The microRNA *miR-18a* links proliferation and inflammation during photoreceptor regeneration in the injured zebrafish retina

Evin Magner², Pamela Sandoval-Sanchez¹, Peter F. Hitchcock³, Scott M. Taylor^{1*}

*Correspondence to Scott M. Taylor, staylor1@uwf.edu, ORCID ID 0000-0002-5823-5043

¹ Department of Biology, University of West Florida, 11000 University Parkway,
Pensacola, FL 32514

² Plant and Microbial Biology, University of Minnesota, 1479 Gortner Avenue, St. Paul,
MN 55108

³ Department of Ophthalmology and Visual Sciences, University of Michigan, W. K.
Kellogg Eye Center, 1000 Wall Street, Ann Arbor, MI 48105

ABSTRACT

In mammals, photoreceptor loss causes permanent blindness, but in zebrafish (*Danio rerio*), Müller glia function as intrinsic stem cells, producing progenitor cells that regenerate photoreceptors and restore vision. MicroRNAs (miRNAs) critically regulate neurogenesis in the brain and retina, but the roles of miRNAs in injury-induced neuronal regeneration are largely unknown. The miRNA *miR-18a* regulates photoreceptor differentiation in the embryonic retina. The purpose of the current study was to determine the function of *miR-18a* during injury-induced photoreceptor regeneration. RT-qPCR, in-situ hybridization (ISH) and immunohistochemistry (IHC) showed that *miR-18a* expression increases throughout the retina by 1-day post-injury (dpi) and continues to increase through 5 dpi. Bromodeoxyuridine (BrdU) labeling showed that at 7 and 10 dpi, when regenerated photoreceptors are normally differentiating, there are more proliferating Müller glia-derived progenitors in homozygous *miR-18a* mutant (*miR-18a^{mi5012}*) retinas compared with wild type (WT), indicating that *miR-18a* negatively regulates injury-induced proliferation. At 7 and 10 dpi, *miR-18a^{mi5012}* retinas have fewer mature photoreceptors than WT, but there is no difference at 14 dpi, revealing that photoreceptor regeneration is delayed. BrdU labeling showed that the excess progenitors in *miR-18a^{mi5012}* retinas migrate to other retinal layers besides the photoreceptor layer. Inflammation is critical for photoreceptor regeneration and RT-qPCR showed that, in the absence of *miR-18a*, inflammation is prolonged. Suppressing inflammation with dexamethasone rescues the *miR-18a^{mi5012}* phenotype. Together, these data show that during injury-induced photoreceptor regeneration, *miR-*

18a regulates proliferation and photoreceptor regeneration by regulating key aspects of the inflammatory response during photoreceptor regeneration in zebrafish.

KEYWORDS: Danio rerio, Neuroinflammation, Müller glia, Stem cells, Neurogenesis, CNS

DECLARATIONS

Funding: NIH 1R15EY031089-01 (SMT), NIH T32EY013934 (SMT); P30EYO7003 (PFH), NIH R01EY07060 (PFH), and an unrestricted grant from the Research to Prevent Blindness, New York (PFH).

Conflicts of interest/Competing interests: The authors have no conflicts or competing interests

Availability of data and material: All data and *miR-18a^{mi5012}* fish are freely available upon request

Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Evin Magnier, Pamela Sandoval-Sanchez and Scott M. Taylor. The first draft of the manuscript was written by Evin Magnier and Scott M. Taylor and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Code availability: N/A

Ethics approval: All experimental procedures were approved by the University of West Florida Institutional Animal Care and Use Committee

Consent to participate: N/A

Consent for publication: N/A

INTRODUCTION

Photoreceptor loss in the human retina causes permanent blindness, but in the injured retina of zebrafish, *Danio rerio*, Müller glia (MG) are reprogrammed and divide, producing neuronal progenitors that fully regenerate the lost photoreceptors [1-4]. This regenerative capacity has established the zebrafish retina as an outstanding model for investigating photoreceptor degeneration and regeneration [5]. Mechanisms identified in the zebrafish retina that govern the reprogramming of Müller glia and neuronal regeneration have been used to develop methods to stimulate a limited MG-derived regeneration response in the mouse [6-8]. Understanding the mechanisms that govern photoreceptor regeneration in zebrafish could, therefore, be critical for developing regenerative therapies to treat human blindness.

Recent research has improved our understanding of the molecular pathways that regulate neuronal regeneration in the zebrafish retina [reviewed in 4,3,9,10]. The primary focus of this research has been on identifying the transcriptional mechanisms involved in neuronal regeneration, but recent studies show that post-transcriptional regulation by non-coding RNAs, and specifically microRNAs (miRNAs), also play critical roles in retinal neuronal regeneration [11-15]. Of the more than 2600 mature miRNAs coded by the vertebrate genome [16], the functional roles of a very small number have been established in retinal regeneration. MicroRNAs are likely to play key roles in regulating photoreceptor regeneration, and additional studies that determine the roles of miRNAs in photoreceptor regeneration are critically needed.

Inflammation occurs in response to tissue injury, and several recent studies show that activation of neuroinflammatory pathways is both necessary and sufficient to initiate neuronal (including photoreceptor) regeneration in the zebrafish retina [17-22] [reviewed in 23,24]. Inflammatory signals seem to have the opposite effect in the injured mammalian retina, in which removal of microglia reduces inflammatory gene expression and increases the number of regenerated neurons [25]. Understanding mechanisms that control inflammation in the injured retina may, therefore, be critical to unlocking the regenerative potential in the mammalian retina. Importantly, several miRNAs have been identified as key regulators of inflammatory pathways [26] and as biomarkers of inflammatory disease [27], and identifying the roles of miRNAs in the injured retina could be critical for understanding the link between the retinal inflammatory response and neuronal regeneration.

MicroRNAs are small, 18-25 nucleotides long, non-coding RNAs that generally function by binding to the 3' untranslated region of mRNA and inhibit translation and/or promote mRNA degradation [28,29]. The miRNA, *miR-18a*, was recently found to regulate photoreceptor differentiation in larval zebrafish by suppressing levels of the transcription factor, NeuroD [30]. The role of *miR-18a* during photoreceptor regeneration in zebrafish is currently unknown. Bioinformatics tools predict that *miR-18a* can interact directly with mRNAs that encode more than 25 molecules that function in inflammatory pathways (http://www.targetscan.org/fish_62/), suggesting that *miR-18a* might be an important regulator of inflammation. However, *miR-18a* has not been investigated in the context of inflammatory regulation.

The objective of this research was to determine if *miR-18a* governs photoreceptor regeneration by regulating inflammation. qPCR showed that *miR-18a* expression increases between 3 and 5 days post injury (dpi) and then decreases by 7 dpi. *In situ* hybridization for *miR-18a*, combined with green fluorescent protein (GFP) immunolabeling in transgenic *tg(gfap:egfp^{mi2002})* fish [31] with GFP-labeled Müller glia showed that the *miR-18a* expression increases throughout the retina including in Müller glia (MG) by 1 dpi and in MG-derived progenitors by 3 dpi. 5-Bromo-20-Deoxyuridine (BrdU) labeling revealed that relative to wild-type (WT) animals, at 7 and 10 dpi, homozygous *miR-18a* mutants (*miR-18a^{mi5012}*) have significantly more proliferating MG-derived progenitors. The regeneration of rods and cones is initially delayed in *miR-18a^{mi5012}* fish, but numerically matches wild type animals by 14dpi. There was no overproduction of regenerated photoreceptors. BrdU labeling showed that in *miR-18a^{mi5012}* retinas, more Müller glia-derived progenitors migrate to layers of the retina in addition to the outer nuclear layer (ONL), suggesting that the excess progenitors differentiate into a variety of other retinal cell types. RT-qPCR and *in situ* hybridization showed that at 5 and 7 dpi, when inflammation is normally resolving, in *miR-18a^{mi5012}* retinas, the expression of genes encoding pro-inflammatory cytokines and the cytokine regulator, *nfb1*, are significantly elevated. Finally, suppressing inflammation with dexamethasone in *miR-18a^{mi5012}* fish fully rescues both the excess proliferation and the delay in the regeneration of cone photoreceptors. Together, these data show that following photoreceptor injury in zebrafish, *miR-18a* regulates the proliferation of MG-

derived progenitors and photoreceptor regeneration by regulating key aspects of inflammation.

