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

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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-*

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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

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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.

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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.

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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, *nfkb1*, 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-

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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,

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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-

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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—aattaaccctcactaaagggCTTCGAAGGGGTTCTTGCCGC) and cones *arr3a* (F—GAAGACCAGTGGAAATGGCCAG, R—aattaaccctcactaaagggTCAGAGGCAGCTCTACTGTCAC). *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.

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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 ΔΔCT method was used to calculate expression relative to WT (unlesioned or control) and datawere normalized to *gpia* as the housekeeping gene. Sequences for the standard qPCR primers used were as follows: *gpia* F—TCCAAGGAAACAAGCCAAGC, R—TTCCACATCACACCCTGCAC; *nfkb1* F—CAGCTGGTGACCAACTCTCAG, R—TCCTGTAGGCCTCCATCATGC; *tnfα* F—CTGGAGAGATGACCAGGACCAGGCC, R—GCTGTGGTCGTGTCTGTGCCCAGTC; *il1β* 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 ΔΔCT method was used to calculate expression at different post-injury time points relative to unlesioned. Statistical significance between

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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 signficant).

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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=0.0.024) 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 ribroprobe 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.

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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 $\pm$ 7.2 SD, WT 87.6 $\pm$ 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 $\pm$ 9.8 SD, WT 30.6 $\pm$ 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 $\pm$ 3.2 SD, WT 14.0 $\pm$ 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.

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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-*

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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 regeneneration 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}

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15.1 ± 2.2 SD, WT 6.2 ± 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 *tnfα* is higher in *miR-18a*^{mi5012} retinas compared with WT (*miR-18a*^{mi5012} 4.51 ± 1.14 SD, WT 2.31 ± 0.99 SD, p=0.033) (Figure 5a). Then at 3 dpi, around the normal peak of MG-derived progenitor proliferation, *il1β* expression

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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*, *il1β*, 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; *il1b 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

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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 ± 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,

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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 photoceptor differentiation in the developing

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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

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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

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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, *tnf*α expression is increased only at 1 dpi, differing markedly from observations in *mmp9* mutant retinas

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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 *nfκb1* 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 *nfκb1* and

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inflammatory cytokines at 5 dpi compared with WT, and the continued higher expression of *nfκb1* 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

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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.

