 14 days post-injury (dpi) *miR-18a* mutant fish generate more cones than WT. Together, these results demonstrate that *miR-18a* regulates the cell cycle and photoreceptor differentiation through molecular control over inflammation.

INTRODUCTION

The World Health Organization (WHO) estimates that over 252.6 million people suffer from some form a severe visual impairment. In response to the issue in 2013, the WHO adopted a program called "Universal Eye Health: A Global Action Plan." The goal of the project was two-fold: (1) reducing visual impairment and (2) increasing treatment options and providing rehabilitation for those with visual impairments. As of 2015, of the 7.33 billion people in the world, nearly 36 million were blind. Additionally, 216.6 million lived with a moderate to severe impairment, and another 188.5 million lived with mild visual impairment (Bourne et al 2017). Visual impairment, no matter the severity, places terrible economic tolls on both individuals and society as they attempt to cope with the loss or impairment (Goldman 2014). Compared to data collected in 1990, the occurrence of blindness by 2015 rose by 17.6%. While a portion of this increase is due to population growth and aging populations, a growing number of cases occur from degenerative conditions or injuries incurred at the workplace (Bourne et al 2017). Both such events can result in photoreceptor degeneration, the leading cause of blindness (Wright et al 2010). Considering that population growth is expected to continue, there needed to be a more significant effort in combating this public health concern, hence forming the Global Action Plan and its drive to develop new treatment options. Developing new technologies to regenerate damaged photoreceptors may be one of the best courses of action. Forms of a new treatment, such as stem-cell-based therapies not only increase the quality of life for patients but, as studies have shown, the investment into alleviating visual impairments in the population can provide the most substantial returns on investments for businesses and communities (Bourne et al 2017).

Blindness and visual impairment result from a loss of cells and neurons in the retina and cause many degenerative diseases in humans. Retinitis pigmentosa, age-related macular

degeneration, diabetic retinopathy, and glaucoma are a few of the most concerning eye conditions affecting the retina and causing blindness (Wright et al 2010; Wan and Goldman 2016). Retinal cell loss in humans, as with other mammals, results in a progressive degeneration that leaves irreversible damage (Goldman 2014). Developmental issues resulting from genetic mutations in the retina likewise damage or permanently alter photoreceptors, resulting in blindness. Research suggests that at least 146 mutations are linked to causing photoreceptor degeneration in the retina (Huet et al 2013).

The Retina

The retina is the layer of tissue located in the rear of the eye and composed, namely of three distinct layers: the (1) ganglion cell layer (GCL), (2) inner nuclear layer (INL), and (3) outer nuclear layer (ONL). Six neural cell types and one glial cell type compose these three layers (Wan and Goldman 2016). With 55 distinct cell types, the retina is a highly complex, yet simplified system. One unique evolutionary characteristic of the retina is that it is highly conserved throughout the vertebrates. That is to say, the overall morphology and function of the retina are relatively unchanged from fish to humans (Brockerhoff and Fadool 2011). Figure 1 illustrates the basic anatomy of the zebrafish retina, including the two neural cell types known as photoreceptors. Photoreceptors are the neuroepithelial cells in the retina used to convert light into neural signals (Horton et al 2015). Photoreceptors in the eyes are divided into rods and cones. These highly polarized neural cell types, located in the distal layer of the retina, serve as the first and primary sensory cells in the detection of light. Rods detect wavelengths in low light

settings, while cones primarily function to detect color in high light instances and transform the information into a signal for the brain (Brockerhoff and Fadool 2011).

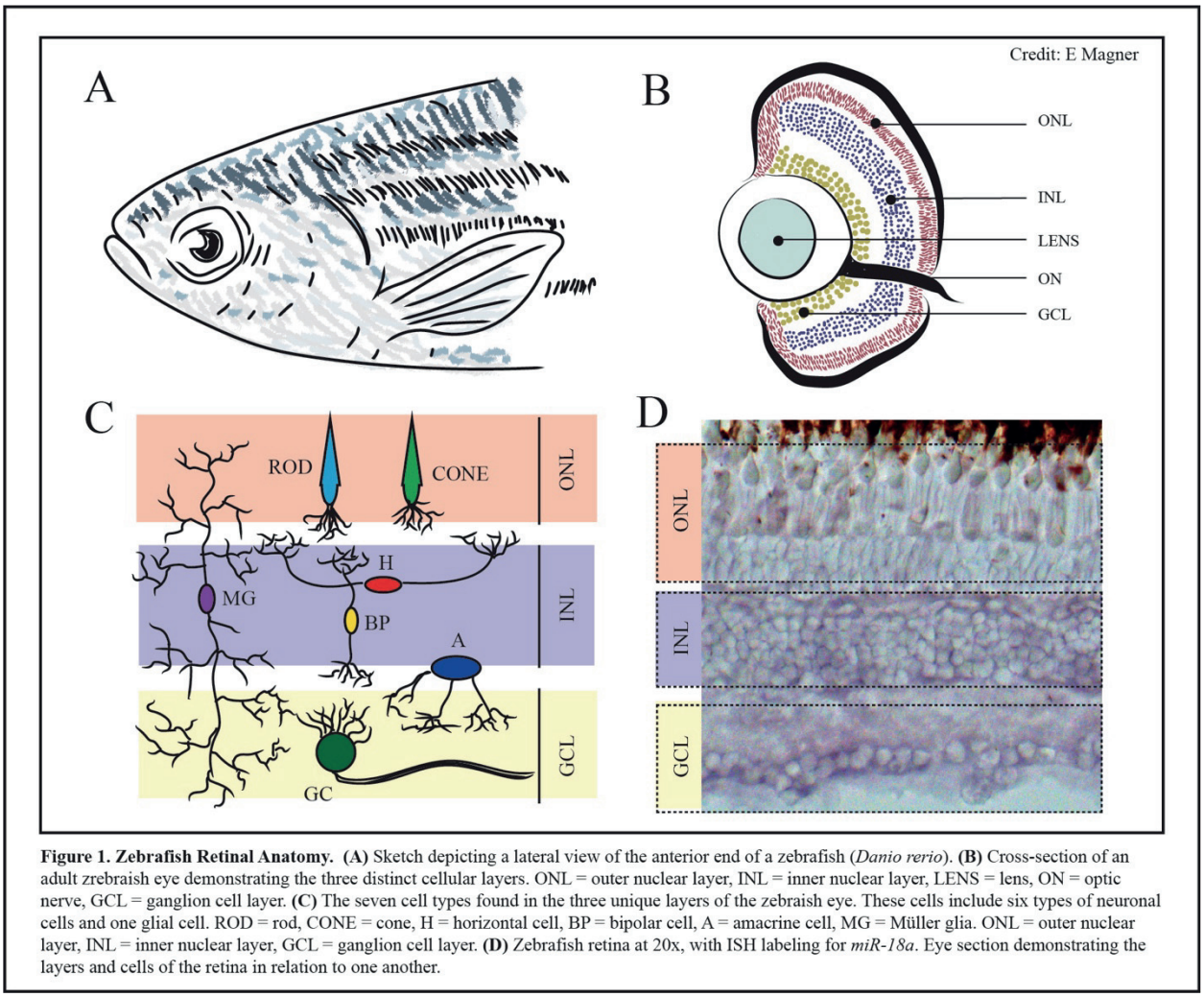


Figure 1. Zebrafish Retinal Anatomy. **(A)** Sketch depicting a lateral view of the anterior end of a zebrafish (*Danio rerio*). **(B)** Cross-section of an adult zrebraish eye demonstrating the three distinct cellular layers. ONL = outer nuclear layer, INL = inner nuclear layer, LENS = lens, ON = optic nerve, GCL = ganglion cell layer. **(C)** The seven cell types found in the three unique layers of the zebraish eye. These cells include six types of neuronal cells and one glial cell. ROD = rod, CONE = cone, H = horizontal cell, BP = bipolar cell, A = amacrine cell, MG = Müller glia. ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer. **(D)** Zebrafish retina at 20x, with ISH labeling for *miR-18a*. Eye section demonstrating the layers and cells of the retina in relation to one another.

Injury to the mammalian retina produces a protective response from cells called Müller Glia (MG). This response, termed reactive gliosis, results in a detrimental effect. Following injury, cells within the retina begin to die off without a proliferating response (Goldman 2014; Rajaram et al 2014). Progressive cell die-off causes glial scarring and fibrosis, further damaging the local, healthy cell population. In contrast, MG respond very differently in the teleost fish retina, in which they initiate a robust regenerative response following injury (Wan and Goldman

2017). This rigorous regenerative response (Fig. 2) produces progenitor cells capable of differentiating into the various retinal cell types, including photoreceptors. As progenitors divide, they migrate toward the ONL, differentiating as they move to replace lost and damaged photoreceptors. The reestablishment of healthy photoreceptors eventually restores the vision in the damaged teleost eye. Vital to the regenerative process is the precise control over the innate immune response, exit from the cell cycle, and differentiation to restore lost neurons (Wan and Goldman 2016). By deciphering the mechanisms behind photoreceptor regeneration in zebrafish (*Danio rerio*), it is the hope that these principles could be applied to therapeutically regenerate human photoreceptors.

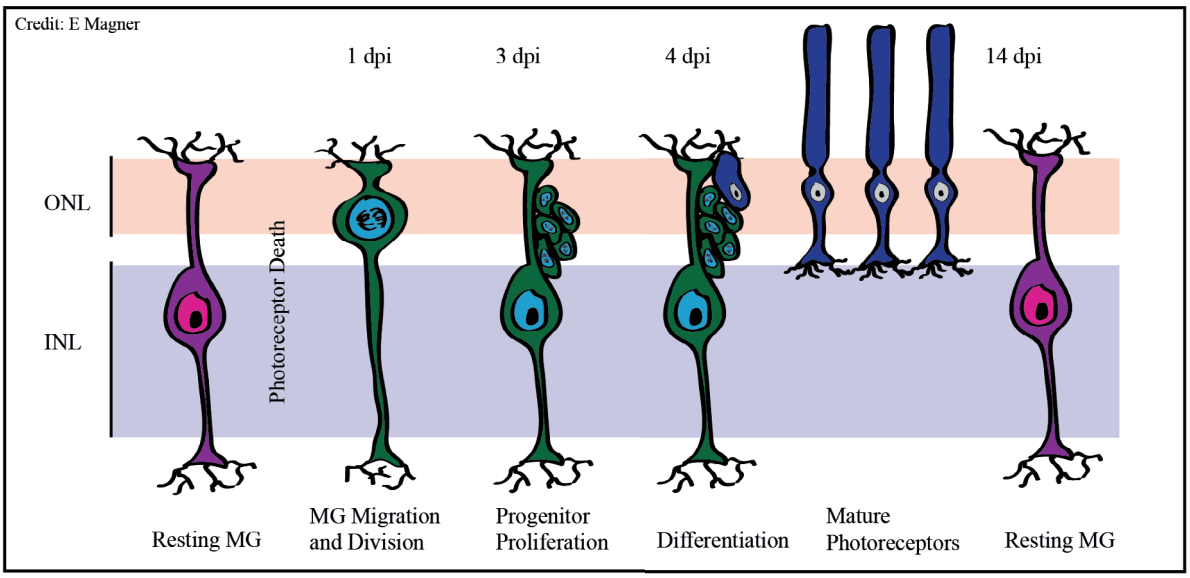


Figure 2. Overview of the Regenerative Response to Injury in the Retina. Upon photoreceptor death, the Müller glia (MG) nucleus respond by migrating from inner nuclear layer (INL) to the outer nuclear layer (ONL) where it re-enters the cell cycle and produces a single multipotent progenitor. Retinal progenitors undergo a substantial proliferation response before migrating to areas of damaged. Once in located in the damaged area, the progenitors differentiate into new neurons.

Müller Glia and Microglia

The zebrafish has profound regenerative abilities, capable of regenerating tissues throughout the body as a mode of restoring function to damaged organs or repairing certain effects of aging (Wan and Goldman 2016). The key to this mechanism is the creation of mitotic

progenitors from multipotent stem cells. In the eye, a unique set of cells, common to all vertebrates, called Müller glia, produces progenitors (Lenkowski et al 2013; Rajaram et al 2014). For zebrafish, the Müller glia function as multipotent retinal stems cells generating missing neurons in damaged areas. Through homeostatic and developmental mechanisms, the MG asymmetrically divide to produce neural progenitors capable of differentiating into any retinal neuron (Bernardos et al 2007). MG cell bodies reside in the inner nuclear layer, but their processes extend through all layers of the retina, allowing them to support and interact with each retinal cell type. In a relaxed state, the MG acts in neural protection, retinal homeostasis, and retinal structure (Wohl et al 2017). Once damaged though, by mechanical forces, chemical toxicity, phototoxicity, or genetic lesioning (damage caused to the structure or base-pairing of DNA), the MG undergo a massive reprogramming event, causing changes to the morphology, biochemistry, and physiology of the cell (Brockerhoff and Fadool 2011). This reprogramming event causes large-scale gene expression alterations accompanied by a partial change in the genome's methylation and the activation of genes associated with multipotency (Wan and Goldman 2016). This cascade of events stimulates an increase in the proliferation of progenitor cells in both the INL and ONL, regenerating damaged photoreceptors and restoring vision. The mammalian response, while similar, only involves the upregulation of progenitor markers and entry into the cell cycle. The produced progenitors, however, do not differentiate (Mitchell et al 2018). Zebrafish, having otherwise similar retinas to humans, thus serve as useful models in retinal neurobiology. In studying the innate Müller glia cell-mediated regenerative response, newly discovered mechanisms may allow humans to undergo a similar regenerative process (Brockerhoff and Fadool 2011).

Microglia cells are macrophages found alongside Müller glia in the retina. Serving as the resident immune cells, microglia are the first line of defense for the eye (Rashid et al 2018). Found throughout the majority of the retina, except for in the ONL, these cells are on a constant mission to maintain homeostasis within the neuro-retinal environment (Okunuki et al 2018). When an injury, disease, or genetic mutation begins to damage or deteriorate the parenchyma, the microglia are the first to respond. Past studies have shown that microglia initiate and propagate the immune response within the retina and other central nervous system (CNS) locations (Madeira et al 2015). Upon activation, the cell quickly loses its protrusions to the surrounding area and begins to proliferate. As proliferation continues, the progenitors begin to migrate towards the damaged tissue (Rashid et al 2018). Moving toward the damaged tissue, the microglia begin to orchestrate the neuroinflammatory response as a whole. Then once near the damage, the cell begins to alter its function to prevent further damage. These macrophages, acting as they would in other tissues of the body, begin to remove debris and dead cells from the affected area. This action not only limits the damage, but studies demonstrate it increases the survivability of the surrounding cells as well (Okunuki et al 2018). In tandem with the MG, these two cell types are critical in the neurogenesis and regeneration of photoreceptors and have, therefore, been chosen to be the primary focus of this investigation (Lenkowski et al 2013).

MicroRNA (miRNA)

Recent research has helped to broaden our understanding of the molecular pathways that regulate neural regeneration in the zebrafish retina (Lenkowski et al 2013; Goldman 2014). Currently lacking, however, is an understanding of the roles of non-coding RNAs in regulating these pathways. MicroRNAs (miRNA) are a specific set of small, usually between 18-25 nucleotides long, non-coding RNAs (Rajman and Schratt 2017) that have the potential to

substantially regulate neural regeneration. The generalized role of miRNA is to regulate mRNA stability and translation throughout the body (Wan and Goldman 2016). By binding with the complementary binding sites in the 3' untranslated region (UTR), the miRNA can suppress translation or promote mRNA degradation (Huntzinger and Izaurralde 2011; Rajaram et al 2014).

miRNAs play several vital roles in the development and homeostasis of the eye (Lenkowski et al 2013; Taylor et al 2019). In development, miRNAs act in the spatial-temporal regulation of neuronal gene expression. This regulation controls neural differentiation, circuit development, and modifications of neural circuits (Rajman and Schratt 2017). MG cells require miRNA in order to maintain glial homeostasis and retinal construction (Wohl et al 2017). Recent studies have shown that miRNAs play a crucial role in retinal development and regeneration (Rajaram et al 2014; Taylor et al 2019). Newly published data demonstrates that the microRNA *miR*-18a regulates NeuroD, in turn, regulating the differentiation of photoreceptors in the developing retina of zebrafish (Taylor et al 2019). Furthermore, miRNAs regulate regeneration in the fin, heart, and spinal cord of zebrafish. Therefore, it is likely that additional miRNAs may regulate other mechanisms critical for photoreceptor regeneration. Identifying and understanding the mechanisms controlling regeneration will further the design of therapies to trigger photoreceptor regeneration in the human retina (Rajaram et al 2014).