METHODS

Fish husbandry, photolytic lesions and tissue preparation

All fish were maintained at 28.5°C on a 14/10-h light/dark cycle under standard husbandry conditions [32]. AB wild-type (WT) strain zebrafish, purchased from the Zebrafish International Research Center (ZIRC; University of Oregon, Portland, OR, USA), were used for control experiments. The *miR-18a^{mi5012}* has a 25 bp insertion in the sequence coding for the primary transcript *pri-miR-18a*, and homozygous mutant fish, used for all experiments here, do not produce mature *miR-18a* [30]. The transgenic line, *Tg(gfap:egfp)^{mi2002}* [31], expresses Enhanced Green Fluorescent Protein (EGFP) in Müller glia and MG-derived progenitors, and was used in conjunction with *in situ* hybridization to determine the cellular expression of *miR-18a*.

As previously described, photolytic lesions were used to selectively kill photoreceptors [33]. Briefly, fish were exposed to ultra-high intensity light (>120,000 lux) in a 100 ml beaker for 30 minutes, using a SOLA SE II 365 White Light Engine (Lumencor, Beaverton, OR, USA). Following photolytic lesions, fish were maintained in normal system water and exposed to the standard 14/10-h light cycle.

Prior to collecting tissues, fish were euthanized in 0.1% Tricaine Methanesulfonate (MS-222) and then decapitated. For histology, eyes were removed,

fixed (overnight at 4°C) in 4% paraformaldehyde, infiltrated with 20% sucrose (in PBS) and then in a 2:1 mixture of 20% sucrose and optimal cutting temperature (OCT) compound, and then finally embedded and frozen in OCT. Sections, 10µm in thickness, were collected through the center of the eye and thaw mounted onto glass slides. For qPCR, retinas were isolated from two fish per biological replicate (4 retinas), and total RNA, including small RNAs, were purified using the miRvana miRNA purification kit (Invitrogen, Carlsbad, CA, USA).

Systemic labeling with BrdU, dexamethasone treatment, immunohistochemistry and *in situ* hybridization

Cells in the S-phase of the cell cycle were labeled with BrdU by swimming fish in 5 mM BrdU for 24 hours. Fish were then either sacrificed immediately or at variable timepoints thereafter. For BrdU immunolabeling, sections were incubated in 100°C sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 minutes to denature DNA and cooled at room temperature for 20 minutes. Sections then were subjected to standard immunolabeling as described below.

Fish treated with dexamethasone to inhibit inflammation were exposed in system water to 15 mg/L dexamethasone (Sigma-Aldrich, Corp, D1756) diluted in 0.1% MetOH [21]. Control fish were treated with 0.1% MetOH only. Fish were treated between 2-6 dpi. All solutions were changed daily, and fish were fed brine shrimp every other day.

Standard immunolabeling was performed using previously published protocols [34]. The primary and secondary antibodies and dilutions used here were: mouse anti-

BrdU 1:100 (347580; BD Biosciences, Franklin Lakes, NJ, USA), Zpr-1 1:200 (anti-Arrestin 3, red-green double cones, ZIRC), Zpr-3 1:200 (anti-Rhodopsin, rods, ZIRC), rabbit anti-GFP (ab290; Abcam, Cambridge, UK), goat anti-mouse Alexa Fluor 555 1:500 (Invitrogen), and goat anti-rabbit Alexa Fluor 488 (Invitrogen). Following immunohistochemistry, when applicable, TUNEL labeling was performed using the Click-iT Plus TUNEL assay (C10617, Invitrogen).

In situ hybridizations were performed using previously published protocols [35,36]. Riboprobes were generated from PCR products using the following primers and by adding a T3 polymerase sequence on the reverse primer (lowercase letters) [37]. Probes were generated for rods *rhodopsin* (F—GAGGGACCGGCATTCTACGTG, R—aaataaccctcactaaagggCTTCGAAGGGGTTCTTGCCGC) and cones *arr3a* (F—GAAGACCAGTGGAAATGGCCAG, R—aaataaccctcactaaagggTCAGAGGCAGCTCTACTGTAC). *In situ* hybridization for mature *miR-18a* was performed using a miRCURY LNA detection probe (Exiqon/Qiagen, Germantown, MD), labeled with DIG at the 5' and 3' ends. Standard *in situ* hybridization methods were used for *miR-18a*, as described above, but using a 0.25 µM probe working concentration at a hybridization temperature of 58°C. For comparisons of relative expression across post-injury time points or between WT and *miR-18a^{mi5012}* fish, all tissue sections were placed on the same slides and/or developed for identical periods of time.

Reverse transcriptase quantitative real-time PCR (RT-qPCR)

As described above, for RT-qPCR, retinas were removed and total RNA purified from the eyes of 2 fish (4 retinas) per biological replicate. Three to five biological replicates were collected for each experimental group, treatment or time point. Reverse transcription was performed using the Superscript III First Strand cDNA Synthesis System (18080051, Invitrogen). Each biological replicate was run in triplicate using 20 ng cDNA and the Applied Biosystems PowerUp Sybr Green Master Mix (A25741, Applied Biosystems, Invitrogen), on an Applied Biosystems StepOnePlus 96-well Real-Time PCR System. The $\Delta\Delta CT$ method was used to calculate expression relative to WT (unlesioned or control) and data were normalized to *gpia* as the housekeeping gene.

Sequences for the standard qPCR primers used were as follows: *gpia* F—

TCCAAGGAAACAAGCCAAGC, R—TTCCACATCACACCCTGCAC; *nfk1* F—

CAGCTGGTGACCAACTCTCAG, R—TCCTGTAGGCCTCCATCATGC; *tnf α* F—

CTGGAGAGATGACCAGGACCAGGCC, R—GCTGTGGTCGTGTCTGTGCCCAGTC;

il1 β F—GCATGAGGGCATCAGGCTGGAGATG, R—

TCCGGCTCTCAGTGTGACGGCCTGC; *il6* F—CCTGTCTGCTACACTGGCTAC, R—

CACTTCTGCCGGTCGCCAAGG. To quantify mature *miR-18a* expression, a TaqMan

custom qPCR assay was designed for mature *miR-18a* and for the small nuclear RNA

U6, to be used as the housekeeping gene for data normalization (ThermoFisher

Scientific, Halethorp, MD, USA). The $\Delta\Delta CT$ method was used to calculate expression at

different post-injury time points relative to unlesioned. Statistical significance between

time points and between WT and *miR-18a^{mi5012}* values for each gene was determined using a Student's t-test ($p < 0.05$)

Cell counts and data analysis

All counts of BrdU-labeled progenitors and rod and cone photoreceptors were performed at 200x magnification across 0.3 mm of linear retina. Measurements and cell counts were done using ImageJ analysis software [38]. For each biological replicate (each fish), cells were counted in 3 non-adjacent retinal cross-sections in the vicinity of the optic nerve. For each analysis, 3-5 biological replicates were used per treatment, time point or genotype. All comparisons were pairwise (e.g. treated vs. control) and Student's t-tests were used to determine statistical significance (p values less than 0.05 were considered statistically significant).

RESULTS

Following photoreceptor injury, *miR-18a* is expressed in both the inner and outer nuclear layers, including in Müller glia and Müller glia-derived progenitors

RT-qPCR and *in situ* hybridization were performed to determine the temporal and spatial expression of *miR-18a* following photoreceptor injury. Taqman qPCR showed that, compared with control uninjured retinas, *miR-18a* expression is significantly higher at 3 days (2.15 ± 0.44 SD fold higher, $p=0.003$), 5 days (2.06 ± 0.69 SD fold higher, $p=0.0024$) and 7 days (1.54 ± 0.30 SD fold higher, $p=0.045$) post-retinal injury (dpi) (Figure 1a). *In situ* hybridization using an LNA riboprobe for mature *miR-18a*, combined with immunolabeling for green fluorescent protein (GFP) in *Tg(gfap:egfp)^{mi2002}* fish, showed that at in control uninjured retinas, there is only very slight expression of *miR-18a* (Figure 1b) but by 24 hpi, around the time that Müller glia divide once to produce a neuronal progenitor [see 39], *miR-18a* is expressed in many cells throughout the inner nuclear layer (INL), including Müller glia (Figure 1c). Then at 3 dpi, during the peak of progenitor proliferation, *miR-18a* remains strongly expressed in the INL and ONL, including in Müller glia and MG-derived progenitors (Figure 1d). Finally, by 7 dpi, when MG-derived progenitors are normally exiting the cell cycle, *miR-18a* expression is again similar to the expression pattern seen between 0 and 24 hpi (Figure 1e, f). Together, these results show that *miR-18a* expression is upregulated in the retina during the time periods when Müller glia and then MG-derived progenitors divide.