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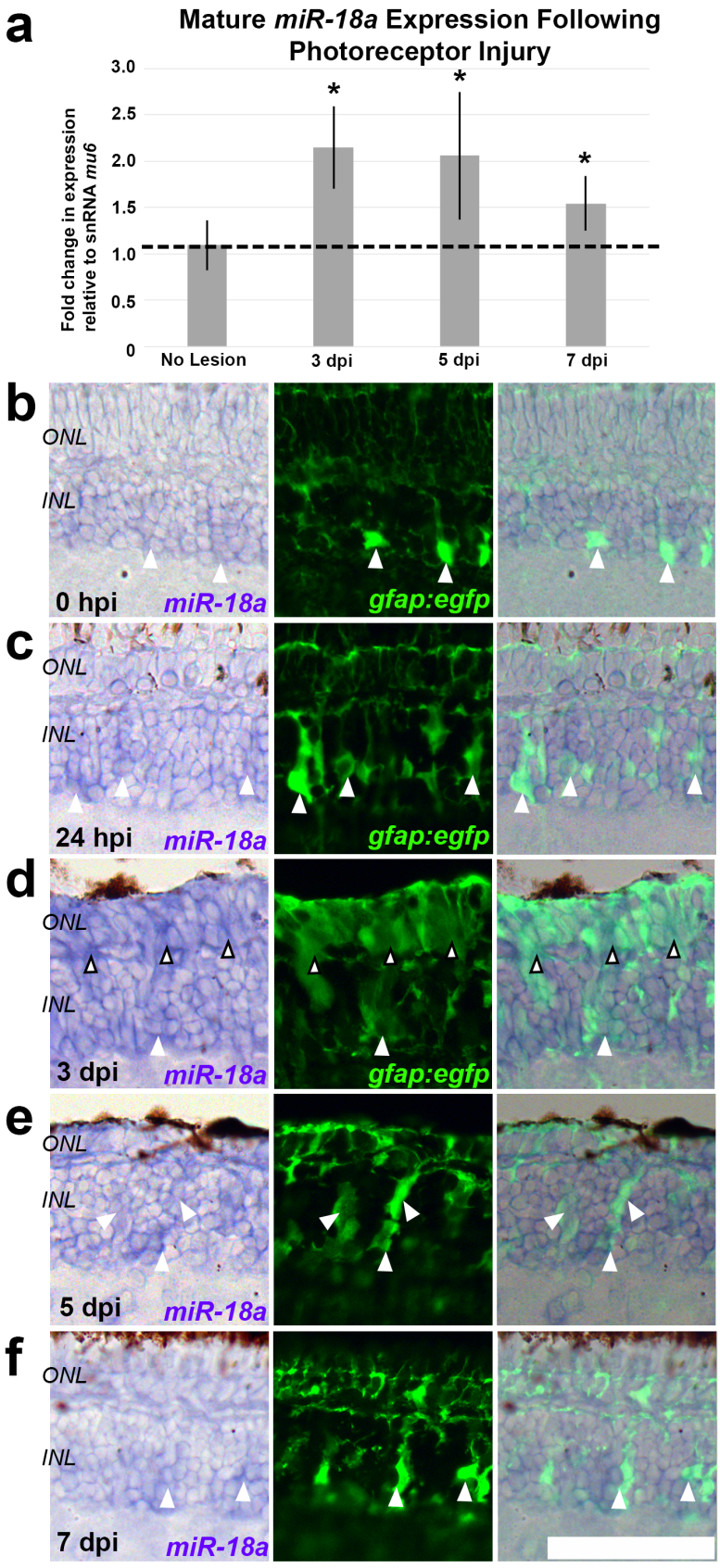

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Fig. 1 Expression of *miR-18a* in the retina following photoreceptor injury. (a) Taqman RT-qPCR showing retinal expression of *miR-18a* in control (no lesion) retinas compared with 3, 5 and 7 days post-injury (dpi). Expression (fold differences) are calculated using the ΔΔCT 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

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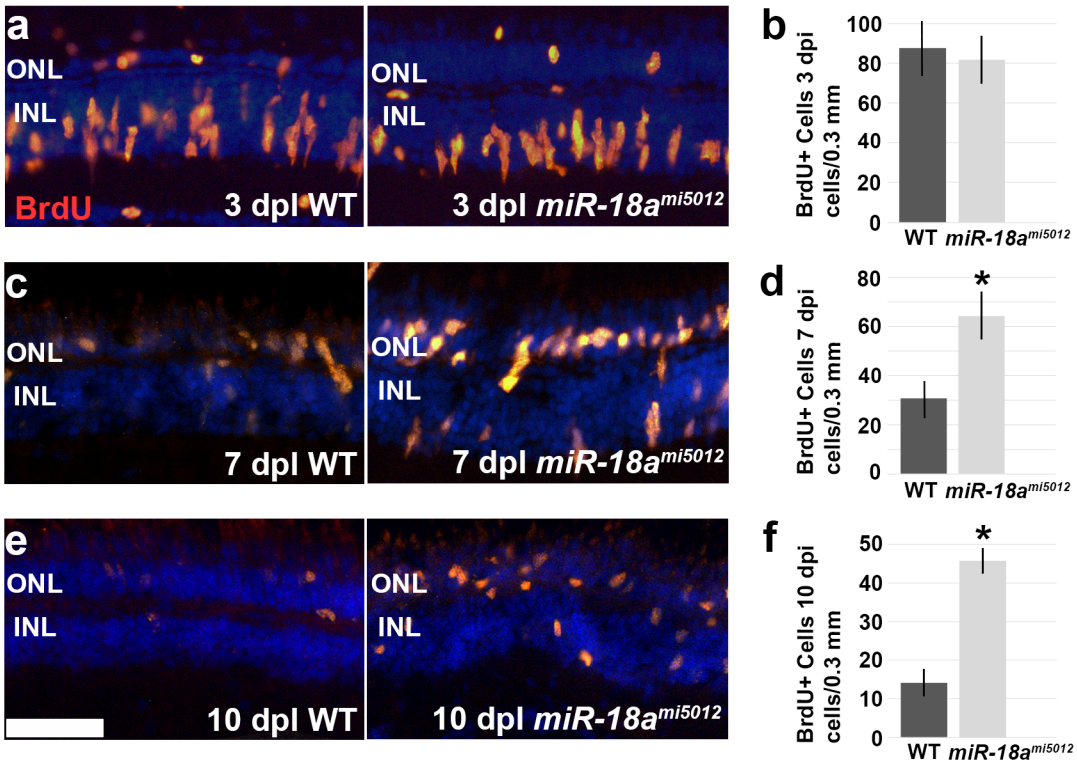