Inflammation

Inflammation plays a vital role in the development, repair, and homeostasis of the retina as it modulates both immune and nonimmune function. Recent studies have likewise demonstrated it as critical to the retinal regeneration response, as well (Silva et al 2020). The immune system (macrophages) typically initiates inflammation following an injury or infection.

This response initiates the activation of immune cells and the production of cytokines. Critical to this process is the microglia and their production of pro-inflammatory and anti-inflammatory signals. While this initially appears counterintuitive, it is necessary to balance inflammation. Initially, an inflammatory response will elicit recovery. However, an accumulation of pro-inflammatory markers leads to chronic inflammation, which can contribute to disease or further scarring, as in reactive gliosis (Arroba et al 2018).

In zebrafish, cytokines can stimulate the proliferation and regeneration of MG by activating several molecular pathways (Wan et al 2014). In mammals, inflammation, as a result of photoreceptor damage, inhibits the regenerative response, and develops into irreversible scarring (Mitchell et al 2018). For zebrafish, inflammation initiates the retinal regenerative response but, beyond approximately 2 days following retinal injury, it suppresses regeneration (Lenkowski et al 2013; Silva et al 2020). Previous studies, using transgenic fish, investigated the timing of inflammation and showed that prolonged inflammation past 2 days post-injury (dpi) reduced the number of progenitors and affected the differentiation of photoreceptors. However, by chemically suppressing inflammation at this time point, typical proliferation and differentiation returned (White et al 2017; Silva et al 2020). This balance of inflammation demonstrates the imperative nature of the proper regulation of inflammation in the retinal regenerative process.

Aims

Although damage to photoreceptor cells, in mammals, is irreversible, new technology is attempting to change that (Goldman 2014). Regrowing photoreceptors in-vitro from human embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) is one such treatment. Once grown to maturity, medical professionals transplant the photoreceptors into the patient's retina,

hoping they might replace lost receptors (Stern et al 2018). A second new procedure attempts to reprogram the endogenous Müller glia cells into replacing the damaged photoreceptors (Pollak et al 2013; Sanges et al 2016). Both treatments are currently inefficient and have severe limitations due to the lack of information surrounding regeneration mechanisms. The development of new treatments is only possible by expanding our knowledge of the mechanisms behind the degeneration and regeneration of photoreceptors.

Using *Danio rerio* as a model system, the long-term goal of this research is to characterize the molecular mechanisms that control photoreceptor regeneration. Past investigations have demonstrated that microRNAs (miRNAs) are essential in photoreceptor development and maintenance by regulating the expression of specific biomolecules (Horton et al 2015; Taylor et al 2019). At present, though, the roles of microRNAs in neural regeneration, and photoreceptor regeneration specifically, are critically lacking. Preliminary data from tests with *miR-18a* mutants indicate that this microRNA critically regulates photoreceptor regeneration, but the mechanisms through which it functions are not known.

The overall objective of this research was to identify the mechanisms through which *miR-18a* regulates neural regeneration in the retina. To determine the mechanisms, the current research tested the central hypothesis that following an injury to the retina, *miR-18a* regulates the cell cycle in stem cells (Müller glia) and photoreceptor progenitors. The rationale is that findings from this investigation will identify critical molecular mechanisms that allow neurons, including photoreceptors, to regenerate. In turn, this information may assist in the design of techniques that stimulate the Müller glia to reprogram and regenerate damaged retinas in humans.

METHODS

Housing and Breeding Zebrafish

AB wild-type (WT), *miR-18a* mutants, and transgenic (Tg) zebrafish were reared and housed in an Aquaneering Zebrafish Aquatic Housing System in Dr. Scott M. Taylor's lab in Building 58 on the University of West Florida's campus. The temperature, lighting, and filtration are controlled by the system to provide optimal environmental conditions at 28.5 °C with a 14 hour (hr) light and 10 hr dark cycle. Individual tanks allow for the separation of transgenic/mutant lines and sexes. Separating the sexes enables control over the population and the planned development of new fish lines.

Generating CRISPR/Cas9 Mutants and Transgenic Fish

Control experiments used AB wild-type zebrafish acquired from the Zebrafish International Resource Center (ZIRC; University of Oregon, Portland, OR). Several transgenic lines of fish allowed for the investigation into different components of the regenerative pathways (Bernardos and Raymond 2006). Taylor et al. (2019) previously generated *miR-18a* mutant fish using CRISPR/Cas9 to target the *miR-18a* precursor sequence and described methods for generating and characterizing these fish. Briefly, a *miR-18a* stable mutant fish line was established with a 25 base-pair (bp) insertion in the *pre-miR-18a* sequence, and Taqman RT-qPCR showed that homozygous mutants completely lack mature *miR-18a*. Homozygous fish are identified by PCR amplifying the genomic sequence around the *miR-18a* locus and then using an AleI restriction digest to identify the mutant sequence that lacks this cut site.

In addition, two established lines of transgenic lines of fish [Tg(*gfap:egfp*^{mi2002}) and Tg(*mpeg1:egfp*)] were used to visualize the expression of *miR*-18a in relation to responding cells. Both transgenic lines exhibit enhanced green fluorescent protein (EGFP), with the only

difference being the location of expression. Tg(*gfap:egfp*^{mi2002}) fish allow for the precise examination of the Müller glia (Bernardos and Raymond 2006), while Tg(*mpeg1:egfp*) track the microglia (macrophages) (Ellett et al 2011).

In order to maintain the mutant and transgenic lines, zebrafish were placed in breeding boxes with males and females and situated in an incubation chamber. The chamber allows for more precise control of temperature and light to encourage breeding. Embryo collection occurred within 15 min of spawning and housed in an incubator with matching parameters as the adult housing system. Collected embryos were grown in small petri dishes before transferring to the adult housing system.

Photolytic Lesioning

Photolytic lesioning selectively damages photoreceptors in the zebrafish retina in order to elicit a regeneration response (Taylor et al 2012). Figure 3 exemplifies the type of system used for this procedure. Zebrafish, up to three at a time, were placed in 100-mL glass beakers with water from the system. Wrapping the beaker in reflective insulation prevents light from escaping, while a small window cut into the side allows for the input of the light source. The window allows the ultra-high intensity light to penetrate the glass beaker. Fish were subsequently exposed to >120,00 lux for 30 minutes. Past studies have shown this to be sufficient to destroy photoreceptors and induce the regenerative response (Taylor et al 2012; Lenkowski et al 2013). Then, at previously determined time points following lesioning, fish were sacrificed for examination. Identifying proliferating Müller glia and neural progenitors was possible by exposing the fish to 5-bromo-2'-deoxyuridine (BrdU), as to label cells in the S-phase of the cell-cycle. To label proliferating cells, fish were exposed to BrdU (5mM) for 1 hr, prior to (Tang et al 2007).

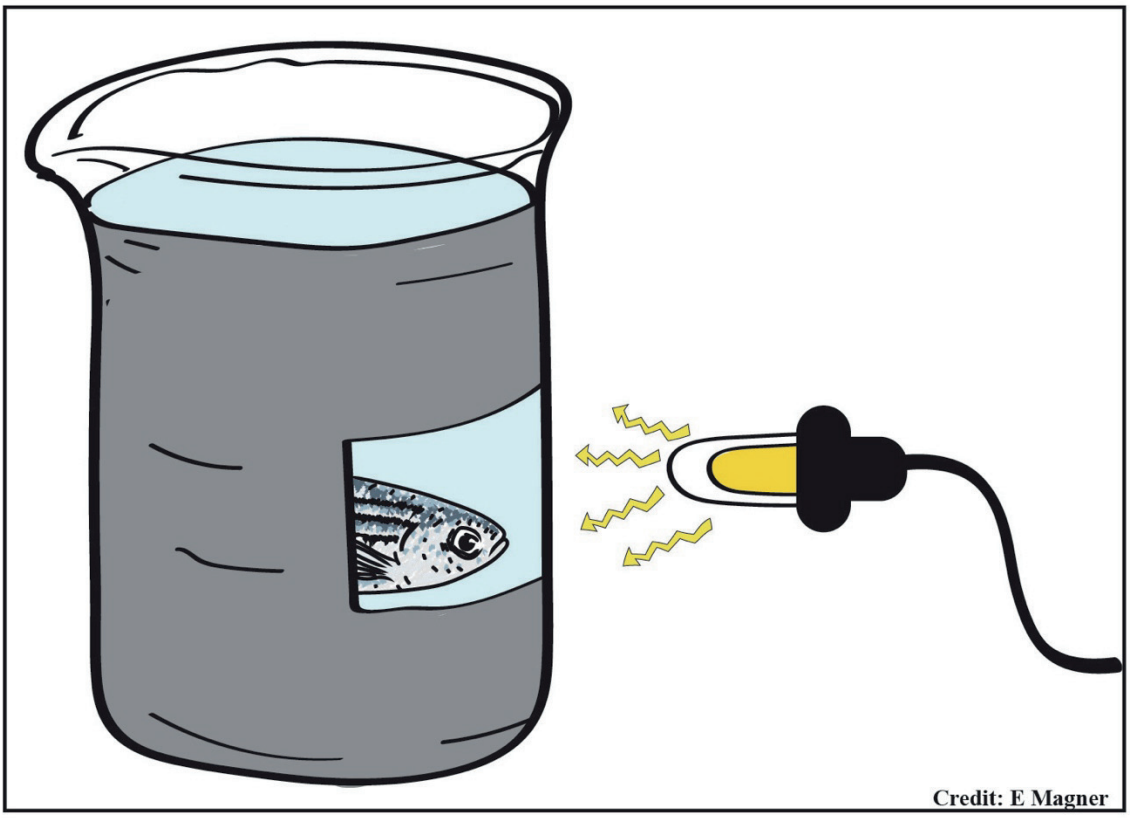


Figure 3. Photolytic Lesioning. Depicted above is the photolytic lesioning system used to lesion the retinas of zebrafish, effectively destroying photoreceptors. Up to three fish are placed in a 100-mL beaker. The beaker is wrapped in relective insulation with a window cut into the material to allow for high intensity light to penetrate. Fish are left exposed to high intensity light for 30 minutes. The entire system shown above is placed in a light box to contain the high-intensity light .

Tissue Collection and Preparation

Whole eyes were surgically harvested from sacrificed zebrafish and fixed in 4% paraformaldehyde. Over the next few days, the eyes were infiltrated with 20% sucrose before being frozen in optimal cutting temperature (OCT) compound. Tissue samples were stored at -80°C until sectioned. Embedded eyes were sectioned dorso-ventrally into 10 µm thick sections using a cryostat (Leica CM1520; Leica, Buffalo Grove, IL). Sectioned retinas were then thaw-mounted on glass slides and ready for subsequent immunolabeling procedures (Superfrost Plus;

Fischer Scientific, Pittsburgh, PA) for immunohistochemical and in-situ hybridization (Taylor et al 2015).

Labeling Using Immunohistochemistry, 5-Bromo-2'Deoxyuridine (BrdU), and Proliferating Cell Nuclear Antigen (PCNA)

Immunohistochemical labeling (IHC) and in-situ hybridization (ISH) were performed on the collected retinas of zebrafish (Hitchcock and Kakuk-Atkins 2004; Taylor et al 2015). For IHC procedures, antibodies localize specific antigens (proteins) in the sectioned tissue, based on their antigen-antibody interaction. Published protocols for immunolabeling were performed on the cross-sectioned retinas, through the central section of the retina (Luo et al 2012).

For immunohistochemical labeling, slides were incubated in 100°C sodium citrate buffer (10 mM sodium citrate,0.05% Tween 20, pH 6.0) for 30 min and then allowed to cool to room temperature for 20 minutes. Slides were then incubated with primary antibodies overnight at 4°C. The primary antibodies [ratio] used in this experiment were mouse anti-PCNA monoclonal antibody [1:200] (Sigma-Aldrich, St. Louis, MO), mouse anti-BrdU [1:50,1:100] (BD Biosciences, San Jose, CA), rabbit green fluorescent protein [1:1000] (ThermoFisher Scientific, Waltham, MA). Following primary antibody incubation, sections were washed three times in PBS/TX100 before incubation in the secondary antibody at room temperature. Secondary antibodies [ratio] were goat anti-mouse Alexa Fluor 488 [1:500] (Life Technologies, Carlsbad, CA), rabbit GFP 488 (1:500), and nuclei were counterstained using 20mM Hoeschst 33342 [1:1000] (Thermo Fischer Scientific, Waltham, MA).

Proliferating Müller glia and neural progenitors were labeled using 5-Bromo-2'Deoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA). PCNA more broadly labels proliferating cells due in part because it has a longer labeling duration and includes cells

from different phases. Cells in the G1, S, G2, and M stages all express PCNA at high levels and are easily labeled. Alternatively, BrdU only labels cells in the S phase, as this is when the molecule is taken up by cells and incorporated synthesized into the DNA. Furthermore, BrdU is more precisely controlled since the labeling duration is limited to the time of exposure. Therefore, we intended to use BrdU for more specific labeling of the progenitors producing rods and cones. BrdU was diluted into aquarium water and kept at room temperature. Fish, requiring the BrdU label, were subsequently exposed to 5 mM BrdU for 1 hr, immediately before collection. Immunolabeling, following exposure, proceeded with standard immunolabeling procedures on sectioned tissue (Luo et al 2012).

In-Situ Hybridization

Previously published studies concluded that in-situ hybridization is a more effective procedure for labeling and counting newly differentiated photoreceptors than immunolabeling (Taylor et al 2015). In-situ hybridization (ISH) procedures employ Digoxigenin(DIG)-labeled riboprobes generated from polymerase chain reaction (PCR) products. Developed probes label specific mRNA expressed in cells (David and Wedlich 2001). To concurrently identify cells in tissue sections that express EGFP, immunolabeling for EGFP followed ISH. Also, following ISH, immunolabeling for BrdU (5mM) or PCNA identified cells in S-phase of the cell cycle (Tang et al 2007).

DIG-labeled antisense riboprobes, for in-situ hybridization, were generated from PCR products. For similar primer lengths, a T3 polymerase promoter sequence (lowercase, underlined) was added on the reverse primer, while a T7 polymerase promoter sequence was added to the forward primer. In-situ hybridization with *rhodopsin (Rho)* was used to identify rod

photoreceptors, and *pde6c* was used to identify cone photoreceptors. Following primer generation, previously described in-situ hybridization protocols were performed.

Rhodopsin (Rod Photoreceptors)

T7F:taatacgactcactatagggGAGGGACCGGCATTCTACGTG

T3R:aattaaccctcactaaagggCTTCGAAGGGGTTCTTGCCGC

Pde6c (Cone Photoreceptors)

T7F: taatacgactcactatagggGGACGTGAGCCTGTCTGAAG

T3R: aattaaccctcactaaagggCCTGCCATCAGGAGTTCGG

Labeling for *miR-18a* in retinal sections was accomplished using a miRCURY LNA detection probe (Exiqon/Qiagen, Germantown, MD), which hybridized with mature *miR-18a.* The detection probe was designed with DIG-labels at the 5' and 3' ends. Once again, standard in-situ hybridization procedures followed as previously described, using a 1:200 concentration of the probe at a temperature of 55°C (Taylor et al 2019).