During photoreceptor regeneration, *miR-18a* regulates proliferation among MG-derived progenitors

To determine if *miR-18a* regulates cell proliferation among MG-derived progenitors, BrdU labeling and immunostaining were used to quantitatively compare the number of proliferating cells in WT and *miR-18a^{mi5012}* retinas. In the injured WT retina, progenitor proliferation peaks around 3 dpi, some MG-derived progenitors normally stop dividing between 4 and 5 dpi, and the first regenerated photoreceptors can be detected between 5 and 6 dpi [see 40]. By 7 dpi, many new photoreceptors have normally regenerated and fewer progenitors continue to proliferate and, by 10 dpi, very few progenitors normally continue to proliferate. Compared with WT retinas, the number of proliferating progenitors in *miR-18a^{mi5012}* retinas did not differ at 3 dpi (*miR-18a^{mi5012}* 81.7 ± 7.2 SD, WT 87.6 ± 8.1 SD cells/0.3 mm, $p=0.60$) (Figure 2a, b), indicating that the initial proliferative response is unaltered in *miR-18a^{mi5012}* retinas. At 7 dpi, however, there were significantly more BrdU-positive cells in the *miR-18a^{mi5012}* retinas than in WT (*miR-18a^{mi5012}* 64.3 ± 9.8 SD, WT 30.6 ± 7.2 SD cells/0.3 mm, $p=0.001$) (Figure 2c, d). In *miR-18a^{mi5012}* retinas, there were also significantly more proliferating cells at 10 dpi (*miR-18a^{mi5012}* 45.7 ± 3.2 SD, WT 14.0 ± 3.6 SD cells/0.3 mm, $p<0.001$) (Figure 2e, f). These data show that following photoreceptor injury, in the absence of *miR-18a*, the timing of the initial proliferative response is unchanged, but that MG-derived progenitors continue to proliferate longer than in WT retinas.

During photoreceptor regeneration, *miR-18a* regulates the timing, but not the extent, of photoreceptor regeneration

In *miR-18a^{mi5012}* retinas, the prolonged period of proliferation among MG-derived progenitors suggests that photoreceptor regeneration might be delayed and/or that more photoreceptors might be produced. To determine if *miR-18a* regulates the timing and/or extent of photoreceptor regeneration, *in situ* hybridization was used to differentially label mature cones and rods in the retina at 7 dpi, when large numbers of newly differentiated photoreceptors can first be detected, at 10 dpi, when most new photoreceptors have been normally regenerated, and 14 dpi, when photoreceptor regeneration is normally complete. *In situ* hybridization for mature cones (*arr3a*) and rods (*rho*) (Figure 3a, b) and quantification of these cells (Figure 3c, d) showed that at 7 dpi, *miR-18a* retinas have fewer mature cones than WT (*miR-18a^{mi5012}* 32.3 ± 14.7 SD, WT 77.5 ± 4.5 SD cells/0.3 mm, $p=0.001$), but the number of mature rods does not differ (*miR-18a^{mi5012}* 98.5 ± 38.6 SD, WT 127.1 ± 72.1 SD cells/0.3 mm, $p=0.51$). Hoechst labeling of cone nuclei at 7 dpi also showed that *miR-18a^{mi5012}* retinas have fewer cone nuclei than WT (*miR-18a^{mi5012}* 86.8 ± 9.2 SD, WT 101.3 ± 5.2 SD cells/0.3 mm, $p=0.033$). Together, these data indicate that both cone photoreceptor maturation and regeneration are delayed. At 10 dpi, *miR-18a* retinas have fewer mature cones and rods than WT (cones *miR-18a^{mi5012}* 76.4 ± 2.5 SD, WT 107.6 ± 6.5 SD cells/0.3 mm, $p=0.001$; rods *miR-18a^{mi5012}* 146.4 ± 46.7 SD, WT 275.6 ± 32.5 SD cells/0.3 mm, $p=0.017$) but, at 14 dpi, the number of mature photoreceptors does not differ (cones *miR-18a^{mi5012}* 125.7 ± 11.4 SD, WT 120.3 ± 14.7 SD cells/0.3 mm, $p=0.462$; rods *miR-*

$18a^{mi5012}$ 237.7 ± 28.8 SD, WT 261.3 ± 32.1 SD cells/0.3 mm, $p=0.241$) (Figure 3c, d).

Taken together, these data show that in $miR-18a^{mi5012}$ retinas compared with WT, the same overall numbers of photoreceptors are regenerated by 14 dpi, but photoreceptor regeneration and maturation are delayed.

Following photoreceptor injury, $miR-18a$ regulates the number of neuronal progenitors that are produced

In $miR-18a^{mi5012}$ retinas compared with WT, MG-derived progenitors proliferate for a longer period of time, suggesting that excess neuronal progenitors are produced. However, since extra photoreceptors are not generated, these excess progenitors might either die or migrate to other retinal layers, possibly differentiating into other cell types. To investigate this, TUNEL labeling was used to label dying cells at 10 dpi, when the largest differences in photoreceptor numbers were identified between WT and $miR-18a^{mi5012}$ retinas. This experiment showed that, at 10 dpi, there were no differences in the number of TUNEL-positive cells between WT and $miR-18a^{mi5012}$ retinas (data not shown), indicating that excess progenitors in $miR-18a^{mi5012}$ retinas are not eliminated by cell death. To establish the potential fates of the excess MG-derived progenitors in $miR-18a^{mi5012}$ retinas, fish were exposed to 5 mM BrdU from 3 to 4 dpi during the peak of cell proliferation, and then fish were sacrificed at 14 dpi when photoreceptor regeneration is complete. Immunolabeling for BrdU at 14 dpi shows that compared with WT, $miR-18a^{mi5012}$ retinas have more BrdU⁺ cells in both the INL ($miR-18a^{mi5012}$ 22.7 ± 4.3 SD, WT 11.0 ± 3.2 SD cells/200 mm, $p=0.019$) and ganglion cell layer (GCL) ($miR-18a^{mi5012}$

15.1 $\pm$ 2.2 SD, WT 6.2 $\pm$ 1.9 SD cells/200 mm, p=0.006) (Figure 4), indicating that the excess progenitors produced at 3-4 dpi either remain in the INL or migrate to the GCL, suggesting that they may differentiate into a variety of other cell types.

Following photoreceptor injury, *miR-18a* regulates the extent and duration of the inflammatory response

The effect of *miR-18a* on the cell cycle during photoreceptor regeneration is strikingly different from embryonic development, in which *miR-18a* regulates photoreceptor differentiation but not cell proliferation [30]. This indicates that in the injured retina, *miR-18a* regulates pathways that are specific to the post-injury response. Silva *et al.* [21] showed that in injured *mmp9* mutant retinas, there was increased inflammation resulting in excess proliferation among MG-derived progenitors. This finding helped lead to the rationale that increased or prolonged inflammation might cause the excess proliferation observed in *miR-18a^{mi5012}* retinas. Indeed, the miRNA target database TargetscanFish (http://www.targetscan.org/fish_62) predicts that *miR-18a* interacts with mRNA for many molecules involved in inflammatory pathways. To determine if *miR-18a* regulates inflammation, RT-qPCR was used to compare the mRNA expression levels of key inflammatory molecules at 1, 3, 5 and 7 dpi in WT and *miR-18a^{mi5012}* retinas. These data show that at 1 dpi, around the time that Müller glia normally begin to divide, expression of *tnf α* is higher in *miR-18a^{mi5012}* retinas compared with WT (*miR-18a^{mi5012}* 4.51 $\pm$ 1.14 SD, WT 2.31 $\pm$ 0.99 SD, p=0.033) (Figure 5a). Then at 3 dpi, around the normal peak of MG-derived progenitor proliferation, *il1 β* expression

is higher in *miR-18a^{mi5012}* retinas compared with WT (*miR-18a^{mi5012}* 1.61 ± 0.21 SD, WT 0.97 ± 0.06 SD, $p=0.007$) (Figure 5b). At 5 dpi, when many photoreceptor progenitors normally stop proliferating and begin to differentiate, expression of *il6*, *il1 β* , and the cytokine regulator Nuclear Factor Kappa B 1 (*nfkb1*) are higher in *miR-18a^{mi5012}* retinas compared with WT (*il6* *miR-18a^{mi5012}* 1.32 ± 0.26 SD, WT 0.85 ± 0.25 SD, $p=0.042$; *il1 β* *miR-18a^{mi5012}* 1.71 ± 0.47 SD, WT 0.97 ± 0.08 SD, $p=0.028$; *nfkb1* *miR-18a^{mi5012}* 1.68 ± 0.23 SD, WT 1.19 ± 0.17 SD, $p=0.021$) (Figure 5c). Finally, at 7 dpi, when many photoreceptor progenitors have normally stopped proliferating and have fully differentiated, *nfkb1* expression is still higher in *miR-18a^{mi5012}* retinas (*miR-18a^{mi5012}* 1.33 ± 0.22 SD, WT 1.03 ± 0.07 SD, $p=0.044$) (Figure 5d). Taken together, these results indicate that, compared with WT, there is both a higher level of inflammatory pathway activity and a prolonged inflammatory response in *miR-18a^{mi5012}* retinas following photoreceptor injury and death.