Fig. 2 Comparison of the number of proliferating cells in WT and *miR-18a*^{mi5012} retinas at 3, 7 and 10 days post-photoreceptor injury (dpi). (a) BrdU immunolabeling in 3 dpi retinas in which fish were immersed in 5 mM BrdU from 2 to 3 dpi; BrdU^{+} cells (S phase of the cell cycle) are shown in orange-red 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

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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

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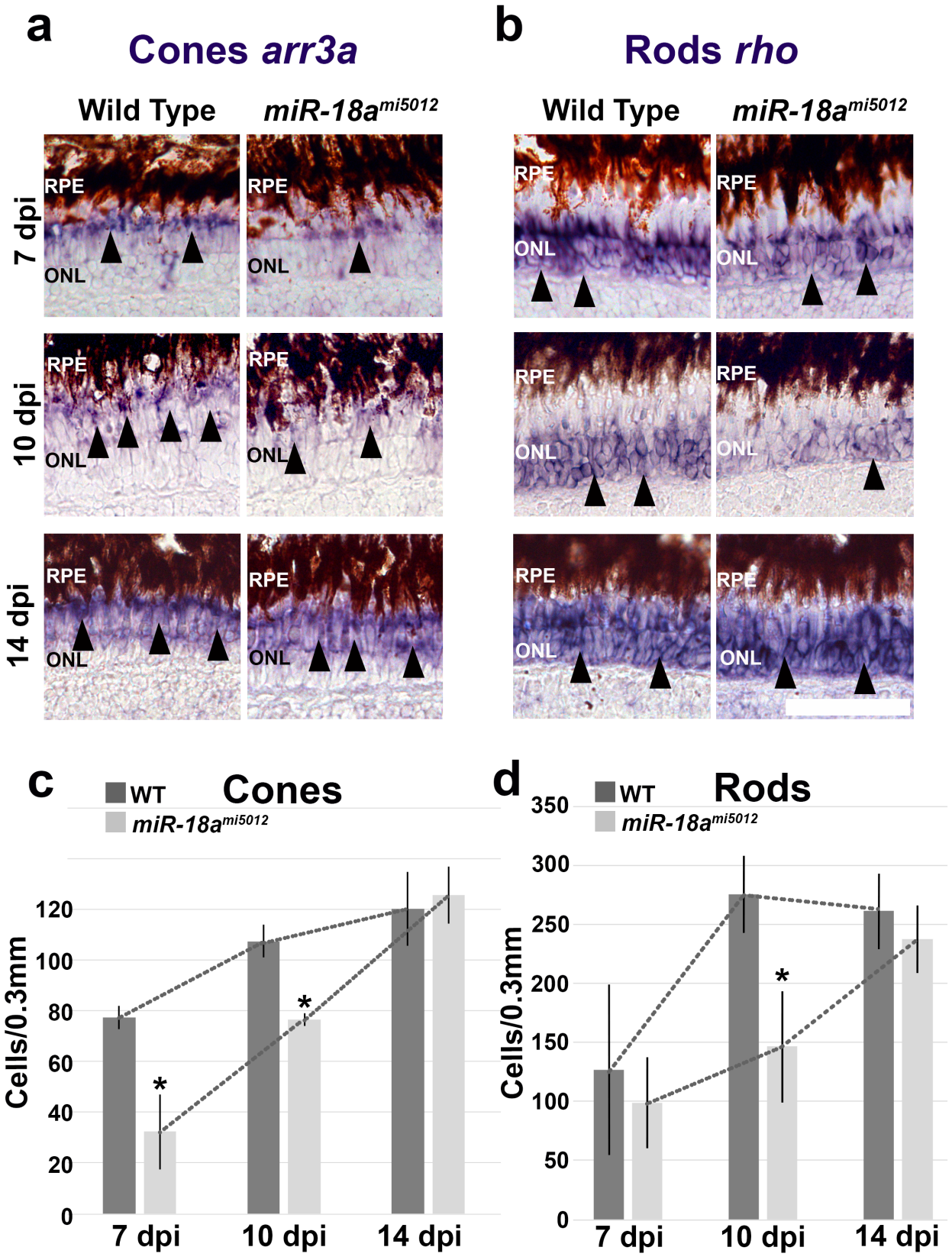