Cell Counts and Imagery

For imagery and cell counts, only central-dorso retinal sections containing optic nerve sections were used. All cell counts were done in three non-adjacent cross-sections of the eye and repeated in 3-4 eyes. This data acts as technical replicates. Each eye used was collected from different individual fish and therefore act as the biological replicates. Student's t-test and Analysis of Variance (ANOVA) were used to compare the cell counts between the mutant and wild-type fish.

Imaging the slides used a combination of bright light and fluorescent imaging. For epifluorescence microscopy, an Olympus AX70 microscope (Olympus, Tokyo, Japan) with an attached digital camera was used to photograph the slides. Images were taken at 10X and 20X

predominately; however, 40X was utilized for closer examination. CY3, FITC, and DAPI filters allowed for observation of different labeling techniques. Images were analyzed and cells counted using ImageJ software (National Institute of Health and the Laboratory for Optical and Computational Instrumentation, Bethesda, MD). Photoshop (Adobe, San Jose, CA) was subsequently used to merge images for comparing the location of expression in relation to other cells.

Dexamethasone Treatment

To further investigate the role of *miR-18a* in controlling inflammation and the innate immune response, we tested if chemical suppression of inflammation would rescue the phenotype in the *miR-18a* mutants. Recently published work demonstrates that inflammation can be pharmacologically suppressed in zebrafish using dexamethasone (Dex) (White et al 2017). Dexamethasone is a glucocorticoid receptor agonist used to suppress and identify inflammatory signaling as a factor that contributes to the central nervous system regeneration, particularly in zebrafish. For this experiment, a concentration of 15 mg/L (38 uM) Dex was used to chemically suppress inflammation. Initially, a stock solution of 15 g/L was dissolved in 0.1% methanol and stored in aliquots at -20°C. For treatment, aliquots were thawed in a water bath and diluted 1:1000 with aquarium system water to reduce shock (White et al 2017). In rescuing the phenotype, this suggests inflammation as the mechanism for control of cell cycle exit and differentiation during regeneration.

This experiment needed to repeat a second time due to a failure on the first attempt to rescue the phenotype in the *miR-18a* mutants successfully (Fig. 14). In the initial experiment, *miR-18a* and WT fish received treatment beginning at 4 dpi and continuing to 6 dpi, to coincide with peak expression of *pre-miR-18a*. Fish were exposed to BrdU (5mM) prior to

sacrifice and collected at 7 dpi. This timing was based on previous research and the understanding of differentiation occurring between days 4 and 5 post-injury. However, this first attempt did not have a significant effect on progenitor proliferation or the regeneration of photoreceptors in mutants; thus the experiment needed adjusting. In the second attempt to rescue the phenotype, the treatment period expanded to include earlier time points. Based on preliminary data and crucial periods during regeneration, we began treatment at 2 dpi and continued through 6 dpi. Once more, fish (n = 4 fish for each) were exposed to BrdU (5mM) and collected at 7 dpi. Control fish were exposed to a 0.01% methanol solution for the same period. Comparisons then used Student's t-test to analyze the data.

Statistics

Cell count analysis involved 3-4 biological replicates depending on the experiment. One eye from an individual fish was treated as a single biological replicate. Each experiment has the noted number of replicates (either 3 or 4) used for statistics, in the corresponding graphs. All biological replicate contained 3 technical replicates each. With technical replicates, cells were counted in 3 separate sections from the same eye. The statistical analysis for cell counts was calculated using a two-way ANOVA (SPSS, IBM, Armonk, New York) to determine if there were significant differences among treatments. Following this evaluation, we used a t-test (SPSS, IBM, Armonk, New York) for pairwise comparison. A p-value of $< 0.05$was considered significant for both measures.

RESULTS

Injury to the Retina Induces Expression of *miR-18a* in Retinal Cells

Before developing into their functional form, miRNAs exist in the form of precursor-miRNA (pre-miRNA) (Rajman and Schratt 2017). Preliminary data demonstrates the primary transcript *pre-miR-18a* is upregulated within 8 hours and eventually peaks 5 dpi in zebrafish. 3 dpi, expression of *miR-18a* has increased five-fold, and at 5 dpi, it has risen to a seven-fold increase. This time point is precisely when the photoreceptors are regenerating, but after 5 dpi, the expression begins to taper off. Figure 4 illustrates the expression levels of *pre-miR-18a* following photoreceptor damage.

Interestingly, the regenerative process of the eye follows a similar timeline. Around the 8 hr mark, Müller glia are in the early stages of asymmetrically dividing to produce a multipotent progenitor. Then at the 5 d mark, neural progenitors are beginning to differentiate into new photoreceptors. Therefore, *miR-18a* may regulate both crucial stages of regeneration.

We tested the working hypothesis that following an injury to the photoreceptors, *miR-18a* expression increases in the same manner as the *pre-miR-18a* expression in retinal tissue. ISH was used to determine if cells express *miR-18a* in a similar pattern as previously reported for *pre-miR-18a* following retinal injury. Fish (n = 3 for each) were collected at 0, 1, 3, 5, and 7 dpi. Following collection, ISH labeled the retinal sections for mature *miR-18a*. Our results reveal that mature *miR-18a* expression closely follows changes in *pre-miR-18a* expression in the retinas of WT fish (Fig. 4). Which, as previously mentioned, is consistent with *miR-18a* being involved in a MG guided regenerative response.

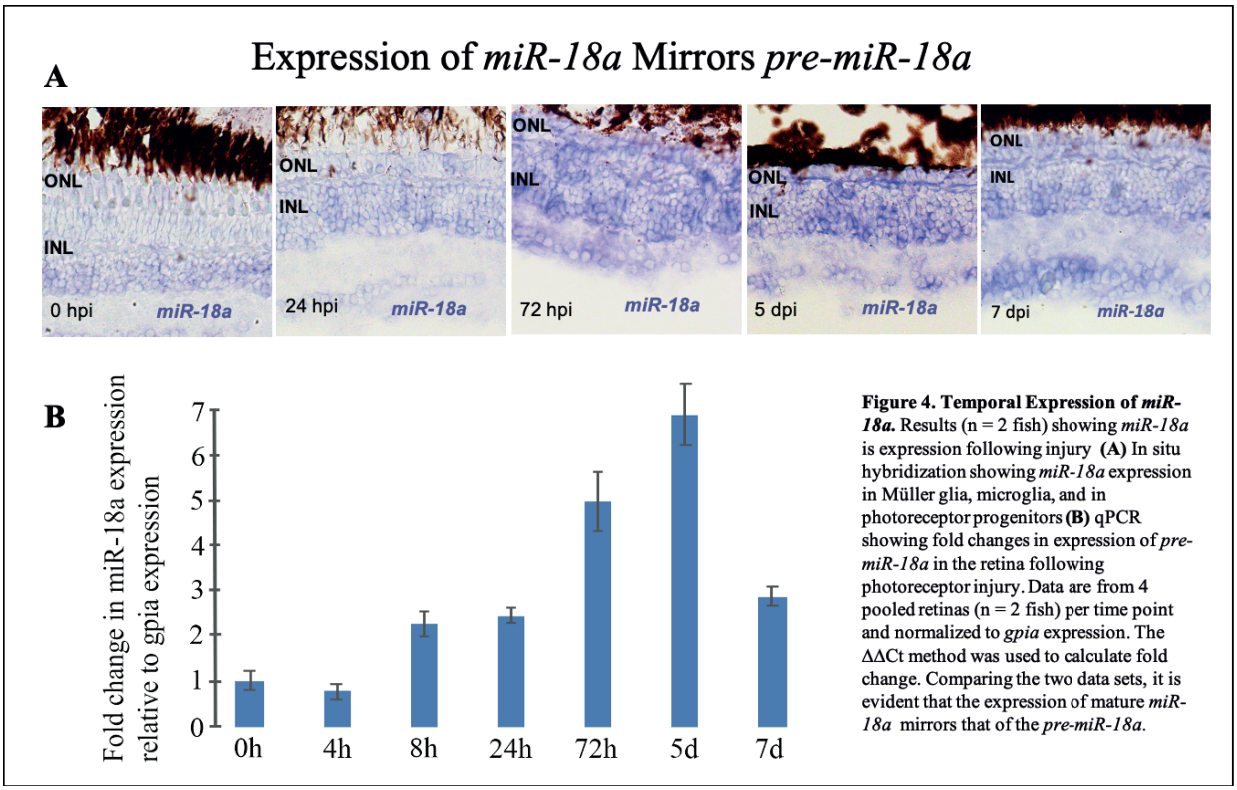


Figure 4. Temporal Expression of *miR-18a*. Results (n = 2 fish) showing *miR-18a* is expression following injury **(A)** In situ hybridization showing *miR-18a* expression in Müller glia, microglia, and in photoreceptor progenitors **(B)** qPCR showing fold changes in expression of *pre-miR-18a* in the retina following photoreceptor injury. Data are from 4 pooled retinas (n = 2 fish) per time point and normalized to *gpia* expression. The ΔΔCt method was used to calculate fold change. Comparing the two data sets, it is evident that the expression of mature *miR-18a* mirrors that of the *pre-miR-18a*.

Microglia, Müller Glia, and Müller Glia Derived Progenitors Express *miR-18a*

Following an injury to zebrafish photoreceptors, microglia and Müller glia (MG) are activated and begin to immediately respond to the stress (Nagashima et al 2013; Rashid et al 2018). Microglia begin by orchestrating the inflammatory response within the damaged tissue. This inflammatory response limits the damage and increases the survivability of the remaining cells (Madeira et al 2015). MG, in comparison, provide the damaged tissue with a population of multipotent progenitors. Functioning as stem cells within the retina, between about 24 and 48 hpi, each MG asymmetrically divides once to produce a neural progenitor near the ONL (Nagashima et al 2013). The previous results, showing that *miR-18a* expression increases substantially in the retina, and particularly in the inner nuclear layer where MG reside, suggests that *miR-18a* might play a role in the MG-mediated regeneration response.

To investigate this further, we tested the working hypothesis that following an injury to the photoreceptors, *miR-18a* expression increases in microglia, Müller glia, and photoreceptor progenitors. In order to determine the temporal and cellular expression of *miR-18a*, ISH was used to label cells expressing *miR-18a* at different post-injury time points, in transgenic fish (n = 3 for each) retinas in which Müller glia [Tg(*gfap:egfp*)] or microglia [Tg(*mpeg1:egfp*)] are labeled with green fluorescent protein (GFP). Fish eyes were collected at 0, 1, 3, and 5 dpi. In Tg(*gfap:egfp*) fish, MG strongly express GFP, but progenitors derived from MG also take some GFP with them but do not label as strongly. Distinctions between MG and progenitors were also identified as the progenitors migrated into the ONL while MG stayed within the INL. ISH on Tg(*gfap:egfp*) retinas identified *miR-18a* expression, which was followed by immunolabeling of GFP-labeled MG and progenitors. Using the same procedure on retinas from Tg(*mpeg1:egfp*) fish, ISH showed *miR-18a* expression relative to microglia (macrophages). Figure 5.1 demonstrates the temporal and spatial expression of *miR-18a* in microglia, Müller glia, and progenitors.

ISH (Fig. 5.1) illustrates the association between *miR-18a* expression and the location of microglia, MG, and MG progenitors. Microglia, following the regenerative response, express *miR-18a* at 3 dpi. The data further suggests, that prior to this time point and then beginning again at 5 dpi, the microglia do not express any level of *miR-18a*. In comparison, the MG express the gene at low levels in the unlesioned retina. Then by 3 dpi, both MG and the MG progenitors express the *miR-18a* at relatively higher levels. In comparing the two time points, expression levels increase following injury and demonstrate the shift in expression towards the progenitors. At 5 dpi, expression levels continue to increase, and labeling confirms expression in the MG and MG progenitors. These results indicate that the photoreceptor injury induces the

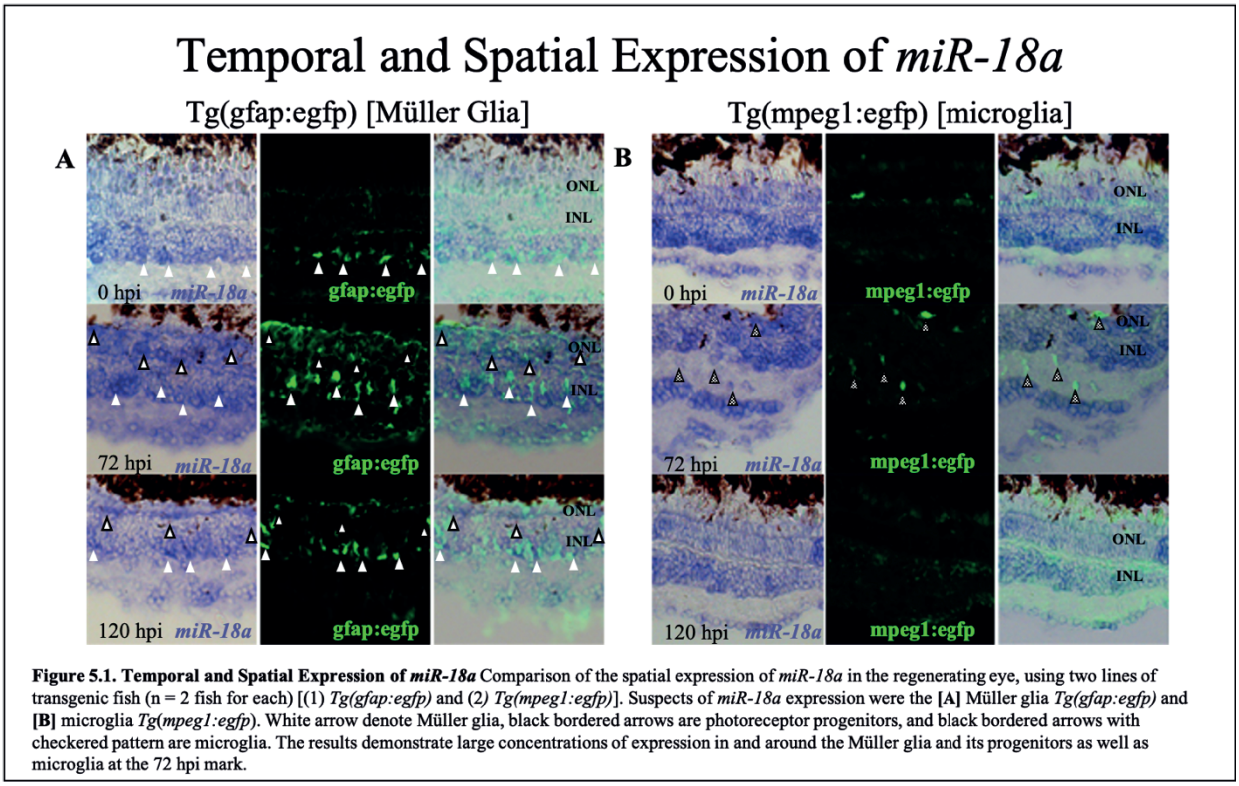


Figure 5.1. Temporal and Spatial Expression of *miR-18a* Comparison of the spatial expression of *miR-18a* in the regenerating eye, using two lines of transgenic fish (n = 2 fish for each) [(1) *Tg(gfap:egfp)* and (2) *Tg(mpeg1:egfp)*]. Suspects of *miR-18a* expression were the **[A]** Müller glia *Tg(gfap:egfp)* and **[B]** microglia *Tg(mpeg1:egfp)*. White arrow denote Müller glia, black bordered arrows are photoreceptor progenitors, and black bordered arrows with checkered pattern are microglia. The results demonstrate large concentrations of expression in and around the Müller glia and its progenitors as well as microglia at the 72 hpi mark.

expression of *miR-18a* in both microglia and Müller glia. Furthermore, this expression persists in the progenitors of the Müller glia. This expression suggests that *miR-18a* plays a significant role in the photoreceptor regeneration response in microglia, MG, and progenitors.