Supressing inflammation in *miR-18a* mutants rescues both the excess proliferation and delayed photoreceptor regeneration

The increased and prolonged expression of inflammatory genes in the injured *miR-18a^{mi5012}* retina led to the hypothesis that, in the absence of *miR-18a*, prolonged and excessive inflammation is causally related to the excess proliferation and delayed photoreceptor maturation observed in *miR-18a* mutants. To test this hypothesis, dexamethasone was used to suppress inflammation in WT and *miR-18a^{mi5012}* fish from 2 to 6 dpi, the time range during which *miR-18a* expression is upregulated in injured

retinas (see Figure 1a, b) and when inflammatory molecules are expressed higher than WT in *miR-18a^{mi5012}* retinas (see Figure 5). Fish were then exposed to 5 mM BrdU from 6 to 7 dpi, to label proliferating cells. BrdU immunolabeling was used to label BrdU⁺ cells in S-phase of the cell cycle, and *in situ* hybridization was used to label mature rod or cone photoreceptors. Compared with controls, the dexamethasone treatment fully rescued the excess proliferation in *miR-18a^{mi5012}* retinas, reducing the number of BrdU⁺ cells to that observed in controls (*miR-18a^{mi5012}* control 65.0 ± 13.5 cells/0.3 mm vs. WT control 42.5 ± 3.5 cells/0.3 mm $p=0.009$; *miR-18a^{mi5012}* dexamethasone treated 36.2 ± 7.7 cells/0.3 mm vs. WT control $42.5 \pm 3.5/0.3$ mm $p=0.096$). Dexamethasone treatment also reduced the number of BrdU⁺ cells in WT retinas by a smaller amount (WT control 42.5 ± 3.5 cells/0.3 mm vs. WT dexamethasone treated 29.2 ± 9.0 cells/0.3 mm $p=0.020$) (Figure 6a, b). Further, dexamethasone treatment rescued the delay in cone maturation and regeneration at 7 dpi, increasing the number of *arr3a*-expressing mature cones to WT control levels (*miR-18a^{mi5012}* control 41.8 ± 3.1 cells/0.3 mm vs. WT control 57.1 ± 6.6 cells/0.3 mm $p=0.015$; *miR-18a^{mi5012}* dexamethasone treated 63.1 ± 7.6 cells/0.3 mm vs. WT control 57.1 ± 6.6 cells/0.3 mm $p=0.314$), and also increasing the number of Hoechst-labeled cone nuclei to WT control levels (*miR-18a^{mi5012}* control 57.2 ± 11.4 cells/0.3 mm vs. WT control 76.7 ± 9.2 cells/0.3 mm $p=0.021$; *miR-18a^{mi5012}* dexamethasone treated 76.3 ± 4.04 cells/0.3 mm vs. WT control 76.7 ± 9.2 cells/0.3 mm $p=0.451$). Dexamethasone treatment had no effect on the number of *arr3a*-expressing mature cone cells in WT retinas (WT control 57.1 ± 6.6 cells/0.3 mm vs. WT dexamethasone treated 56.5 ± 12.6 cells/0.3 mm $p=0.933$) (Figure 6c, d). Together,

these data indicate that in the injured retina, *miR-18a* regulates MG-derived progenitor proliferation and photoreceptor regeneration by regulating inflammation.

DISCUSSION

Recent studies have dramatically improved our understanding of the mechanisms that govern neuronal regeneration in the injured zebrafish retina from dedifferentiated Müller glia [reviewed in 4,41,10]. Some of this information has led to studies showing that mammalian Müller glia possess latent regenerative potential that can be augmented by reprogramming these cells, and that MG-derived progenitors can regenerate some neurons (including photoreceptors) [7,25,8,42-44], but this neuronal regeneration is very inefficient [9,41]. In the injured zebrafish retina, inflammation is necessary and sufficient for neuronal regeneration to begin [45,17,18,46,20,19,23,21,22], but in the injured mammalian retina, inflammation prevents Müller glia from initiating a regenerative response [25]. When inflammation is mis-regulated in the injured zebrafish retina, this leads to aberrant proliferation of MG-derived progenitors and alters photoreceptor regeneration [21,20], showing the importance of precise inflammatory regulation in controlling these events.

Several miRNAs have been identified as important regulators of inflammation and could be key neuroinflammatory regulators in the injured retina [26,27], but studies linking miRNAs with retinal inflammation are lacking. The miRNA *miR-18a* was recently identified as an important regulator of photoreceptor differentiation in the developing

embryonic retina [30], and *miR-18a* is predicted to interact with mRNAs of more than 25 inflammation-related molecules (http://www.targetscan.org/fish_62), suggesting that *miR-18a* could regulate neuroinflammation in the retina, but the roles of *miR-18a* in injury-induced inflammation and photoreceptor regeneration had not been previously investigated.

The objective of the current study was to determine the role of *miR-18a* in photoreceptor regeneration and to determine if it regulates inflammation during this response. Following photoreceptor injury, *miR-18a* is expressed in the INL and ONL, including in dividing Müller glia as early as 1 dpi and in both Müller glia and proliferating MG-derived progenitors at 3-5 dpi, indicating that *miR-18a* functions during key times of cell division during photoreceptor regeneration. In *miR-18a^{mi5012}* retinas compared with WT, more cells continue to proliferate at 7 and 10 dpi, when most photoreceptor progenitors have normally exited the cell cycle, indicating that *miR-18a* regulates proliferation of MG-derived progenitors. This differs from findings in the developing embryonic retina in which *miR-18a* regulates the timing of photoreceptor differentiation but does not regulate cell proliferation [30]. The function of *miR-18a* to limit progenitor proliferation also differs from the general function of *miR-18a* in cancer, in which it typically promotes the proliferation of tumor cells [47,48] including glioblastoma cells [49]. Also, in the developing mouse neocortex, the *miR-17-92* cluster, which includes *miR-18a*, has been shown to promote proliferation of neuronal progenitors [50]. Interestingly, however, *miR-18a* has also been shown to suppress cell proliferation in other contexts such in pancreatic progenitor cells [47] and myoblast cells [51], and even

has anti-proliferation/anti-tumor effects in colorectal and breast cancers [52,48,53]. The effects of *miR-18a* on the cell proliferation are, therefore, dependent on the cell type and injury/disease state of the tissue involved.

In *miR-18a^{mi5012}* retinas, the prolonged proliferation among neuronal progenitors results in a delay in photoreceptor regeneration and maturation, and causes excess neuronal progenitors to be produced. At least some of these excess progenitors remain in the INL or migrate to the GCL, suggesting that they may differentiate into other types of neurons besides photoreceptors. This is consistent with data showing that following damage to retinal neurons, Müller glia derived progenitors are multipotent and generate multiple cell types even though they preferentially generate the neurons that were lost [54-56]. The presence of additional MG-derived cells in the INL and GCL could also be due to aberrant migration of progenitor cells. Chemokines, produced in response to inflammatory cytokines, guide the migration of neural progenitors [57]. While it is unknown if *miR-18a* regulates chemokines directly, the altered cytokine expression observed in *miR-18a^{mi5012}* retinas could possibly lead to altered chemokine expression and aberrant progenitor migration, resulting in more progenitors failing to migrate to the ONL.