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Fig. 3 *In situ* hybridizations and quantification of mature cone and rod photoreceptors in WT and *miR-18a*^{mi5012} retinas at different post-injury time points. (a) *In situ* hybridization for mature cones (*arr3a*) in WT and *miR-18a*^{mi5012} 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

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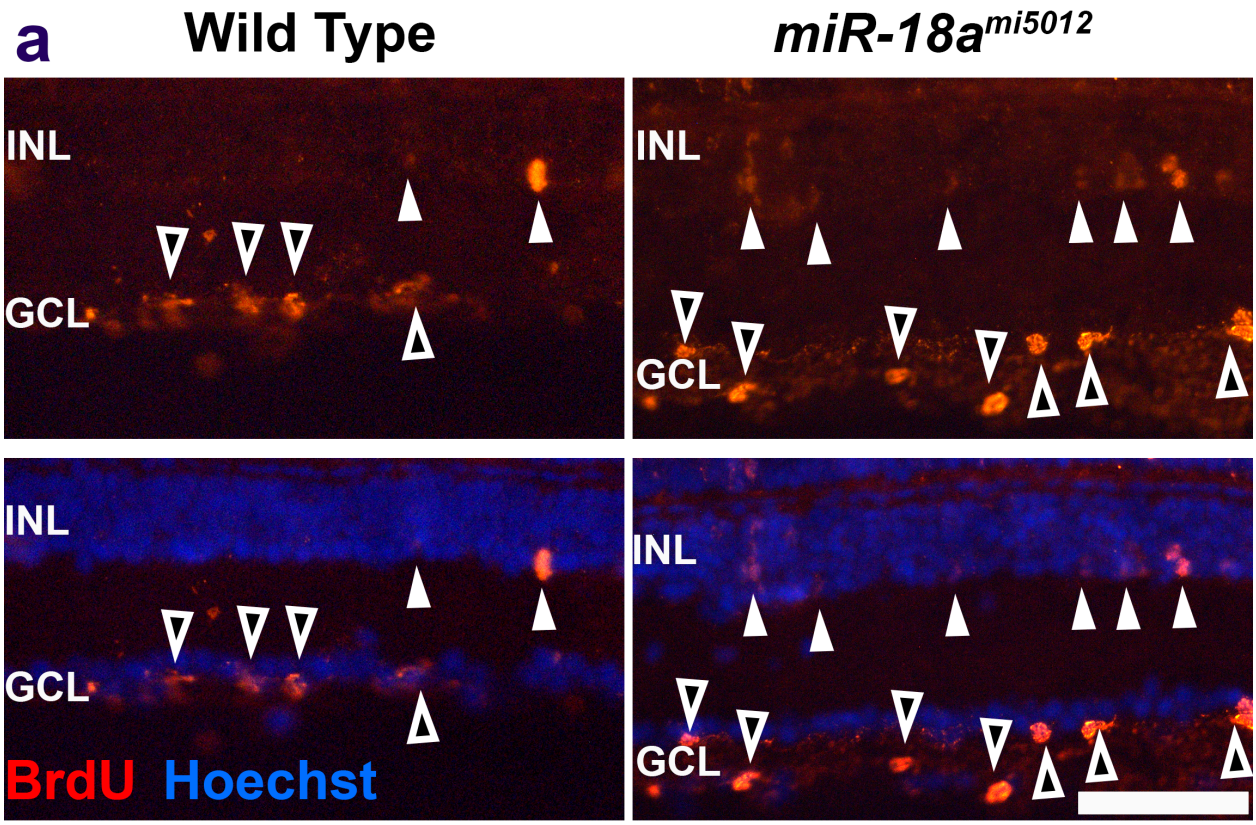