Tg(*gfap:egfp*) fish were used to investigate the expression of *miR-18a* in MG and MG progenitors through the entire regenerative process. To determine the expression of *miR-18a*, ISH was used to label cells expressing *miR-18a* at different post-injury time points in the Tg(*gfap:egfp*) fish. Fish eyes (n = 2 for each) were collected at 0, 1, 3, 5, 7, 10, and 14 dpi. Afterward, ISH localized *miR-18a* and immunolabeling identified GFP-labeled MG and MG progenitors. Figure 5.2 illustrates how expression starts rapidly after injury in the MG, around the time that they divide at 24 hpi, and continues to be expressed in MG and the progenitors through 5-7 dpi during the time that new photoreceptors differentiate. In the unlesioned retina (0 hpi), MG express *miR-18a* at nominal levels. Following injury, however, labeling shows that

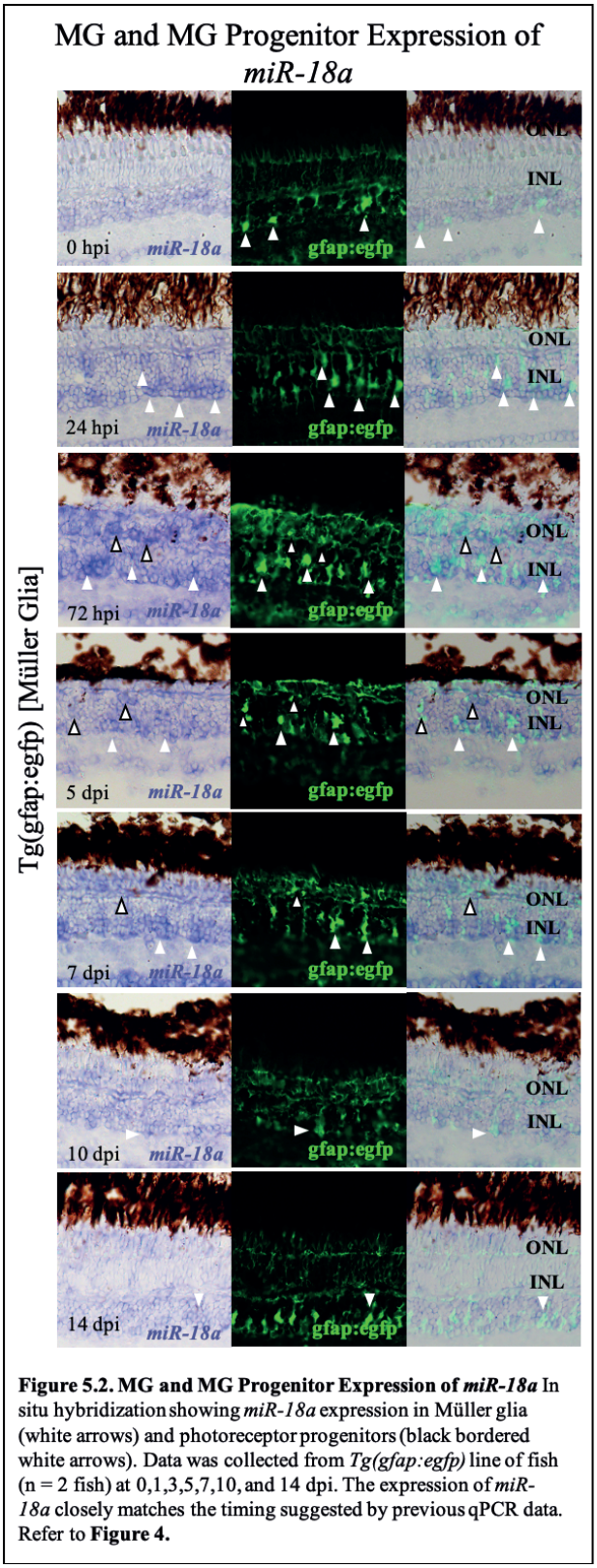


Figure 5.2. MG and MG Progenitor Expression of *miR-18a* In situ hybridization showing *miR-18a* expression in Müller glia (white arrows) and photoreceptor progenitors (black bordered white arrows). Data was collected from *Tg(gfap:egfp)* line of fish (n = 2 fish) at 0,1,3,5,7,10, and 14 dpi. The expression of *miR-18a* closely matches the timing suggested by previous qPCR data. Refer to **Figure 4.**

MG increase the expression levels by 24 hpi, as MG prepare to divide. At 72 hpi, *miR-18a* is strongly expressed in both the MG and the recently produced progenitors in the ONL. Expression of *miR-18a* continues through 5 dpi and into 7 dpi in the MG and the photoreceptor progenitors. However, by 7 dpi, we begin to see a decrease in expression. Following 7 dpi, progenitors (now mature photoreceptors) and MG begin to decrease expression levels of *miR-18a* and return to homeostatic states. At 10 and 14 dpi, the data shows that the only expressing cells are MG found in the outer edged of the INL once more. These results suggest *that miR-18a* is crucial in both the early and late stages of retinal regeneration, functioning in both MG and photoreceptor progenitors.

The Photoreceptor Regeneration Response is Substantially Altered in the *miR-18a* Mutants

Homozygous *miR-18a* mutants were created to further investigate the role of *miR-18a* in photoreceptor regeneration. These mutants, created using CRISPR/Cas9 gene editing, were exhibited to completely lack mature *miR-18a* (Taylor et al 2019). Preliminary data produced by the Taylor Lab showed that these *miR-18a* mutants exhibit approximately twice as many proliferating cells as the wild-type. Compared with wild-type fish immunolabeled for PCNA at 7 and 10 dpi, *miR-18a* mutants have a drastic increase in proliferating cells (Fig. 6). Combined with the data on *pre-miR-18a* expression and expression in the MG and progenitors from the time of injury to differentiation, these findings support the hypothesis *miR-18a* plays a critical role in the mechanisms that regulate both early and late stages of the cell cycle and differentiation.

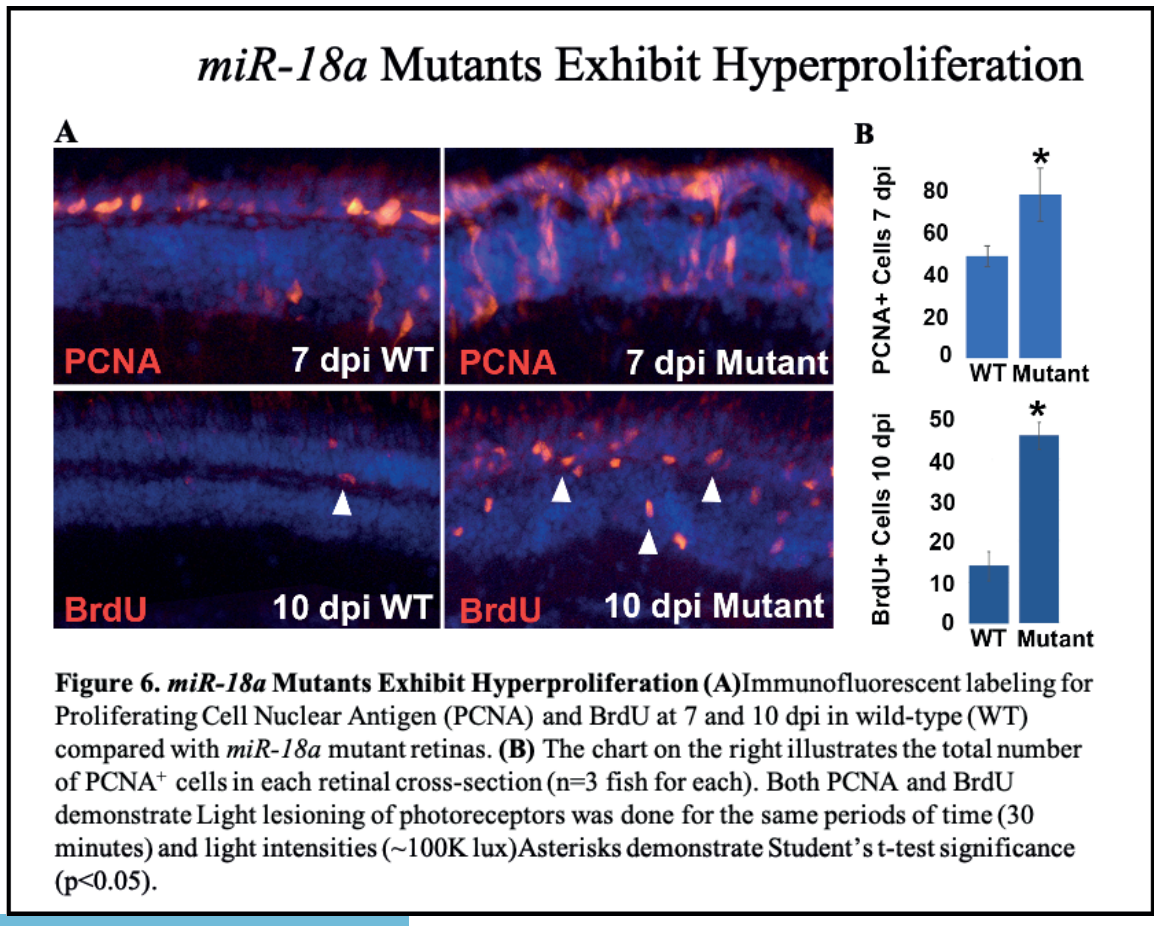


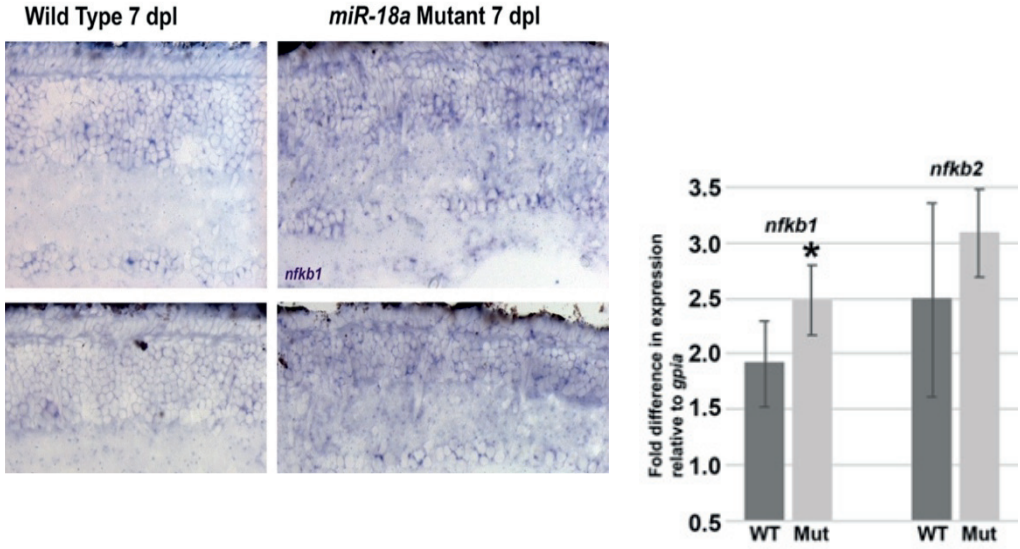
Figure 6. *miR-18a* Mutants Exhibit Hyperproliferation (A)Immunofluorescent labeling for Proliferating Cell Nuclear Antigen (PCNA) and BrdU at 7 and 10 dpi in wild-type (WT) compared with *miR-18a* mutant retinas. **(B)** The chart on the right illustrates the total number of PCNA^{+} cells in each retinal cross-section (n=3 fish for each). Both PCNA and BrdU demonstrate Light lesioning of photoreceptors was done for the same periods of time (30 minutes) and light intensities (~100K lux)Asterisks demonstrate Student's t-test significance ($p<0.05$).

Inflammation is Prolonged in *miR-18a* Mutants

In the retina, photoreceptor injury and death induce an innate immune response to limit the damage and initiate tissue recovery (Arroba et al 2018). As photoreceptors are damaged and die-off, studies show, the surrounding cells begin a rapid expression of genes associated with inflammation. Cytokines are one group of pro-inflammatory signals released by the microglia (macrophages) during this event. Such cytokine signals induce a prompt inflammatory response, which is required for photoreceptor regeneration (Madeira et al 2015). As inflammation rises in the tissue, MG nuclei migrate towards the ONL and enter the cell cycle. Microglia, during this time, begin to proliferate and migrate towards the damaged area (Nelson et al 2013; Rashid et al 2018).

In zebrafish retinas, this type of inflammatory cytokine signaling initiates the immune response and the regenerative process (Silva et al 2020). Past investigations have shown, *tnf-a, tnf-b, nfkb1*, and *nfkb2* are all linked to the regeneration of tissue in zebrafish (Gorsuch and Hyde 2013; Nelson et al 2013; Karra et al 2015). Moreover, the timing of these inflammatory mediators' expression closely follows the well-documented timing of retinal regeneration (Fig. 2). Past studies demonstrate that inflammatory gene expression shadows that of the Müller glial response, progenitor proliferation, and photoreceptor regeneration (Gorsuch and Hyde 2013). However, while inflammation initiates the regeneration, sustained inflammation is detrimental to the retina, as with mammals. Zebrafish have adapted mechanisms of control over inflammation, such as through the use of miRNAs, to prevent chronic inflammation (Rajaram et al 2014; Taylor et al 2019).

Online database predictions (TargetScanFish, MIT, Cambridge, MA) indicate *miR-18a* may regulate mRNA involved in several inflammation pathways, including the transforming growth factor-beta (*TGFβ*) signaling pathway and the tumor necrosis factor (*TNFα*) pathway. With indications of interactions with the inflammatory pathways, we needed to determine if the lack of *miR-18a* resulted in an altered inflammatory response. To test this, we compared the expression of *nfkb1* and *nfkb2* in wild-type and mutant fish. The results indicate that in the absence of *miR-18a,* inflammatory markers, and therefore inflammation, are significantly elevated. While *nfkb1* and *nfkb2* have been shown crucial to neuroregeneration, past studies indicate that persistent inflammation may lead to hyperproliferation (Gorsuch and Hyde 2013; Karra et al 2015; Silva et al 2020). In comparison to the wild-type, *miR-18a* mutant fish express



expression of *nfκB1* (top) and *nfκB2* (bottom) at 7 dpi in wild type (WT) and *miR-18a* mutant retinas (n = 3 fish for each). **(B)** Quantitative PCR showing significantly higher expression of *nfκB1* at 7 dpi in miR-18a mutant retinas. *Student's t-test ($p<0.05$), expression calculated using t.

the inflammatory genes *nfkb1* and *nfkb2* longer (Fig. 7), indicating *miR-18a* functions by controlling inflammation.