The excess proliferation of MG-derived progenitors and higher expression of key inflammatory molecules in *miR-18a^{mi5012}* retinas compared with WT is similar to what is observed in *mmp9* mutant retinas, but there are also some important differences. First, the excess progenitors in *miR-18a^{mi5012}* retinas do not generate excess photoreceptors, and this differs from what is observed in *mmp9* mutant retinas, in which excess

progenitors do generate excess photoreceptors [21]. This difference indicates that *miR-18a* and *Mmp9* may also differentially regulate molecules downstream of pathways that regulate inflammation and cell proliferation. In the subventricular zone of the CNS, *Mmp9* has been found to regulate differentiation of neuronal progenitor cells [58], indicating that it has some capacity to regulate neuronal differentiation. As a microRNA, *miR-18a* likely regulates several molecules involved in different steps of the regeneration response, and members of the *miR-17-92* cluster of miRNAs, which includes *miR-18a*, are key regulators of neurogenesis, involved in cell proliferation, progenitor fate determination and differentiation [reviewed in 59]. Previous work found that *NeuroD* governs the cell cycle and differentiation among photoreceptor progenitors during both embryonic development and regeneration [60,40] and that *miR-18a* regulates *NeuroD* protein levels in the embryonic retina [30]. Downstream of inflammatory pathways, *miR-18a* is also likely to regulate *NeuroD* in the injured retina, and this could affect differentiation of photoreceptors and other cells. The dominant phenotype in injured adult *miR-18a^{mi5012}* retinas, however, is the increased inflammation and cell proliferation, and pathways downstream of inflammation have not yet been investigated.

A second key difference between the *miR-18a^{mi5012}* and *mmp9* mutant retinal phenotypes is that, although both mutations result in an elevated inflammatory response following photoreceptor injury, they result in higher expression of different inflammatory molecules and at different time points. In *miR-18a^{mi5012}* retinas, *tnfα* expression is increased only at 1 dpi, differing markedly from observations in *mmp9* mutant retinas

that have increased expression of *tnfα* (but not other inflammatory molecules) at all post-injury time points [21]. Also in contrast to *mmp9* mutants, *miR-18a^{mi5012}* retinas have increased expression of other cytokines (*il6* and *il1b*) and the cytokine regulator *nfkb1* at 5 dpi, a time point when inflammation is typically subsiding and progenitors are beginning to differentiate. Further, *nfkb1* expression continues to be higher in *miR-18a^{mi5012}* retinas at 7dpi, when widespread differentiation is typically occurring among photoreceptor progenitors. These data indicate that, like *Mmp9*, *miR-18a* negatively regulates inflammation, leading to a converging phenotype (excess neuronal progenitors). However, *miR-18a* regulates different inflammatory molecules than *Mmp9* and, in *miR-18a^{mi5012}* retinas, inflammation is prolonged.

The prolonged expression of important inflammatory molecules in *miR-18a^{mi5012}* retinas during photoreceptor regeneration indicates that *miR-18a* functions during the resolution phase of the inflammatory response, potentially regulating a negative feedback loop that resolves inflammation and restores homeostasis. Resolving neuroinflammation promotes normal tissue repair and if acute inflammation remains unresolved, it can result in inadequate repair or further neuronal damage [61]. Following photoreceptor injury, expression levels of *nfkb1* and certain cytokines are normally upregulated and peak between 24 and 48 hpi and then their expression levels decrease, returning to homeostasis sometime after 7 dpi [see 21]. The removal of NFκB activity and inflammatory cytokine signaling are critical steps in resolving inflammation [62], indicating that the time frame between 48 hpi and 7 dpi in the retina is key for regulating this process. In *miR-18a^{mi5012}* retinas, the higher expression of *nfkb1* and

inflammatory cytokines at 5 dpi compared with WT, and the continued higher expression of *nfkβ1* at 7 dpi, indicate that resolution of the inflammatory response is delayed and that *miR-18a* regulates this process. Treatment of *miR-18a^{mi5012}* fish with dexamethasone from 48 hpi to 6 dpi was a means to chemically resolve retinal inflammation during the normal time frame and, because this fully rescued the *miR-18a^{mi5012}* phenotype (excess proliferation and delayed photoreceptor regeneration), this indicates that *miR-18a* functions during the resolution phase to regulate key aspects of inflammation. This function is in line with functions of several other miRNAs, including *miR-9*, *miR-21*, *miR-146* and *miR-155*, which also play important roles in resolving inflammation by regulating pathways involving macrophages [63]. Importantly, to our knowledge, this is the first study to show that *miR-18a* regulates inflammation, and the first to show that any miRNA regulates inflammation in the injured retina.

Inflammation in the retina following photoreceptor death is generated by the release of cytokines from microglia [64], Müller glia [65], retinal pigmented epithelium (RPE) [reviewed in 66] and dying cells [17]. An underlying assumption is that these cells are responsible for the elevated and prolonged inflammation in the *miR-18a^{mi5012}* retinas, however, given that *miR-18a* expression is elevated relatively ubiquitously in the injured retina, the increased and prolonged inflammation in the mutants could originate from other cellular types. Müller glia are known to secrete inflammatory cytokines in response to retinal injury [23,17,19] and cytokine signals from microglia and/or dying cells are necessary for Müller glia to divide [17,19]. The early expression of *miR-18a* in Müller glia and other retinal cells (by 1 dpi) might, therefore, be a mechanism to limit the

level of retinal inflammation by regulating the secretion and responses to certain inflammatory cytokines. The highest expression of *miR-18a* occurs between 3 and 5 dpi, during the resolution phase of retinal inflammation [see 21]. Based on its 7-base seed sequence (CACCUUA), *miR-18a* is predicted to interact directly with mRNAs of more than 25 molecules that function in inflammatory pathways (http://www.targetscan.org/fish_62), some of which are cytokines and other intercellular signaling molecules (e.g. *Il-16*, *cxcl12a*, *bmp6*), but many of which are transmembrane receptors or molecules that function downstream of inflammatory cytokines (e.g. *Il-7r*, *cxcr4a*, *tnfaip3*). It is therefore probable that *miR-18a* not only reduces inflammatory signals from Müller glia and other cells (e.g. microglia, dying photoreceptors), but also reduces the effects of those inflammatory signals on the MG-derived progenitors.

In conclusion, this study is the first to show that a miRNA regulates inflammation in the injured retina and is the first to show that *miR-18a* is an important inflammatory regulator. This work adds to the growing body of knowledge that miRNAs are key regulators of neurogenesis throughout the central nervous system [67] and neuronal regeneration in the retina [15]. Importantly, the differential responses of Müller glia to inflammation following retinal injury in zebrafish compared with mammals could be key to unlocking the potential for mammalian Müller glia to robustly regenerate neurons. Like *miR-18a*, other miRNAs could be potent inflammatory regulators in the injured retina and may be key to fully augmenting the regenerative potential of the mammalian retina.

Fig. 1 Expression of *miR-18a* in the retina following photoreceptor injury. (a) Taqman RT-qPCR showing retinal expression of *miR-18a* in control (no lesion) retinas compared with 3, 5 and 7 days post-injury (dpi). Expression (fold differences) are calculated using the $\Delta\Delta CT$ method, using the small nuclear protein RNA U6 (*mu6*) as the housekeeping gene. Error bars represent standard deviation and asterisks indicate significant differences between each injury time point and controls (Student's t-test, $p < 0.05$). (b-f) *In situ* hybridizations for *miR-18a* (purple) in retinal cross sections at different post-injury time points in *Tg(gfap:egfp)^{mi2002}* fish, in which Müller glia and MG-derived progenitors express *egfp*, visualized with immunolabeling for EGFP protein (green). White arrowheads show examples of EGFP⁺ Müller glia that express *miR-18a* and black outlined arrowheads show examples of MG-derived photoreceptor progenitors in the ONL that express *miR-18a*. Abbreviations: ONL—outer nuclear layer, INL—inner nuclear layer; *scale bar*: 50 μm