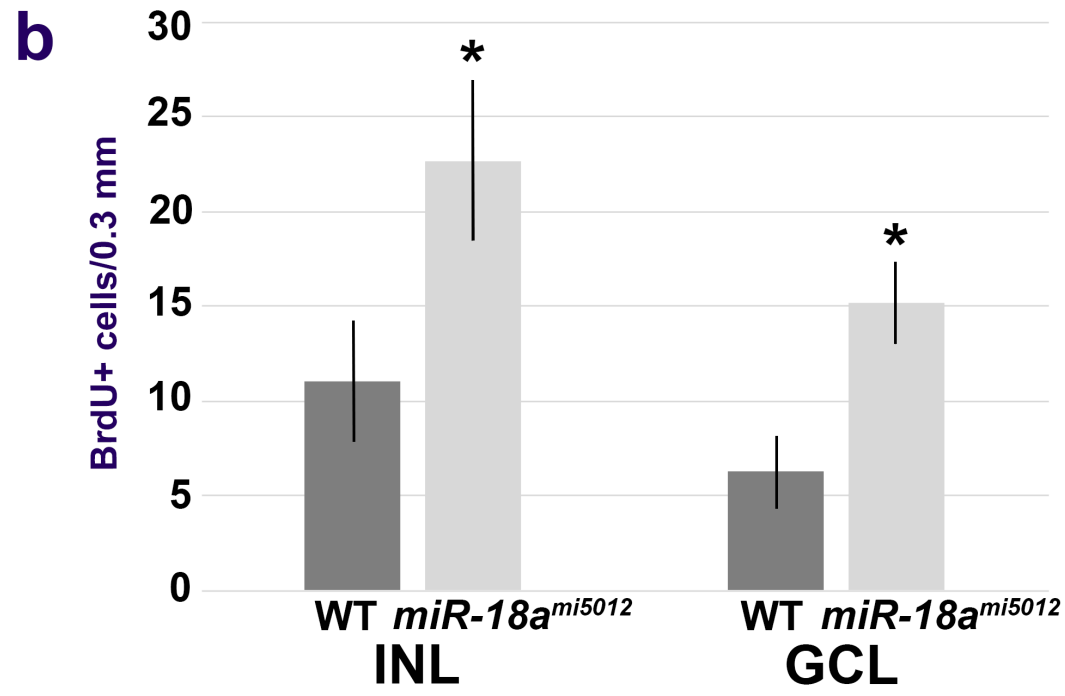

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Fig. 4 The fates of cells in 14 dpi WT and *miR-18a*^{mi5012} retinas that were labeled with BrdU at 3-4 dpi. (a) BrdU immunolabeling of cells exposed to BrdU at 3-4 dpi, in the inner nuclear layer (INL) and ganglion cell layer (GCL) of retinas of 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