Proliferation and Photoreceptor Survivability Require *miR-18a*

At 7 dpi, *miR-18a* mutants exhibit a significant increase in proliferating cells as compared to wild-type fish. However, is it was not well understood if and how photoreceptor regeneration and progenitor differentiation in *miR-18a* mutants differs from the wild-type. To determine if hyperproliferation of cells persisted and affected the regeneration and survivability of photoreceptors due to a lack of *miR-18a*, the regeneration of rods and cones in *miR-18a and wild-type were compared.* ISH labeled rods and cones in both the wild-type and *miR-18a* mutants collected at 7 and 14 dpi. By comparing the mutants and wild-type, this tested the hypothesis that *miR-18a* mutant fish regenerate different proportions of photoreceptors due to the lack of a functioning miRNA. In comparing rod regeneration, our data suggest that *miR-18a* is involved in differentiation and survivability. In the 7 dpi

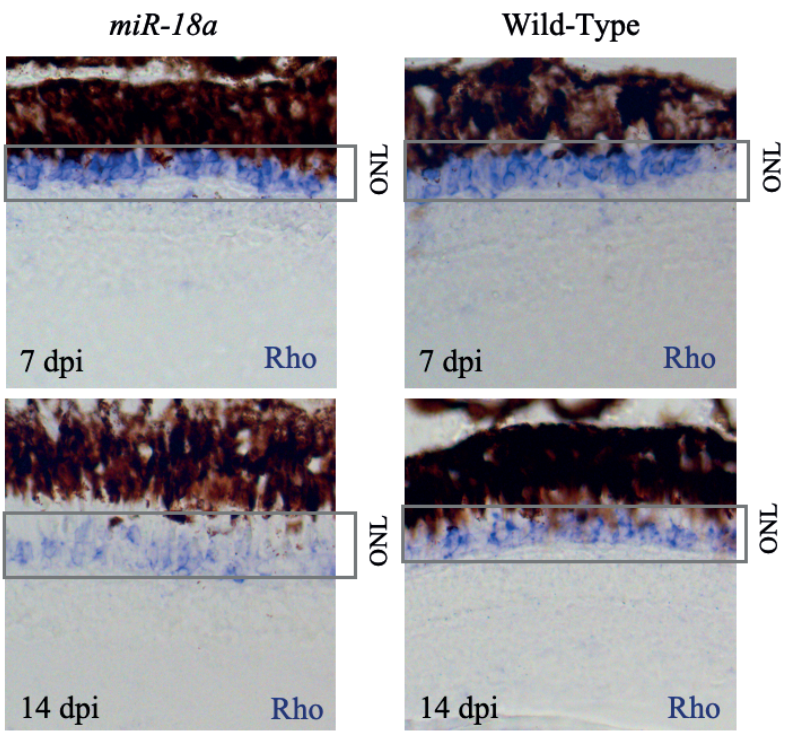


Figure 8.1. Rod Regeneration in *miR-18a* Mutants ISH demonstrates that *miR-18a* mutant fish (n = 2 fish for each) can regenerate mature rods (*Rho*), following lesioning. As compared to the wild-type, the mutants appear to regenerate fewer rods by 14 dpi.

fish, both *miR-18a* and wild-type have similar numbers of regenerated rod photoreceptors. However, at 14 dpi, the *miR-18a* mutants present fewer remaining rods than the wild-type. *miR-18a* mutants additionally exhibit a thicker ONL than the wild-type. Figure 8.1 illustrates the differences seen in labeling for rods. Figure 8.2, in contrast, illustrates the regeneration of cones in the mutants and wild-type. In the 7 dpi retinas, the labeling shows that the relative number and density of cone photoreceptors between the mutant and wild-type fish are similar. At this point in recovery, even the ONL of the two is similar. Nevertheless, by 14 dpi, the effect of hyperproliferation is apparent. Cones in the *miR-18a* mutants at 14 dpi are more densely packed, and the ONL is 45% thinker. These data indicate proper proliferation, differentiation, and survivability of mature photoreceptors require *miR-18a*. Additionally, it suggests *that miR-18a* regulates the differentiation and survivability of rods and cones differently.

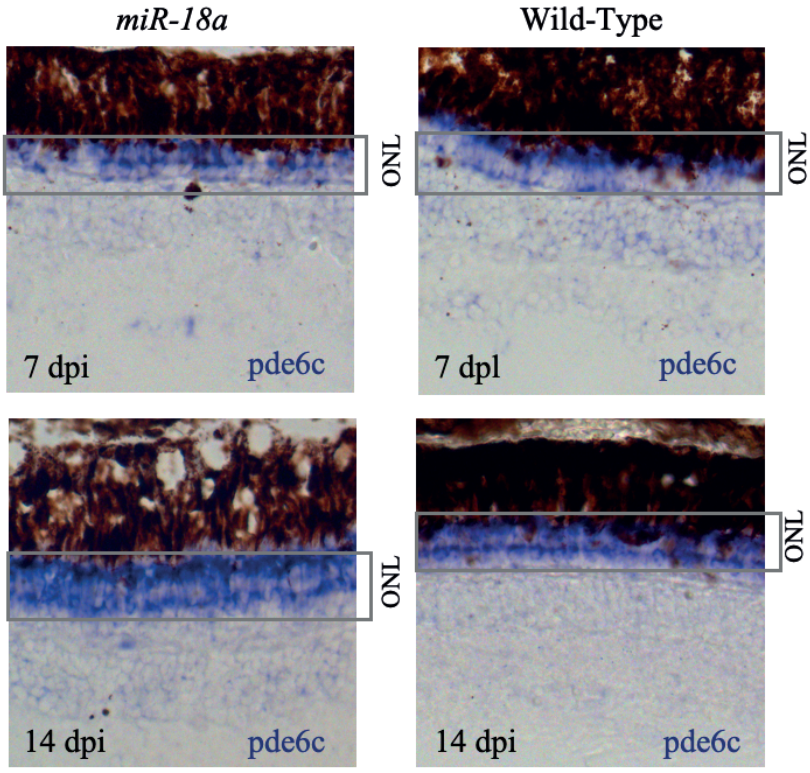


Figure 8.2. Cone Regeneration in *miR-18a* Mutants ISH demonstrates that *miR-18a* mutant fish (n = 2 fish for each) can regenerate mature cones (*pde6c*), following lesioning. As compared to the wild-type, the mutants appear to regenerate cones at different proportions. Mutants appear to produce more cones than wild-type fish.

Suppressing Inflammation Rescues the Aberrant Cell Proliferation in *miR-18a* Mutants

The final experiment of this study tested the working hypothesis that *miR-18a* controls photoreceptor regeneration by regulating inflammation. Figure 7 demonstrated that *miR-18a* mutants exhibit the inflammatory mediators longer. Preliminary data suggested that *miR-18a* regulates the proliferation of progenitors, exit from the cell cycle, and differentiation. *miR-18a* mutants, following injury, exhibit inflammation markers (*nfkb1* and *nfkb2*) for more extended periods and produce fewer mature photoreceptors than AB wild-type fish, as a result. The regulation of inflammation occurs through intracellular signaling pathways. Signaling cells secrete cytokines and other molecules, which consequently activate post-signaling mechanisms (Wan et al 2014; Arroba et al 2018). To examine if *miR-18a* primarily regulates the regenerative response through the suppression of inflammation, we used the chemical suppression of inflammation to impact the photoreceptor replacement. Broad-spectrum inflammation inhibition used at points vital to the regenerative process should regulate the regeneration, if it is a control mechanism.

These results suggest that *miR-18a* works to regulate and suppress inflammation during regeneration. Therefore, it is critical to determine if *miR-18a* is required to regulate regeneration through inflammation suppression. To define the necessity of *miR-18a*, we attempted to rescue the mutant phenotype using inflammation inhibitors.

To determine if suppressing inflammation rescues the mutant phenotype, IHC compared the number of proliferating cells between mutant and wild-type fish at 7 dpi. This collection time point was chosen because most photoreceptor progenitors have typically exited the cell cycle by this stage, and our earlier experiments showed substantially increased cell proliferation in *miR-*

18a mutants at 7 dpi. Here the number of proliferating cells was used to illustrate the transition of exiting the cell cycle and differentiating into mature photoreceptors.

We explored the overall effect of inflammation suppression by investigating the reduction or increase of proliferating progenitors as a result of chemical suppression. As we saw earlier (Fig. 6) in the data, *miR-18a* mutants regularly exhibit hyperproliferation. However, in this final experiment, we aimed to suppress inflammation and rescue the phenotype in the mutants. With proper inflammatory regulation, the goal was to reduce the proliferating progenitors and regenerate standard populations of rod and cone photoreceptors.

Figure 9.1 shows the labeling and comparison for PCNA labeled cells. The data demonstrate that *miR-18a* mutants treated with Dex, show a significant reduction in the number of proliferating progenitors. When compared to the wild-type control, the Dex treated *miR-18a* fish are not significantly different in proliferation, showing a return to normal proliferation. This supports the hypothesis of rescuing the phenotype, as it reports a significant reduction in

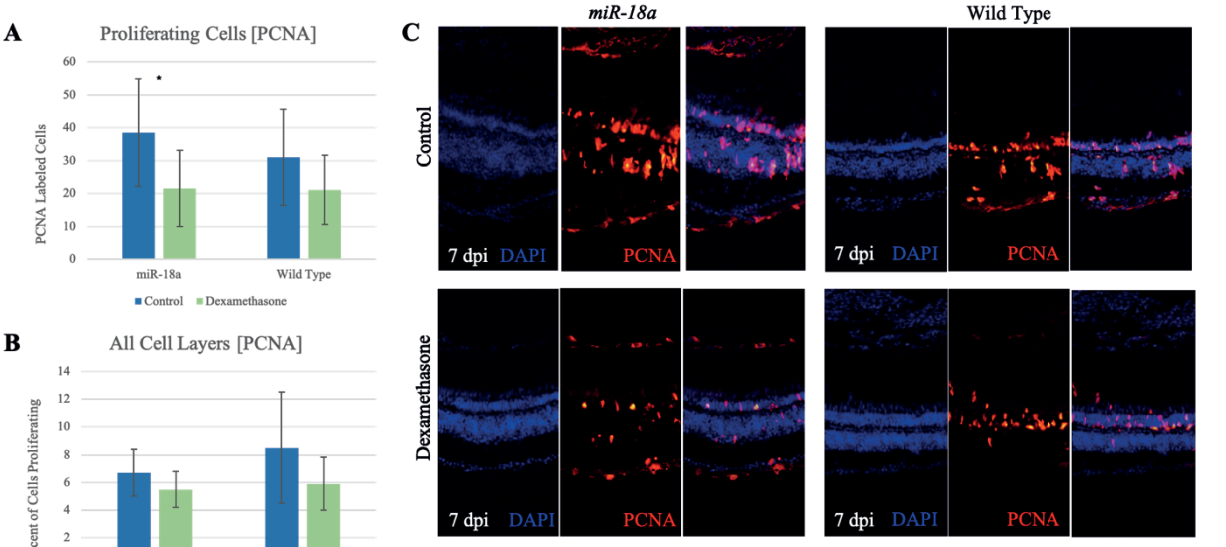


Figure 9.1. Total Proliferating Cells *[PCNA]* Proliferating cells in mutant and WT fish (n = 3 fish for each). **(A)** The number of proliferating cells in the injured retina. Dexamethasone significantly effected the number of proliferating cells in the *miR-18a* fish (*), overall seeing a reduction. Error bars represent standard deviation and data was compared using a Student's t-test and asterisks indicate $p < 0.05$. **(B)** Comparison of the percent of cells proliferating in the outer nuclear layer. **(C)** IHC using DAPI and PCNA to illustrate the divisions and cell cycles of injured retina.

proliferation which closely follows the proliferation of wild-type fish. Further supporting these results is the data from the BrdU labeling. Figure 9.2 illustrates the labeling that resulted from BrdU labeling and the number of labeled cells in each of the treatment and control groups. In comparing the results of inflammation suppression between treated and control groups, BrdU labeled cells demonstrate a significant reduction in proliferation in the *miR-18a* mutants and a return to typical proliferation, as seen in WT fish. Dex treated mutants demonstrated both a reduction in the number of proliferating cells and the proportion of cells proliferating. Figure 9.2 demonstrates that control groups for each fish begin significantly different. Then, following Dex treatment, there is a significant reduction in the proliferation. This reduction in proliferation restores levels to that of the WT. This is some of the most robust evidence for rescuing the phenotype. These results suggest that, following injury, the lack of *miR*-18a causes the loss of regulation over inflammation. Losing the ability to control inflammation then results in unregulated proliferation. However, by suppressing inflammation, we see a rescue of the

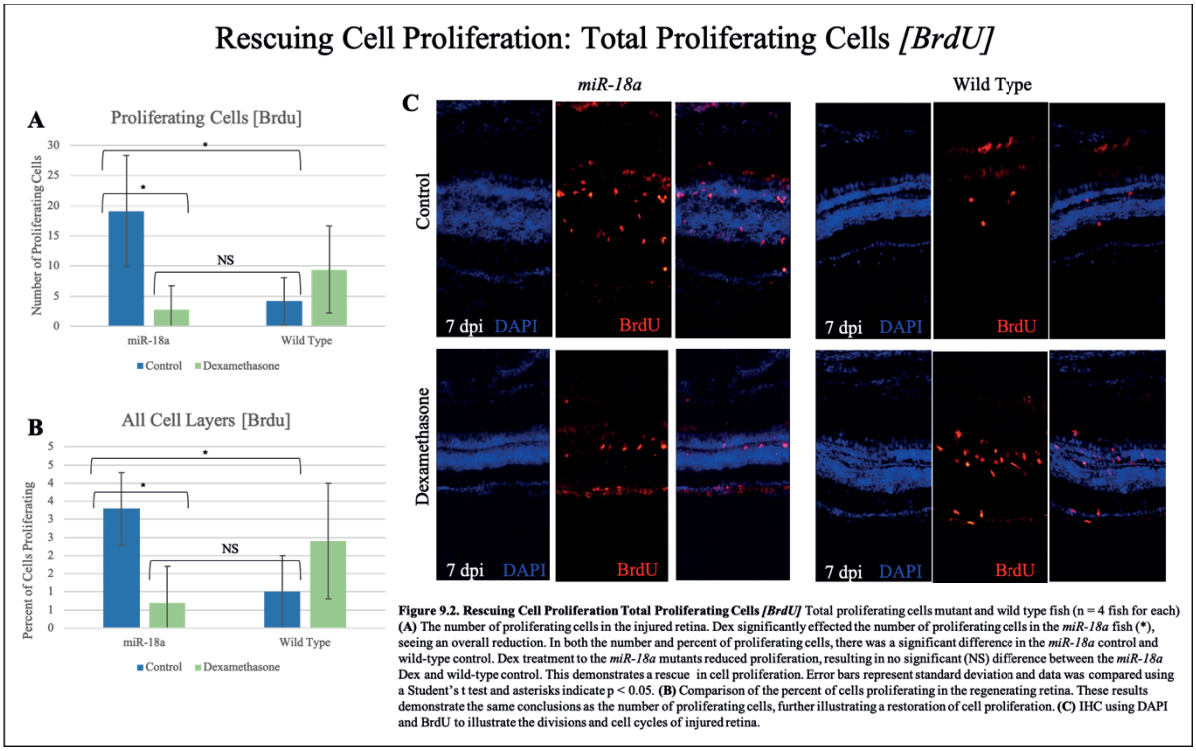


Figure 9.2. Rescuing Cell Proliferation Total Proliferating Cells *[BrdU]* Total proliferating cells mutant and wild type fish (n = 4 fish for each) **(A)** The number of proliferating cells in the injured retina. Dex significantly effected the number of proliferating cells in the *miR-18a* fish (*), seeing an overall reduction. In both the number and percent of proliferating cells, there was a significant difference in the *miR-18a* control and wild-type control. Dex treatment to the *miR-18a* mutants reduced proliferation, resulting in no significant (NS) difference between the *miR-18a* Dex and wild-type control. This demonstrates a rescue in cell proliferation. Error bars represent standard deviation and data was compared using a Student's t test and asterisks indicate $p < 0.05$. **(B)** Comparison of the percent of cells proliferating in the regenerating retina. These results demonstrate the same conclusions as the number of proliferating cells, further illustrating a restoration of cell proliferation. **(C)** IHC using DAPI and BrdU to illustrate the divisions and cell cycles of injured retina.

aberrant proliferation to control levels. These results indicate that the increased/prolonged inflammation observed in *miR-18a* mutants is responsible for the increased cell proliferation in the injured retinas of these fish, and that by regulating [suppressing] inflammation we were able to restore cell proliferation to WT levels.