Fig. 2 Comparison of the number of proliferating cells in WT and *miR-18a*^{mi5012} retinas at 3, 7 and 10 days post-photoreceptor injury (dpi). (a) BrdU immunolabeling in 3 dpi retinas in which fish were immersed in 5 mM BrdU from 2 to 3 dpi; BrdU⁺ cells (S phase of the cell cycle) are shown in orange-red and Hoechst labeled cell nuclei are blue (b) BrdU⁺ cell counts in these retinas in cells per 0.3 mm of linear retina. (c) BrdU immunolabeling in 7 dpi retinas in which fish were immersed in 5 mM BrdU from 6 to 7 dpi; BrdU⁺ cells (S phase of the cell cycle) are shown in orange-red and Hoechst labeled cell nuclei are blue; and (d) BrdU⁺ cell counts in these retinas in cells per 0.3 mm of linear retina. (e) BrdU immunolabeling in 10 dpi retinas in which fish were immersed in 5 mM BrdU from 9 to 10 dpi; BrdU⁺ cells (S phase of the cell cycle) are

shown in orange-red and Hoechst labeled cell nuclei are blue; and (f) BrdU⁺ cell counts in these retinas in cells per 0.3 mm of linear retina. Error bars represent standard deviation and asterisks indicate significant differences (Student's t-test, $p < 0.05$); Abbreviations: ONL—outer nuclear layer, INL—inner nuclear layer; *scale bar*: 50 μm

Fig. 3 *In situ* hybridizations and quantification of mature cone and rod photoreceptors in WT and *miR-18a^{mi5012}* retinas at different post-injury time points. (a) *In situ* hybridization for mature cones (*arr3a*) in WT and *miR-18a^{mi5012}* retinas at 7, 10 and 14 days post-injury (dpi). Arrowheads show examples of labeled cones. (b) *In situ* hybridization for mature rods (*rho*) in WT and *miR-18a^{mi5012}* retinas at 7, 10 and 14 days post-injury (dpi). Arrowheads show examples of labeled rods. (c) Cone photoreceptor counts and (d) rod photoreceptor counts in retinal cross sections (cells per 0.3 mm of linear retina). Error bars represent standard deviation and asterisks indicate significant differences (Student's t-test, $p < 0.05$). Dotted lines on the graph connect the tops of the bars to show the trends. Abbreviations: ONL—outer nuclear layer, RPE—retinal pigmented epithelium; *scale bar*: 50 μm

Fig. 4 The fates of cells in 14 dpi WT and *miR-18a^{mi5012}* retinas that were labeled with BrdU at 3-4 dpi. (a) BrdU immunolabeling of cells exposed to BrdU at 3-4 dpi, in the inner nuclear layer (INL) and ganglion cell layer (GCL) of retinas of 14 dpi, in WT and *miR-18a^{mi5012}* fish. White arrowheads show BrdU⁺ cells in the INL and white outlined arrowheads show BrdU⁺ in the GCL. (b) Counts of BrdU⁺ cells in the INL and GCL of these retinas; cells were counted in retinal cross sections across 0.3 mm of linear retina. Error bars represent standard deviation and asterisks indicate significant differences (Student's t-test, $p < 0.05$); *scale bar*: 50 μm

Fig. 5 RT-qPCR showing expression of inflammatory molecules in WT and *miR-18a^{mi5012}* retinas at 1, 3, 5 and 7 dpi. (a-d) RT-qPCR showing fold differences in expression of the inflammatory genes *tnfα* (*tnfa*), *il6*, *nfkb1* and *il1β* (*il1b*) compared with WT 1dpi, normalized to the housekeeping gene *gpia*, in WT and *miR-18a^{mi5012}* retinas at 1 (a), 3 (b), 5 (c) and 7 dpi (d). Error bars represent standard deviation and asterisks indicate significant differences (Student's t-test, $p < 0.05$)

Fig. 6 Rescue of the 7 dpi *miR-18a^{mi5012}* phenotype by inflammatory suppression with dexamethasone. (a) BrdU immunolabeling (orange-red) in retinal cross sections of WT and *miR-18a^{mi5012}* fish exposed to BrdU from 6-7 dpi in control fish (top panels) and dexamethasone treated (Dex) fish (treated from 2-6 dpi to suppress inflammation) (bottom panels); arrowheads show BrdU⁺ cells in the INL of *miR-18a^{mi5012}* retinas (to right) that are largely absent from WT and Dex treated retinas. (b) Quantification of BrdU⁺ cells in retinal cross sections of WT and *miR-18a^{mi5012}* fish exposed to BrdU from 6-7 dpi in control and dexamethasone treated fish. Cells were counted over 0.3 mm

linear retina; error bars represent standard deviation and asterisks indicate significant differences (Student's t-test, $p < 0.05$). (c) *In situ* hybridization for mature cones (*arr3a*) in retinal cross sections of WT and *miR-18a^{mi5012}* control fish (top panels) and dexamethasone treated fish (bottom panels). (d) Quantification of mature cones (*arr3a⁺*) in retinal cross sections of WT and *miR-18a^{mi5012}* control and dexamethasone treated fish. Cells were counted over 0.3 mm linear retina; error bars represent standard deviation and asterisks indicate significant differences (Student's t-test, $p < 0.05$). Abbreviations: ONL—outer nuclear layer, INL—inner nuclear layer, RPE—retinal pigmented epithelium; *scale bars*: 50 μm

REFERENCE LIST

1. Hitchcock P, Ochocinska M, Sieh A, Otteson D (2004) Persistent and injury-induced neurogenesis in the vertebrate retina. *Prog Retin Eye Res* 23 (2):183-194.
doi:papers3://publication/doi/10.1016/j.preteyeres.2004.01.001
2. Bernardos RL, Barthel LK, Meyers JR, Raymond PA (2007) Late-stage neuronal progenitors in the retina are radial Müller glia that function as retinal stem cells. *Journal of Neuroscience* 27 (26):7028-7040.
doi:papers3://publication/doi/10.1523/JNEUROSCI.1624-07.2007
3. Goldman D (2014) Müller glial cell reprogramming and retina regeneration. *Nat Chem Biol*:1-12. doi:papers3://publication/doi/10.1038/nrn3723
4. Lenkowski JR, Raymond PA (2014) Müller glia: Stem cells for generation and regeneration of retinal neurons in teleost fish. *Prog Retin Eye Res* 40C:94-123.
doi:papers3://publication/doi/10.1016/j.preteyeres.2013.12.007
5. Bockerhoff SE, Fadool JM (2011) Genetics of photoreceptor degeneration and regeneration in zebrafish. *Cell Mol Life Sci* 68 (4):651-659.
doi:papers3://publication/doi/10.1007/s00018-010-0563-8
6. Pollak J, Wilken MS, Ueki Y, Cox KE, Sullivan JM, Taylor RJ, Levine EM, Reh TA (2013) ASCL1 reprograms mouse Muller glia into neurogenic retinal progenitors. *Development* 140 (12):2619-2631. doi:papers3://publication/doi/10.1242/dev.091355
7. Jorstad NL, Wilken MS, Grimes WN, Wohl SG, VandenBosch LS, Yoshimatsu T, Wong RO, Rieke F, Reh TA (2017) Stimulation of functional neuronal regeneration from

Müller glia in adult mice. *Nature* 548 (7665):103-107.

doi:papers3://publication/doi/10.1038/nature23283

8. Yao K, Qiu S, Wang YV, Park SJH, Mohns EJ, Mehta B, Liu X, Chang B, Zenisek D, Crair MC, Demb JB, Chen B (2018) Restoration of vision after de novo genesis of rod photoreceptors in mammalian retinas. *Nature* 560 (7719):484-488.

doi:papers3://publication/doi/10.1038/s41586-018-0425-3

9. Hamon A, Roger JE, Yang X-J, Perron M (2016) Müller glial cell-dependent regeneration of the neural retina: An overview across vertebrate model systems. *Dev Dyn* 245 (7):727-738. doi:papers3://publication/doi/10.1002/dvdy.24375

10. Wan J, Goldman D (2016) Retina regeneration in zebrafish. *Curr Opin Genet Dev* 40:41-47. doi:papers3://publication/doi/10.1016/j.gde.2016.05.009

11. Rajaram K, Harding RL, Bailey T, Patton JG, Hyde DR (2014) Dynamic miRNA expression patterns during retinal regeneration in zebrafish: reduced dicer or miRNA expression suppresses proliferation of Müller glia-derived neuronal progenitor cells. *Dev Dyn* 243 (12):1591-1605. doi:papers3://publication/doi/10.1002/dvdy.24188