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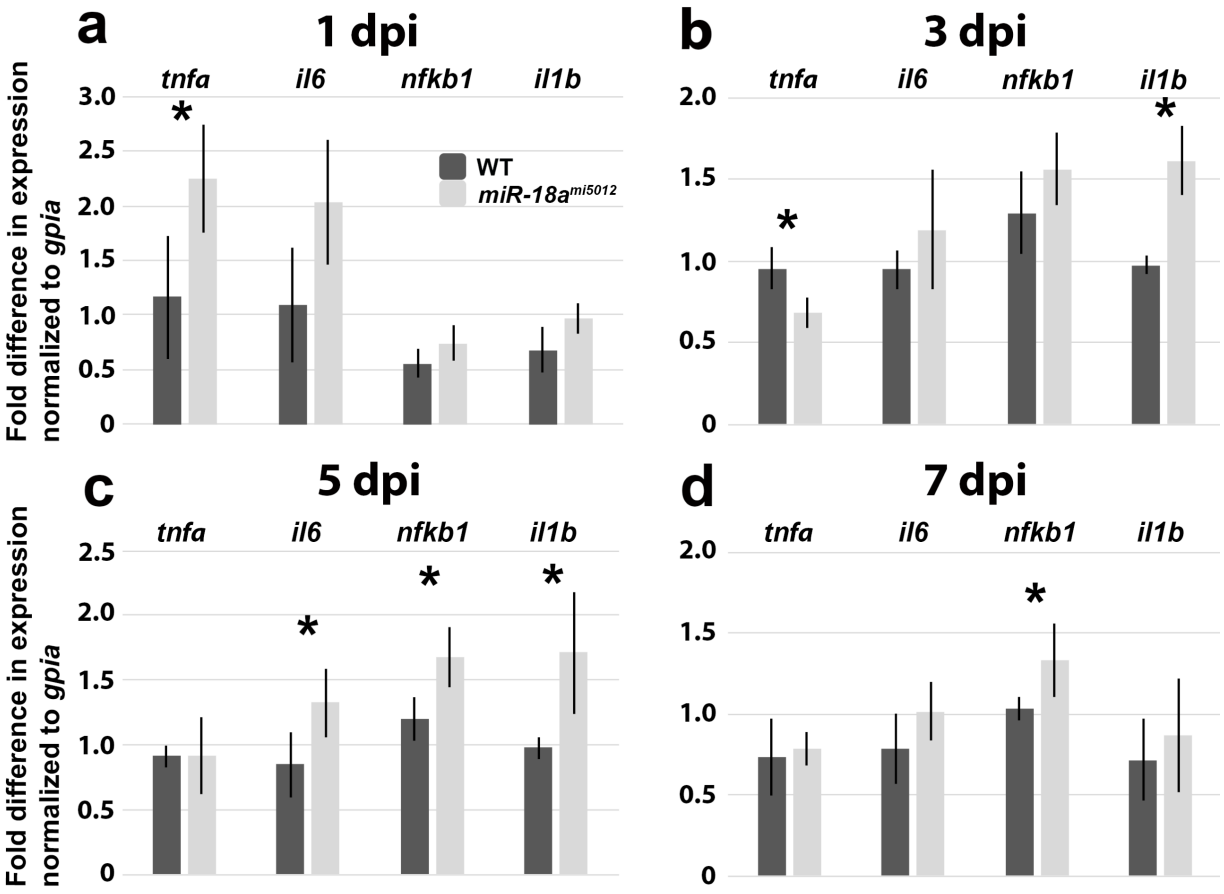

Fig. 5 RT-qPCR showing expression of inflammatory molecules in WT and *miR-18a*^{mi5012} retinas at 1, 3, 5 and 7 dpi. (a-d) RT-qPCR showing fold differences in expression of the inflammatory genes *tnf*α (*tnfa*), *il6*, *nfkb1* and *il1*β (*il1b*) compared with WT 1dpi, normalized to the housekeeping 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, $p<0.05$)