Suppressing Inflammation Partially Rescues Photoreceptor Differentiation in *miR-18a* Mutants

To determine the effect inflammation and *miR-18a* has on the differentiation of mature photoreceptors, we investigated the regeneration of rod and cones following inflammation suppression. Using ISH, rods and cones were labeled in *miR-18a* mutants and wild-type fish. The numbers of regenerated photoreceptors were then compared. Figure 10.1 illustrates the results of attempting to rescue the regeneration of rods in *miR-18a* mutants. The results show that there was no significant difference in the number of rods regenerated in the *miR-18a* mutants between

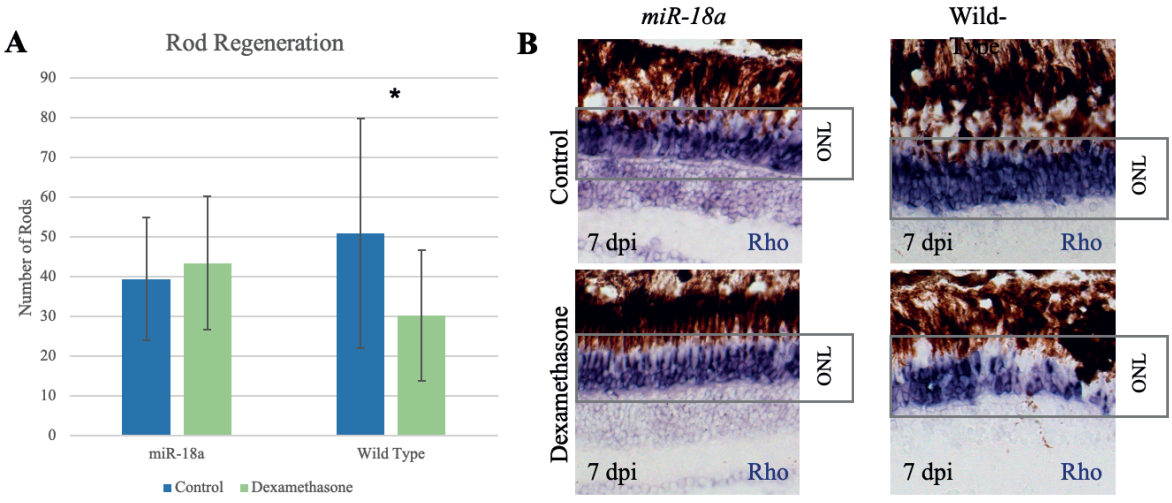


Figure 10.1. Rescue of Rod Regeneration in *miR-18a* Mutants (A) Number of rods regenerated in *miR-18a* and wild-type (n = 4 fish for each) fish following treatment with dexamethasone. Dexamethasone significantly effected the regeneration of rods in WT fish (*). No significant difference in the regeneration of rods in the *miR-18a* fish, although it appears the migration of mature rods into the ONL has become more efficient. Error bars represent standard deviation and data was compared using a Student's t test and asterisks indicate $p < 0.05$. **(B)** ISH, using *Rho*, comparing mature rod photoreceptors in the ONL of 7 dpi *miR-18a* and wild-type eyes.

control and Dex treated fish, though numbers did trend upward following Dex treatment. Of additional note, the organization of rods within the ONL of Dex treated mutants appears more organized and more fully differentiated. In contrast, the wild-type fish demonstrated a significant reduction in the number of rods produced at 7 dpi, following treatment with Dex.

In comparison, Figure 10.2 illustrates the regeneration of cones in the mutant and wild-type fish following the suppression of inflammation. When compared to the results for rod regeneration, these results show a different outcome. In the *miR-18a* mutants, dexamethasone treatment from 2-6 dpi significantly increases the number of cones regenerated at 7 dpi. While wild-type cones are also regenerated at higher numbers following inflammation suppression, they are not significant. These results suggest that while affecting the differentiation of rods and cones differently, *miR-18a* regulates the regeneration of photoreceptors primarily through inflammation suppression.

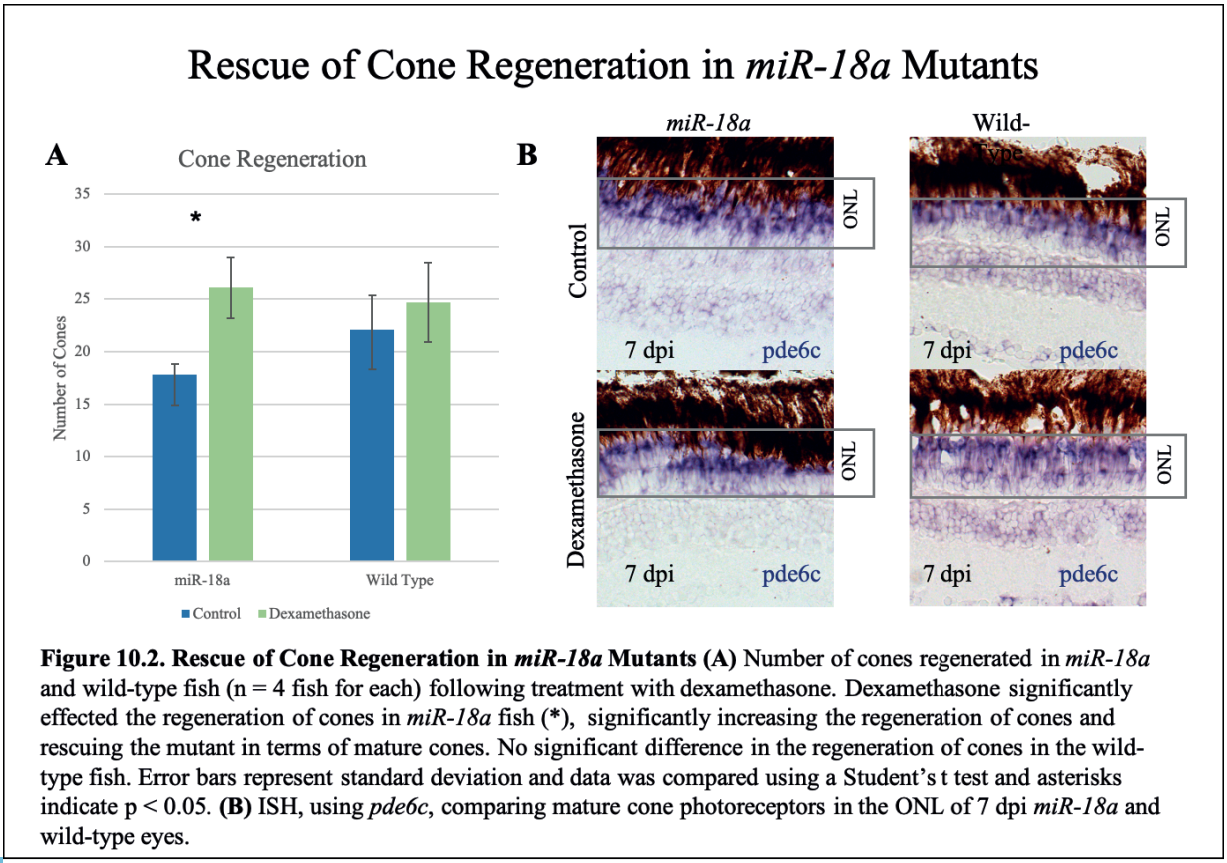


Figure 10.2. Rescue of Cone Regeneration in *miR-18a* Mutants (A) Number of cones regenerated in *miR-18a* and wild-type fish (n = 4 fish for each) following treatment with dexamethasone. Dexamethasone significantly effected the regeneration of cones in *miR-18a* fish (*), significantly increasing the regeneration of cones and rescuing the mutant in terms of mature cones. No significant difference in the regeneration of cones in the wild-type fish. Error bars represent standard deviation and data was compared using a Student's t test and asterisks indicate $p < 0.05$. **(B)** ISH, using *pde6c*, comparing mature cone photoreceptors in the ONL of 7 dpi *miR-18a* and wild-type eyes.

Retinal Call Layers are Differentially Affected by Dexamethasone Treatment in *miR-18a* Mutants and Wild-Type

To further investigate the claims that Dex suppresses inflammation and regulates the proliferation and differentiation of progenitors, the counted cells were identified by the layer they were found in (GCL, INL, or ONL). The comparison between the layers allowed for a closer examination of the role of *miR-18a* and the advancement of progenitors toward differentiation. Working through the three layers, the data shows that the treatment of dexamethasone affected cell proliferation in each layer differentially. Beginning in the ganglion cell layer, PCNA labeling showed a significant difference in the number of cells proliferating in the wild-type fish, following treatment. The *miR-18a* mutants, however, were not significantly affected by the treatment (Fig. 11.1). PCNA broadly labels cells in the G1, S, G2, and M phases. Therefore, we have used it as a more general account of the results while relying more on BrdU. BrdU is more specific in labeling the S phase of the cell cycle and therefore illustrates a more precise window of events. BrdU labeling in the ganglion cell layer showed a significant reduction in the number of proliferating cells in the *miR-18a* mutants as a result of suppressing inflammation. Past investigations have neglected this layer of the retina in regeneration, but we aim to demonstrate that it remains affected through regeneration.

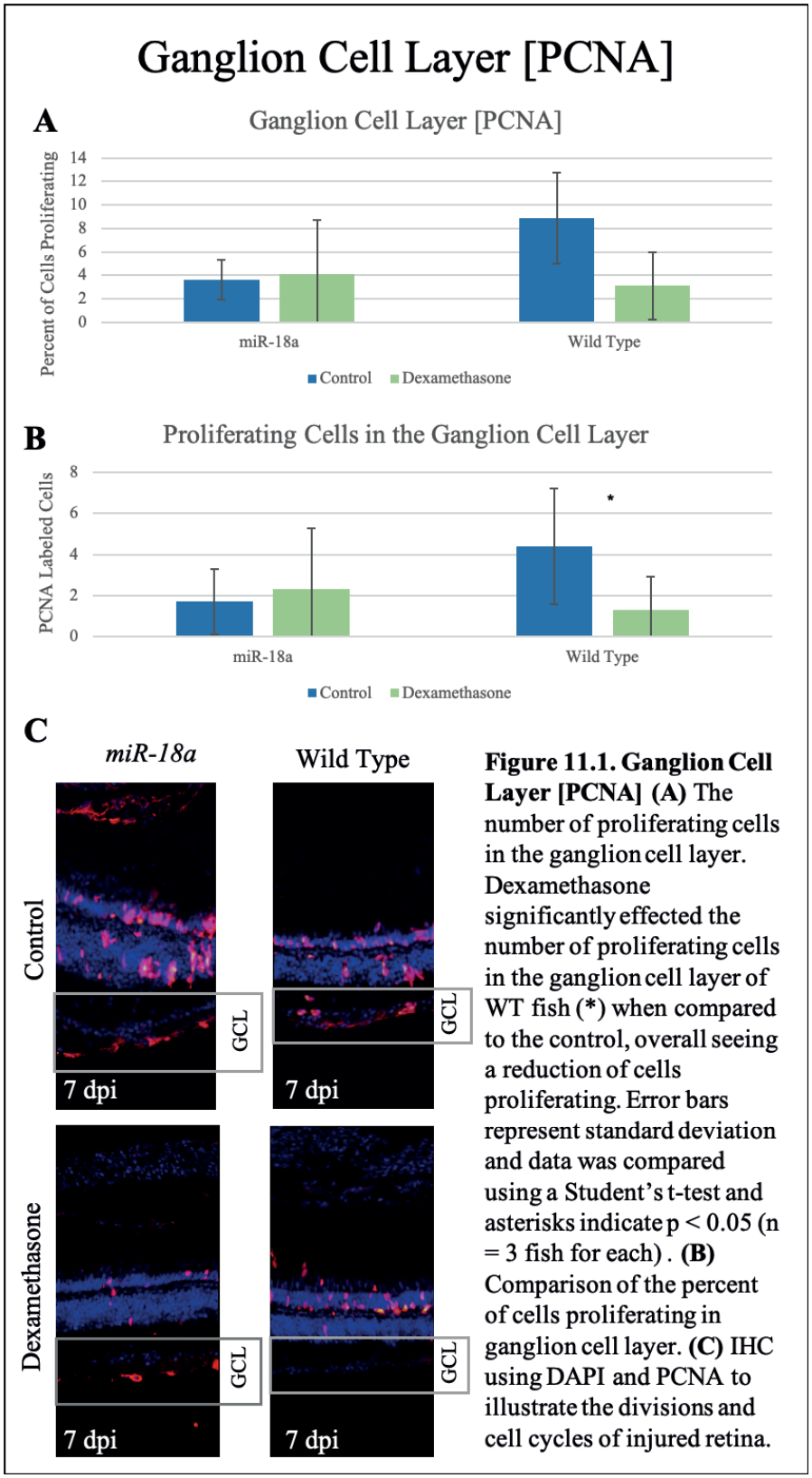


Figure 11.1. Ganglion Cell Layer [PCNA] (A) The number of proliferating cells in the ganglion cell layer. Dexamethasone significantly effected the number of proliferating cells in the ganglion cell layer of WT fish (*) when compared to the control, overall seeing a reduction of cells proliferating. Error bars represent standard deviation and data was compared using a Student's t-test and asterisks indicate $p < 0.05$ ($n = 3$ fish for each) . **(B)** Comparison of the percent of cells proliferating in ganglion cell layer. **(C)** IHC using DAPI and PCNA to illustrate the divisions and cell cycles of injured retina.

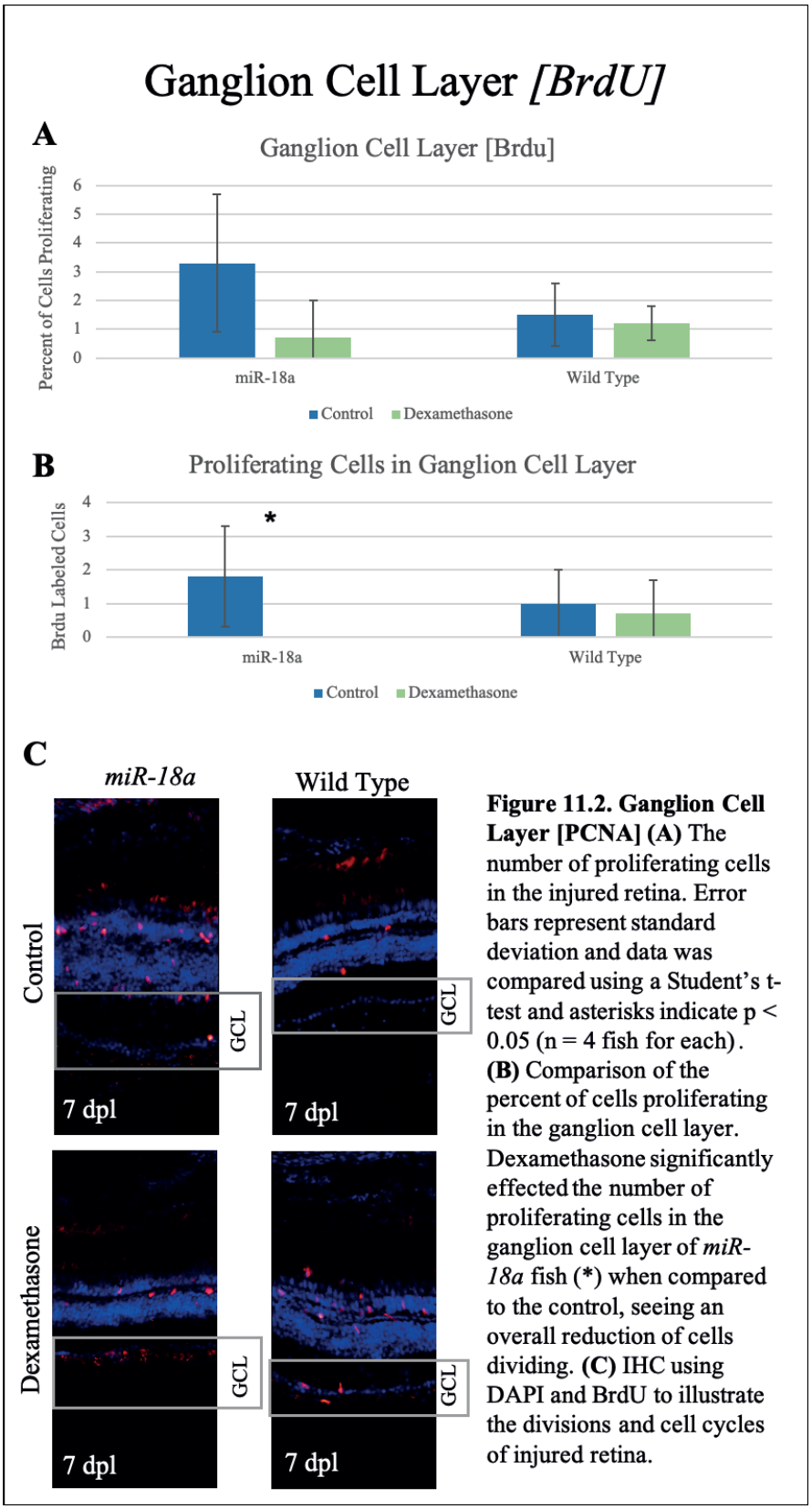


Figure 11.2. Ganglion Cell Layer [PCNA] (A) The number of proliferating cells in the injured retina. Error bars represent standard deviation and data was compared using a Student's t-test and asterisks indicate $p < 0.05$ ($n = 4$ fish for each). **(B)** Comparison of the percent of cells proliferating in the ganglion cell layer. Dexamethasone significantly effected the number of proliferating cells in the ganglion cell layer of *miR-18a* fish (*) when compared to the control, seeing an overall reduction of cells dividing. **(C)** IHC using DAPI and BrdU to illustrate the divisions and cell cycles of injured retina.