12. Rajaram K, Harding RL, Hyde DR, Patton JG (2014) miR-203 regulates progenitor cell proliferation during adult zebrafish retina regeneration. *Dev Biol* 392 (2):393-403. doi:papers3://publication/doi/10.1016/j.ydbio.2014.05.005

13. Kaur S, Gupta S, Chaudhary M, Khursheed MA, Mitra S, Kurup AJ, Ramachandran R (2018) let-7 MicroRNA-Mediated Regulation of Shh Signaling and the Gene Regulatory Network Is Essential for Retina Regeneration. *CellReports* 23 (5):1409-1423. doi:papers3://publication/doi/10.1016/j.celrep.2018.04.002

14. Ramachandran R, Fausett BV, Goldman D (2010) Ascl1a regulates Müller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. *Nat Cell Biol* 12 (11):1101-1107.

doi:papers3://publication/doi/10.1038/ncb2115

15. Konar GJ, Ferguson C, Flickinger Z, Kent MR, Patton JG (2020) miRNAs and Müller Glia Reprogramming During Retina Regeneration. *Front Cell Dev Biol* 8:632632.

doi:papers3://publication/doi/10.3389/fcell.2020.632632

16. Plotnikova O, Baranova A, Skoblov M (2019) Comprehensive Analysis of Human microRNA-mRNA Interactome. *Front Genet* 10:933.

doi:papers3://publication/doi/10.3389/fgene.2019.00933

17. Nelson CM, Ackerman KM, O'Hayer P, Bailey TJ, Gorsuch RA, Hyde DR (2013) Tumor necrosis factor- α is produced by dying retinal neurons and is required for Muller glia proliferation during zebrafish retinal regeneration. *Journal of Neuroscience* 33 (15):6524-6539. doi:papers3://publication/doi/10.1523/JNEUROSCI.3838-12.2013

18. Wan J, Zhao X-F, Vojtek A, Goldman D (2014) Retinal Injury, Growth Factors, and Cytokines Converge on β -Catenin and pStat3 Signaling to Stimulate Retina Regeneration. *Cell Reports* 9 (1):285-297.

doi:papers3://publication/doi/10.1016/j.celrep.2014.08.048

19. Zhao X-F, Wan J, Powell C, Ramachandran R, Myers MG, Goldman D (2014) Leptin and IL-6 family cytokines synergize to stimulate Müller glia reprogramming and retina regeneration. *Cell Reports* 9 (1):272-284.

doi:papers3://publication/doi/10.1016/j.celrep.2014.08.047

20. White DT, Sengupta S, Saxena MT, Xu Q, Hanes J, Ding D, Ji H, Mumm JS (2017) Immunomodulation-accelerated neuronal regeneration following selective rod photoreceptor cell ablation in the zebrafish retina. *Proc Natl Acad Sci USA* 114 (18):E3719-E3728. doi:papers3://publication/doi/10.1073/pnas.1617721114
21. Silva NJ, Nagashima M, Li J, Kakuk-Atkins L, Ashrafzadeh M, Hyde DR, Hitchcock PF (2020) Inflammation and matrix metalloproteinase 9 (Mmp-9) regulate photoreceptor regeneration in adult zebrafish. *Glia* 68 (7):1445-1465. doi:papers3://publication/doi/10.1002/glia.23792
22. Zhang Z, Hou H, Yu S, Zhou C, Zhang X, Li N, Zhang S, Song K, Lu Y, Liu D, Lu H, Xu H (2020) Inflammation-induced mammalian target of rapamycin signaling is essential for retina regeneration. *Glia* 68 (1):111-127. doi:papers3://publication/doi/10.1002/glia.23707
23. Iribarne M (2021) Inflammation induces zebrafish regeneration. *Neural Regen Res* 16 (9):1693-1701. doi:papers3://publication/doi/10.4103/1673-5374.306059
24. Nagashima M, Hitchcock PF (2021) Inflammation Regulates the Multi-Step Process of Retinal Regeneration in Zebrafish. *Cells* 2021, Vol 10, Page 783 10 (4):783. doi:papers3://publication/doi/10.3390/cells10040783
25. Todd L, Finkbeiner C, Wong CK, Hooper MJ, Reh TA (2020) Microglia Suppress Ascl1-Induced Retinal Regeneration in Mice. *CellReports* 33 (11):108507. doi:papers3://publication/doi/10.1016/j.celrep.2020.108507

26. Tahamtan A, Teymoori-Rad M, Nakstad B, Salimi V (2018) Anti-Inflammatory MicroRNAs and Their Potential for Inflammatory Diseases Treatment. *Front Immunol* 9:1377. doi:papers3://publication/doi/10.3389/fimmu.2018.01377
27. Roy S, Benz F, Luedde T, Roderburg C (2015) The role of miRNAs in the regulation of inflammatory processes during hepatofibrogenesis. *Hepatobiliary Surg Nutr* 4 (1):24-33. doi:papers3://publication/doi/10.3978/j.issn.2304-3881.2015.01.05
28. Huntzinger E, Izaurralde E (2011) Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nature Publishing Group*:1-12. doi:papers3://publication/doi/10.1038/nrg2936
29. Jonas S, Izaurralde E (2015) Towards a molecular understanding of microRNA-mediated gene silencing. *Nat Rev Genet* 16 (7):421-433. doi:papers3://publication/doi/10.1038/nrg3965
30. Taylor SM, Giuffre E, Moseley P, Hitchcock PF (2019) The MicroRNA, miR-18a, Regulates NeuroD and Photoreceptor Differentiation in the Retina of Zebrafish. *Dev Neurobiol* 279 (27):28492-28418. doi:papers3://publication/doi/10.1002/dneu.22666
31. Bernardos RL, Raymond PA (2006) GFAP transgenic zebrafish. *Gene Expr Patterns* 6 (8):1007-1013. doi:papers3://publication/doi/10.1016/j.modgep.2006.04.006
32. Avdesh A, Chen M, Martin-Iverson MT, Mondal A, Ong D, Rainey-Smith S, Taddei K, Lardelli M, Groth DM, Verdile G, Martins RN (2012) Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: an introduction. *J Vis Exp* (69):e4196. doi:papers3://publication/doi/10.3791/4196

33. Taylor S, Chen J, Luo J, Hitchcock P (2012) Light-induced photoreceptor degeneration in the retina of the zebrafish. *Methods Mol Biol* 884:247-254.
doi:papers3://publication/doi/10.1007/978-1-61779-848-1_17
34. Luo J, Uribe RA, Hayton S, Calinescu A-A, Gross JM, Hitchcock PF (2012) Midkine-A functions upstream of Id2a to regulate cell cycle kinetics in the developing vertebrate retina. *Neural Dev* 7 (1):33. doi:papers3://publication/doi/10.1186/1749-8104-7-33
35. Barthel LK, Raymond PA (1993) Subcellular localization of α -tubulin and opsin mRNA in the goldfish retina using digoxigenin-labeled cRNA probes detected by alkaline phosphatase and HRP histochemistry. *Journal of Neuroscience Methods* 50 (2):145-152. doi:papers3://publication/doi/10.1016/0165-0270(93)90002-9
36. Hitchcock P, Kakuk-Atkins L (2004) The basic helix-loop-helix transcription factor neuroD is expressed in the rod lineage of the teleost retina. *J Comp Neurol* 477 (1):108-117. doi:papers3://publication/doi/10.1002/cne.20244
37. David R, Wedlich D (2001) PCR-based RNA probes: a quick and sensitive method to improve whole mount embryo in situ hybridizations. *Biotech* 30 (4):769-775.
doi:papers3://publication/uuid/E5750AEF-427A-404F-B8B5-8B198FE51962
38. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Meth* 9 (7):671-675.
doi:papers3://publication/doi/10.1038/nmeth.2089
39. Nagashima M, Barthel LK, Raymond PA (2013) A self-renewing division of zebrafish Müller glial cells generates neuronal progenitors that require N-cadherin to regenerate

retinal neurons. *Development* 140 (22):4510-4521.

doi:papers3://publication/doi/10.1242/dev.090738

40. Taylor SM, Alvarez-Delfin K, Saade CJ, Thomas JL, Thummel R, Fadool JM, Hitchcock PF (2015) The bHLH Transcription Factor NeuroD Governs Photoreceptor Genesis and Regeneration Through Delta-Notch Signaling. *Invest Ophthalmol Vis Sci* 56 (12):7496-7515. doi:papers3://publication/doi/10.1167/iovs.15-17616