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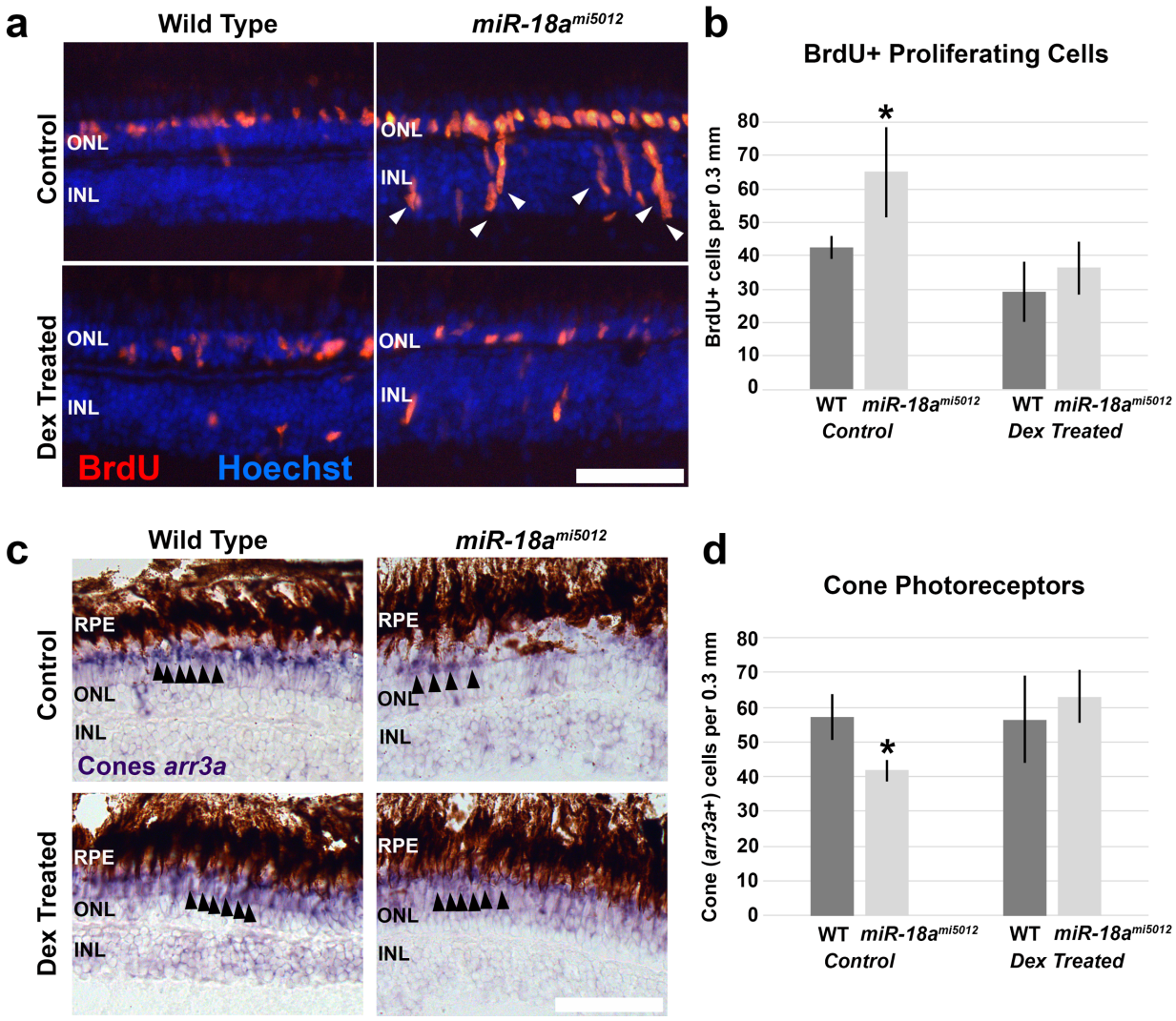

Fig. 6 Rescue of the 7 dpi *miR-18a*^{mi5012} phenotype by inflammatory suppression with dexamethasone. (a) BrdU immunolabeling (orange-red) in retinal cross sections of WT and *miR-18a*^{mi5012} fish exposed to BrdU from 6-7 dpi in control fish (top panels) and dexamethasone treated (Dex) fish (treated 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

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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

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