The inner nuclear layer of the retina is host to a bulk of the cell types in the retina, including the MG. Once the MG divide initially, progenitors begin to proliferate in the INL prior to migrating to the ONL and differentiating into rod and cone photoreceptors. Figure 12.1 demonstrates PCNA labeling in the inner nuclear layer, and the effect dexamethasone had on the mutants and wild-type. According to this data, inflammation suppression significantly reduced the number of proliferating cells in the *miR-18a* mutants and wild-type fish. BrdU labeling further supports these findings. Treatment with Dex significantly reduced the number and the proportion of cells proliferating in the INL. Figure 12.2 illustrates the reduction of proliferating cells in the INL of *miR-18a* mutants. Additionally, although not significant, there was a trend of an increased proportion of cells proliferating in the inner nuclear layer of wild-type fish. The INL of the Dex treated mutants also demonstrated a reduced thickness in the INL and a more orderly arrangement.

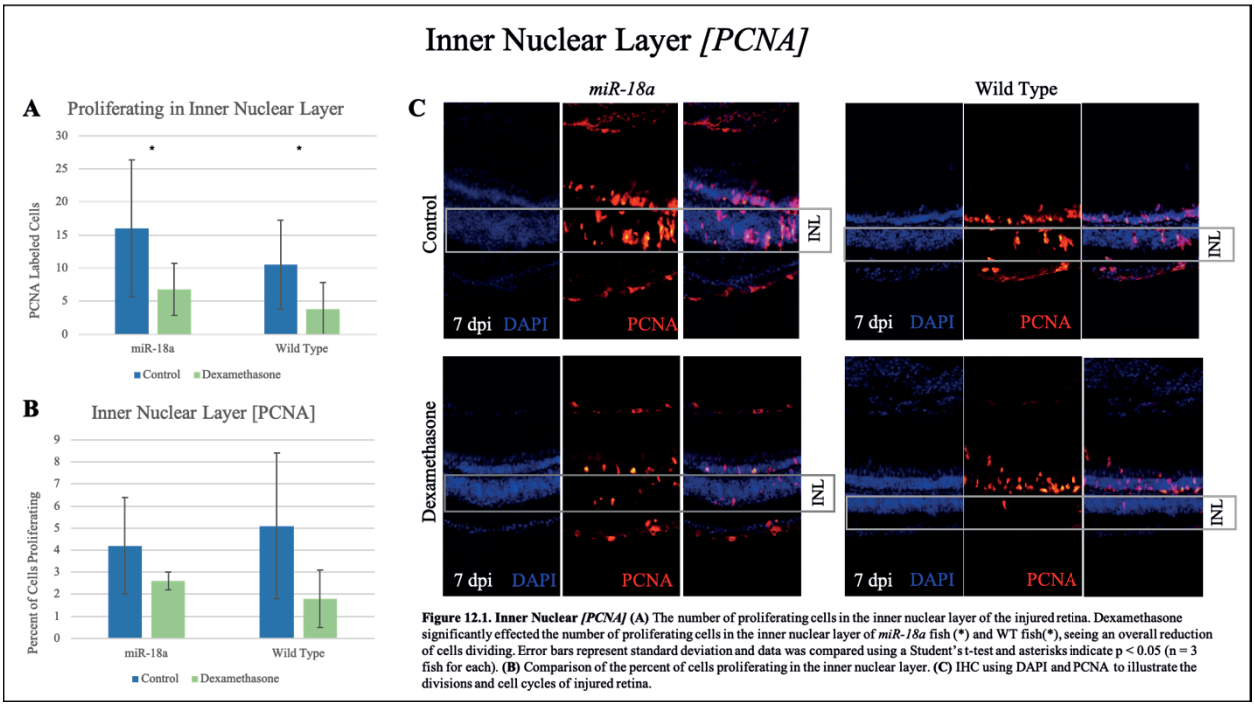


Figure 12.1. Inner Nuclear *[PCNA]* (A) The number of proliferating cells in the inner nuclear layer of the injured retina. Dexamethasone significantly effected the number of proliferating cells in the inner nuclear layer of *miR-18a* fish (*) and WT fish(*), seeing an overall reduction of cells dividing. Error bars represent standard deviation and data was compared using a Student's t-test and asterisks indicate $p < 0.05$ ($n = 3$ fish for each). **(B)** Comparison of the percent of cells proliferating in the inner nuclear layer. **(C)** IHC using DAPI and PCNA to illustrate the divisions and cell cycles of injured retina.

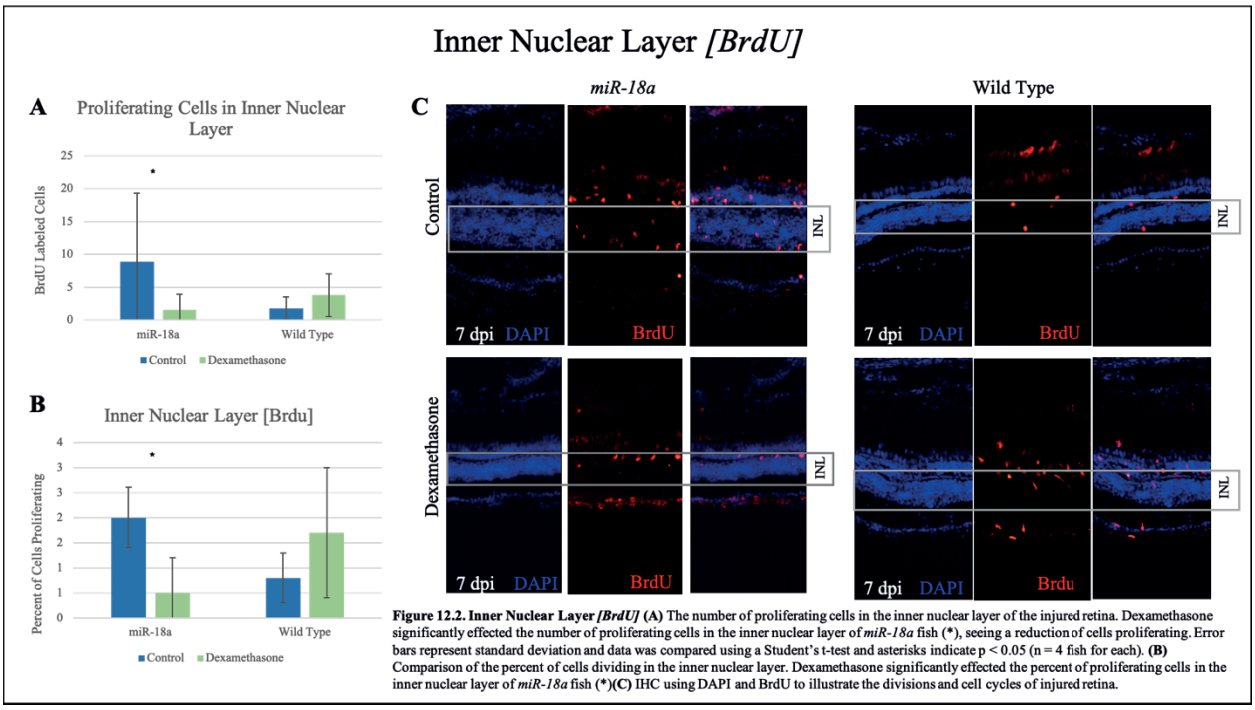


Figure 12.2. Inner Nuclear Layer *[BrdU]* (A) The number of proliferating cells in the inner nuclear layer of the injured retina. Dexamethasone significantly effected the number of proliferating cells in the inner nuclear layer of *miR-18a* fish **(*)**, seeing a reduction of cells proliferating. Error bars represent standard deviation and data was compared using a Student's t-test and asterisks indicate $p < 0.05$ ($n = 4$ fish for each). **(B)** Comparison of the percent of cells dividing in the inner nuclear layer. Dexamethasone significantly effected the percent of proliferating cells in the inner nuclear layer of *miR-18a* fish **(*)(C)** IHC using DAPI and BrdU to illustrate the divisions and cell cycles of injured retina.

The outer nuclear layer of the retina is one of the more critical layers in this study, as it harbors the newly regenerated, differentiating and mature rod and cone photoreceptors. As progenitors begin to differentiate, they migrate towards the ONL and eventually into it (Lenkowski et al 2013). Comparing the number of proliferating cells in this layer illustrates a clearer understanding of the effect of inflammation suppression (Dex) has on the regeneration and differentiation of photoreceptors. Using the data from the PCNA labeling, the data (Fig. 13.1) shows a significant decrease in the number of proliferating cells in the *miR-18a* mutants. Once again, BrdU was used for a shorter window and a more accurate overview of the effects of inflammation suppression. Figure 13.2 demonstrates the BrdU labeling and associated data. Using this set of data, Dex significantly reduced both the number of proliferating cells and the proportion of cells proliferating in the ONL. Taken together, these results indicate that during photoreceptor regeneration, *miR-18a* regulates cell cycle exit and differentiation among photoreceptor progenitors by regulating inflammation.

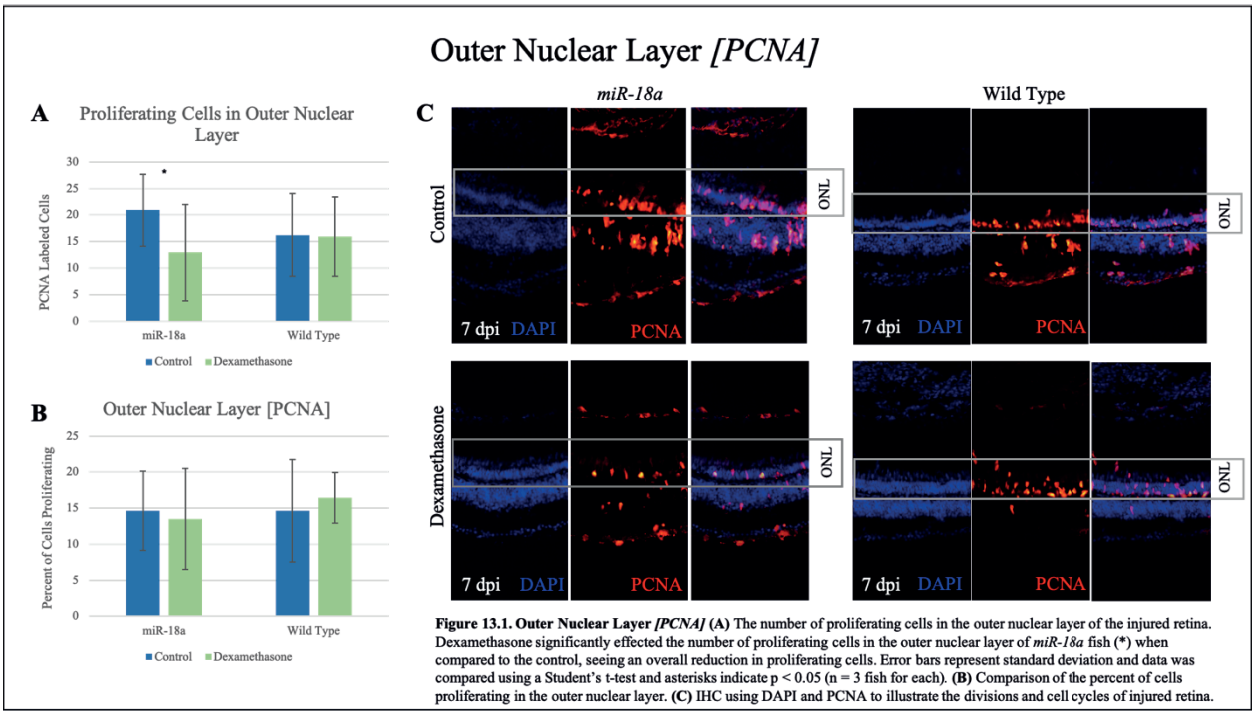


Figure 13.1. Outer Nuclear Layer *[PCNA]* (A) The number of proliferating cells in the outer nuclear layer of the injured retina. Dexamethasone significantly effected the number of proliferating cells in the outer nuclear layer of *miR-18a* fish (*) when compared to the control, seeing an overall reduction in proliferating cells. Error bars represent standard deviation and data was compared using a Student's t-test and asterisks indicate $p < 0.05$ ($n = 3$ fish for each). **(B)** Comparison of the percent of cells proliferating in the outer nuclear layer. **(C)** IHC using DAPI and PCNA to illustrate the divisions and cell cycles of injured retina.