41. Martin JF, Poché RA (2019) Awakening the regenerative potential of the mammalian retina. *Development* 146 (23).

doi:papers3://publication/doi/10.1242/dev.182642

42. Pesaresi M, Bonilla-Pons SA, Simonte G, Sanges D, Di Vicino U, Cosma MP (2018) Endogenous Mobilization of Bone-Marrow Cells Into the Murine Retina Induces Fusion-Mediated Reprogramming of Müller Glia Cells. *EBioMedicine* 30:38-51.

doi:papers3://publication/doi/10.1016/j.ebiom.2018.02.023

43. Sanges D, Romo N, Simonte G, Di Vicino U, Tahoces AD, Fernández E, Cosma MP (2013) Wnt/ β -catenin signaling triggers neuron reprogramming and regeneration in the mouse retina. *CellReports* 4 (2):271-286.

doi:papers3://publication/doi/10.1016/j.celrep.2013.06.015

44. Sanges D, Simonte G, Di Vicino U, Romo N, Pinilla I, Nicolás M, Cosma MP (2016) Reprogramming Müller glia via in vivo cell fusion regenerates murine photoreceptors. *J Clin Invest* 126 (8):3104-3116. doi:papers3://publication/doi/10.1172/JCI85193

45. Conner C, Ackerman KM, Lahne M, Hobgood JS, Hyde DR (2014) Repressing notch signaling and expressing TNF α are sufficient to mimic retinal regeneration by

inducing Müller glial proliferation to generate committed progenitor cells. *Journal of Neuroscience* 34 (43):14403-14419.

doi:papers3://publication/doi/10.1523/JNEUROSCI.0498-14.2014

46. Mitchell DM, Lovel AG, Stenkamp DL (2018) Dynamic changes in microglial and macrophage characteristics during degeneration and regeneration of the zebrafish retina. *J Neuroinflammation* 15 (1):163-120.

doi:papers3://publication/doi/10.1186/s12974-018-1185-6

47. Liu L, Cai X, Liu E, Tian X, Tian C (2017) MicroRNA-18a promotes proliferation and metastasis in hepatocellular carcinoma via targeting KLF4. *Oncotarget* 8 (40):68263-68269. doi:papers3://publication/doi/10.18632/oncotarget.19293

48. Shen K, Cao Z, Zhu R, You L, Zhang T (2019) The dual functional role of MicroRNA-18a (miR-18a) in cancer development. *Clin Trans Med* 8 (1):1-13.

doi:papers3://publication/doi/10.1186/s40169-019-0250-9

49. Jiang Y, Zhou J, Zhao J, Hou D, Zhang H, Li L, Zou D, Hu J, Zhang Y, Jing Z (2020) MiR-18a-downregulated RORA inhibits the proliferation and tumorigenesis of glioma using the TNF- α -mediated NF- κ B signaling pathway. *EBioMedicine* 52:102651.

doi:papers3://publication/doi/10.1016/j.ebiom.2020.102651

50. Bian S, Hong J, Li Q, Schebelle L, Pollock A, Knauss JL, Garg V, Sun T (2013) MicroRNA Cluster miR-17-92 Regulates Neural Stem Cell Expansion and Transition to Intermediate Progenitors in the Developing Mouse Neocortex. *Cell Reports* 3 (5):1398-1406. doi:papers3://publication/doi/10.1016/j.celrep.2013.03.037

51. Liu C, Chen M, Wang M, Pi W, Li N, Meng Q (2018) MiR-18a regulates myoblasts proliferation by targeting Fgf1. PLoS ONE 13 (7):e0201551.
doi:papers3://publication/doi/10.1371/journal.pone.0201551
52. Humphreys KJ, McKinnon RA, Michael MZ (2014) miR-18a inhibits CDC42 and plays a tumour suppressor role in colorectal cancer cells. PLoS ONE 9 (11):e112288.
doi:papers3://publication/doi/10.1371/journal.pone.0112288
53. Zhang N, Zhang H, Liu Y, Su P, Zhang J, Wang X, Sun M, Chen B, Zhao W, Wang L, Wang H, Moran MS, Haffty BG, Yang Q (2019) SREBP1, targeted by miR-18a-5p, modulates epithelial-mesenchymal transition in breast cancer via forming a co-repressor complex with Snail and HDAC1/2. Cell Death Differ 26 (5):843-859.
doi:papers3://publication/doi/10.1038/s41418-018-0158-8
54. D'Orazi FD, Suzuki SC, Darling N, Wong RO, Yoshimatsu T (2020) Conditional and biased regeneration of cone photoreceptor types in the zebrafish retina. J Comp Neurol 528 (17):2816-2830. doi:papers3://publication/doi/10.1002/cne.24933
55. Ranski AH, Kramer AC, Morgan GW, Perez JL, Thummel R (2018) Characterization of retinal regeneration in adult zebrafish following multiple rounds of phototoxic lesion. PeerJ 6:e5646. doi:papers3://publication/doi/10.7717/peerj.5646
56. Powell C, Cornblath E, Elsaiedi F, Wan J, Goldman D (2016) Zebrafish Müller glia-derived progenitors are multipotent, exhibit proliferative biases and regenerate excess neurons. Sci Rep 6:24851. doi:papers3://publication/doi/10.1038/srep24851

57. Belmadani A, Tran PB, Ren D, Miller RJ (2006) Chemokines regulate the migration of neural progenitors to sites of neuroinflammation. *Journal of Neuroscience* 26 (12):3182-3191. doi:papers3://publication/doi/10.1523/JNEUROSCI.0156-06.2006
58. Barkho BZ, Munoz AE, Li X, Li L, Cunningham LA, Zhao X (2008) Endogenous matrix metalloproteinase (MMP)-3 and MMP-9 promote the differentiation and migration of adult neural progenitor cells in response to chemokines. *Stem Cells* 26 (12):3139-3149. doi:papers3://publication/doi/10.1634/stemcells.2008-0519
59. Xia X, Wang Y, Zheng JC (2020) The microRNA-17~92 Family as a Key Regulator of Neurogenesis and Potential Regenerative Therapeutics of Neurological Disorders. *Stem Cell Rev Rep*. doi:papers3://publication/doi/10.1007/s12015-020-10050-5
60. Ochocinska MJ, Hitchcock PF (2009) NeuroD regulates proliferation of photoreceptor progenitors in the retina of the zebrafish. *Mechanisms of Development* 126 (3-4):128-141. doi:papers3://publication/doi/10.1016/j.mod.2008.11.009
61. Dokalis N, Prinz M (2019) Resolution of neuroinflammation: mechanisms and potential therapeutic option. *Semin Immunopathol* 41 (6):699-709. doi:papers3://publication/doi/10.1007/s00281-019-00764-1
62. Fullerton JN, Gilroy DW (2016) Resolution of inflammation: a new therapeutic frontier. *Nat Rev Drug Discov* 15 (8):551-567. doi:papers3://publication/doi/10.1038/nrd.2016.39
63. Alam MM, O'Neill LA (2011) MicroRNAs and the resolution phase of inflammation in macrophages. *Eur J Immunol* 41 (9):2482-2485. doi:papers3://publication/doi/10.1002/eji.201141740

64. Mitchell DM, Sun C, Hunter SS, New DD, Stenkamp DL (2019) Regeneration associated transcriptional signature of retinal microglia and macrophages. *Sci Rep* 9 (1):4768-4717. doi:papers3://publication/doi/10.1038/s41598-019-41298-8
65. Sifuentes CJ, Kim J-W, Swaroop A, Raymond PA (2016) Rapid, Dynamic Activation of Müller Glial Stem Cell Responses in Zebrafish. *Invest Ophthalmol Vis Sci* 57 (13):5148-5160. doi:papers3://publication/doi/10.1167/iovs.16-19973
66. Holtkamp GM, Kijlstra A, Peek R, de Vos AF (2001) Retinal pigment epithelium-immune system interactions: cytokine production and cytokine-induced changes. *Prog Retin Eye Res* 20 (1):29-48. doi:papers3://publication/doi/10.1016/s1350-9462(00)00017-3
67. Rajman M, Schratt G (2017) MicroRNAs in neural development: from master regulators to fine-tuners. *Development* 144 (13):2310-2322. doi:papers3://publication/doi/10.1242/dev.144337