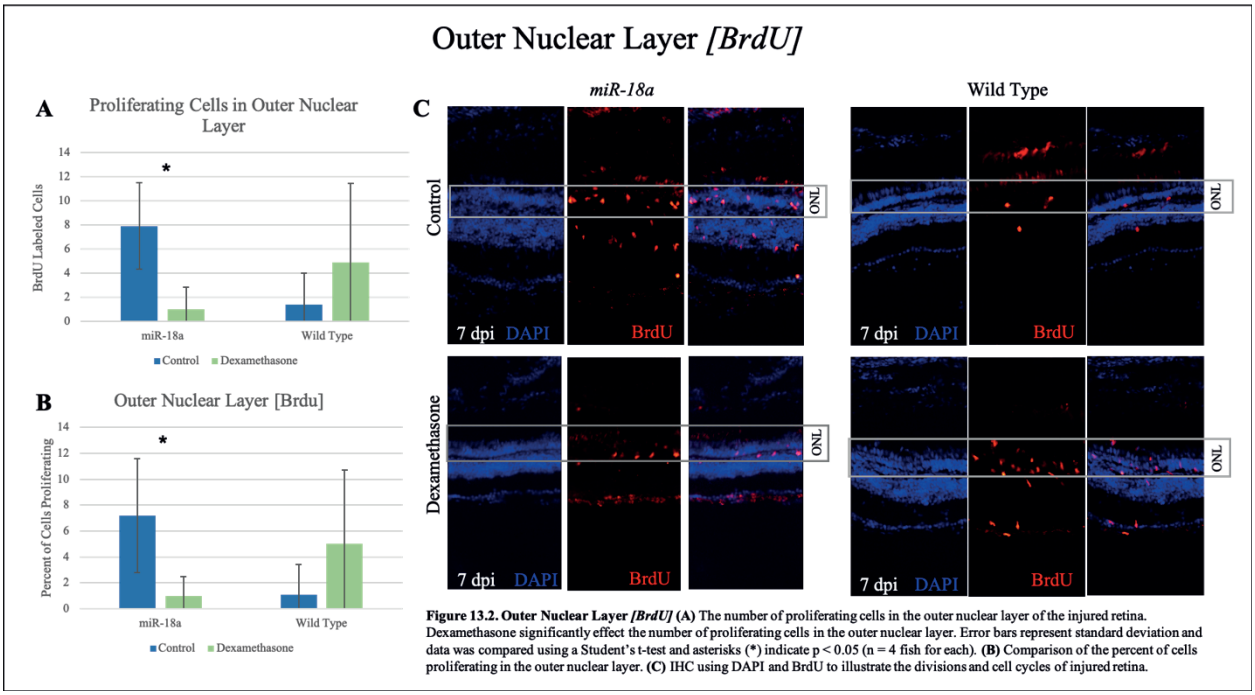


Figure 13.2. Outer Nuclear Layer *[BrdU]* (A) The number of proliferating cells in the outer nuclear layer of the injured retina. Dexamethasone significantly effect the number of proliferating cells in the outer nuclear layer. Error bars represent standard deviation and data was compared using a Student's t-test and asterisks (*) indicate $p < 0.05$ ($n = 4$ fish for each). **(B)** Comparison of the percent of cells proliferating in the outer nuclear layer. **(C)** IHC using DAPI and BrdU to illustrate the divisions and cell cycles of injured retina.

DISCUSSION

The process of retinal regeneration involves a complex cascade of events regulated by mediators; however, in this study, we provide evidence for *miR-18a* as a key regulator for zebrafish retinal regeneration. Regenerating the correct number of rod and cone photoreceptors require a significant amount of control over gene expression and molecular pathways (Lenkowski et al 2013; Wan and Goldman 2017). This level of control involves both the precise temporal and spatial regulation of mechanisms that guide regenerating photoreceptors. This process requires control over the immune response (inflammation), production of progenitors, exiting the cell cycle and differentiating into mature photoreceptors. Each of these crucial steps requires precise gene expression changes that initiate the inflammation, program MG to dedifferentiate, asymmetrically divide the MG, generate progenitors, and guide differentiation (Thummel et al 2011; Lenkowski et al 2013; Wan and Goldman 2017). An overview of the process is found previously illustrated in Figure 2.

In response to the injury or death of photoreceptors, microglia (macrophages) respond by producing inflammation mediators and proliferating near the damaged site (Arroba et al 2018). As the microglia work to clean debris from damaged tissue and bolster survivability of the remaining cells, MG are activated and begin to dedifferentiate (Goldman 2014; Okunuki et al 2018; Rashid et al 2018). Following dedifferentiation, MG asymmetrically divide to produce a single neural progenitor. The multipotent neural progenitor then enters the cell cycle and begins to proliferate (Wan and Goldman 2017). Proliferating progenitors, as time passes, begin to differentiate into the appropriate neural cells (rods and cones) required to restore function. Upon restoration of vision, the previously mentioned cells return to a resting state, and regeneration is complete(Lenkowski et al 2013; Wan and Goldman 2016).

Previous research has demonstrated the action of the microglia and MG, and their partial control over the regenerative process is accomplished through the regulation of mRNA and miRNA expression levels (Huntzinger and Izaurralde 2011; Thummel et al 2011; Rajaram et al 2014; Silva et al 2020). Here we propose *miR-18a* as a potential novel regulator in the regeneration of the retina. Results from this study show that *miR-18a* serves as an essential regulator in the suppression of inflammation, which in turn regulates the cell cycle and differentiation of photoreceptor progenitors. CRISPR/Cas9 gene-editing created a *miR-18a* loss-of-function line of zebrafish. The loss of a functioning *miR-18a* gene resulted in affecting progenitor proliferation and differentiation. ISH for rods and cones demonstrated that *miR-18a* mutant fish generate photoreceptors at lower rates than wild-type. From ISH labeling for *miR-18a* in transgenic *gfap:egfp* fish, results indicate MG, microglia, and photoreceptor progenitors are responsible for the expression of *miR-18a.* Furthermore, the levels and timing of expression follow that of the significant cellular events in retinal regeneration. These findings suggest *miR-18a* expression controls inflammation (avoiding hyperproliferation), and thus the timing of regeneration as it guides the progenitors out of the cell cycle and through differentiation.

To explain the hyperproliferation of progenitors in *miR-18a* mutants, our results suggest MG-derived progenitor proliferation is prolonged and possibly accelerated. Expression of *miR-18a* occurs in both the microglia and the MG, which control the inflammatory response and production of neural progenitors. Therefore, it is possible *miR-18a* regulates cytokines and/or growth factors that regulate the cell cycle in progenitors by expression through microglia and MG. In the absence of *miR-18a,* cell signals for proliferation and cell division persist, causing proliferation to persist for a longer period of time (beyond 5-7 dpi) among the progenitors.

However, this explanation requires additional studies to determine if *miR-18a* directly interacts with cytokines and growth factors, or if it works through other mechanisms to regulate inflammation.

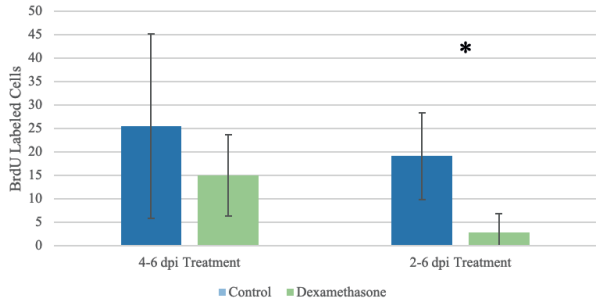
In comparison to the wild-type, *miR-18a* mutant fish express the inflammatory genes *nfkb1* and *nfkb2* longer (Fig. 7), indicating *miR-18a* functions by controlling inflammation. This data, along with online database predictions (TargetScanFish), indicates *miR-18a* may regulate mRNA from two significant pathways. The first pathway is the transforming growth factor-beta (TGFβ) signaling pathway. This pathway is involved in many critical functions from cell growth, differentiation, apoptosis, homeostasis, and more. The second pathway is the tumor necrosis factor (TNFα), which acts as a general inflammatory pathway (Wardle and Wardle 2009). Recent studies have shown Müller glia express TNFα during regeneration, demonstrating MG contribute to inflammation (Nelson et al 2013). Therefore, *miR-18a* may be crucial for the MG. Both the TGFβ and TNFα pathways have been found to regulate the retinal regeneration response in zebrafish and thus considered as possible pathways that *miR-18a* regulates (Lenkowski et al 2013; Nelson et al 2013).

Loss of *miR-18a* function results in the hyperproliferation of progenitors and differential production and survivability of regenerated photoreceptors (Fig. 6, Fig. 8.1, and Fig. 8.2). Recently published work demonstrates that inflammation can be pharmacologically suppressed in zebrafish using dexamethasone (Dex). This recent study also showed that using Dex at 4 dpi promoted photoreceptor regeneration, suggesting that inflammation suppression during this time promotes photoreceptor regeneration (White et al 2017). Inflammation suppression administered from 2-6 dpi rescues the phenotype in the *miR-18a* mutants. Through this study, results show the importance of accurate timing in the control of inflammation. In the first attempt to rescue the

mutant phenotype, fish were treated with Dex from 4-6 dpi. The results from this treatment, however, were insufficient to rescue the mutants. While there was a trend towards a decrease in proliferation, the data concludes it was not sufficient to affect the regeneration significantly. Figure 14 compares the results of the initial rescue attempt to the final attempt, which treated fish from 2-6 dpi. Initially, time points were chosen based off of *pre-miR-18a* expression levels. However, following the failed attempt to rescue the phenotype, we expanded the treatment window to better represent the expression levels of *miR-18a* that were recorded using ISH (Fig. 14). By expanding the suppression of inflammation, we temporally included treatment for additional steps in the regenerative process. That is to say, in the initial attempt, the treatment was affecting the later stages of proliferation and differentiation only. However, by administering suppression at an earlier time point, we begin to affect earlier stages. Studies, bolstering the argument for treatment beginning at 2 dpi, have shown that while inflammation initiates the retinal regeneration, inflammation past 2 dpi suppresses the response. Based on these results, we conclude that inflammation is required for the initiation of the regenerative process but that *miR-18a* suppresses it, beginning at 2 dpi, to control levels of proliferation and guide progenitors through differentiation.

Rescue of *miR-18a* Mutants: Procedural Comparison

A



B

Student's t test [Number of Brd+ Labelled Cells]

Treatment	Data Comparison	P value
4-6 dpi	*miR-18a* Control vs *miR-18a* Dexamethasone	0.314
2-6 dpi	*miR-18a* Control vs *miR-18a* Dexamethasone	0.017*

Figure 14. Rescue of *miR-18a* Mutants: Procedure Comparison (A) Comparing the BrdU+ labeled cells from the two attempts (n = 4 fish for each) to rescue the phenotype. In the first trial, *miR-18a* mutants were exposed to dexamethasone (15 mg/l) from 4 to 6 days post injury. **(B)** This failed to result in a significant change in the number of proliferating cells, and thus failed to rescue the mutant. In the second trial, the treatment was extended to days 2 through 6. The extended window of inflammation suppression significantly effected the regeneration of cells in the injured eye. The second treatment demonstrates the importance of timing in the control of the regeneration process and that the phenotype was rescued in the mutants. Asterisks highlight significant values ($p < 0.05$).

Previously published studies have also demonstrated the importance of timing in inflammation suppression. Depending on the timing, treatment with dexamethasone can either inhibit or enhance the proliferation of progenitors. These effects are then exemplified in the differentiation and regeneration of photoreceptors, particularly rods (White et al 2017). Our results confirm these findings, as we see an effect from the Dex treatment on the rod regeneration of the wild-type fish. Treatment to the *miR-18a* mutants brought them closer to typical rod regeneration; however, it was not sufficient to produce a significant change. Differences in the regeneration of cones in the wild-type were also found not to be significant.

In contrast, treatment with Dex to rescue cone regeneration showed a significant change in the number of cones regenerated. Through the scope of this study, the data suggest inflammation from 2-6 dpi returns cone regeneration to the wild-type state, indicating that *miR-18a* and inflammation significantly regulate cone differentiation but not rod differentiation.

However, additional studies are needed to investigate the survivability of regenerated rods and cones past 7 dpi.

In conclusion, the results presented here demonstrate that during photoreceptor regeneration, *miR-18a* regulates progenitor proliferation, cell cycle exit, and photoreceptor differentiation (Fig. 15). Furthermore, the data indicate *miR-18a* functions through the regulation of inflammation. These findings are consistent with previously published studies involving *miR-18a*. Taylor et al. (2019) demonstrated that *miR-18a* regulated the timing of photoreceptor differentiation through the regulation of NeuroD in the developing retina. The 2019 study showed the importance of *miR-18a* in photoreceptor differentiation; however, it showed no effect on the cell cycle. Here we demonstrate that in regeneration, *miR-18a* functions through inflammation to regulate the cell cycle and differentiation. This study advances our understanding of the role of *miR-18a* in the retina and concludes potentially important understandings that may serve vital to developing technologies for the treatment of human photoreceptor degeneration.

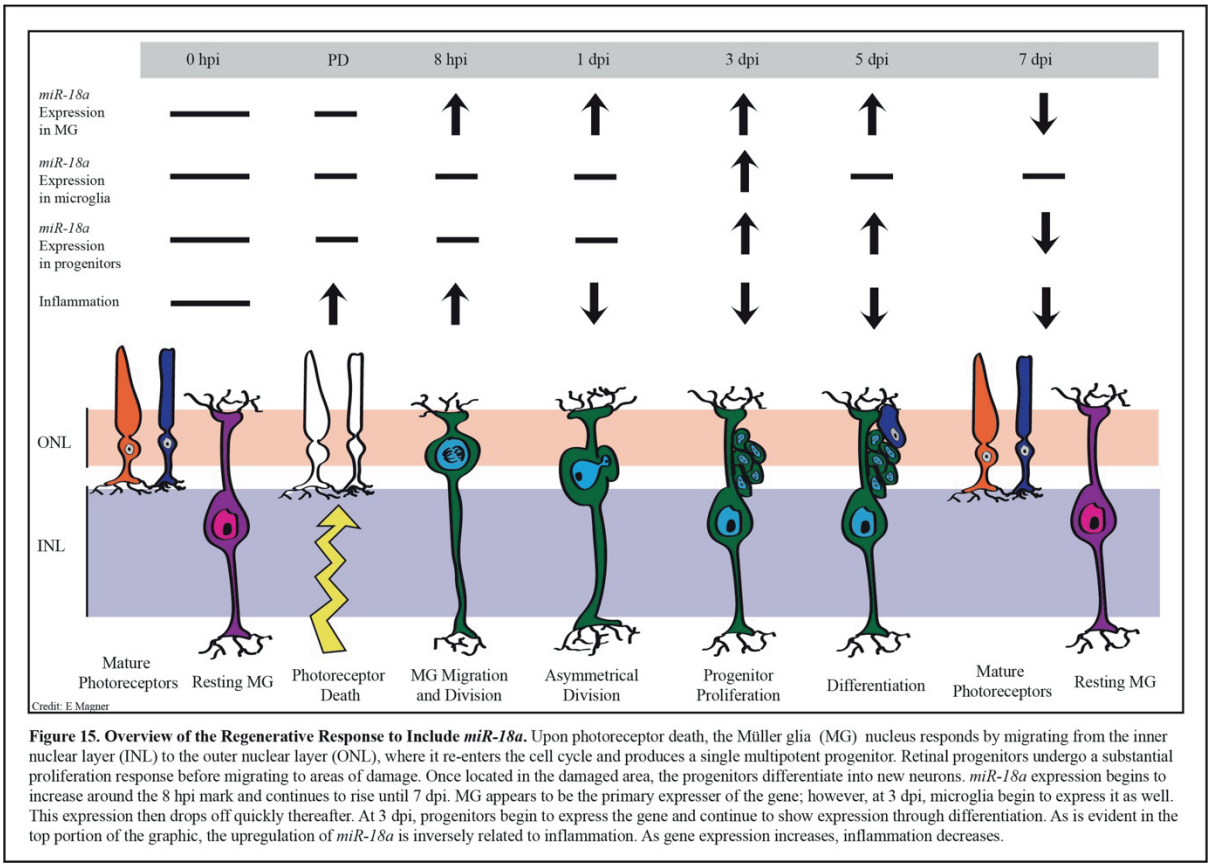


Figure 15. Overview of the Regenerative Response to Include *miR-18a*. Upon photoreceptor death, the Müller glia (MG) nucleus responds by migrating from the inner nuclear layer (INL) to the outer nuclear layer (ONL), where it re-enters the cell cycle and produces a single multipotent progenitor. Retinal progenitors undergo a substantial proliferation response before migrating to areas of damage. Once located in the damaged area, the progenitors differentiate into new neurons. *miR-18a* expression begins to increase around the 8 hpi mark and continues to rise until 7 dpi. MG appears to be the primary expresser of the gene; however, at 3 dpi, microglia begin to express it as well. This expression then drops off quickly thereafter. At 3 dpi, progenitors begin to express the gene and continue to show expression through differentiation. As is evident in the top portion of the graphic, the upregulation of *miR-18a* is inversely related to inflammation. As gene expression increases, inflammation decreases.

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