Published in final edited form as:
Dev Biol. 2014 August 15; 392(2): 393–403. doi:10.1016/j.ydbio.2014.05.005.

miR-203 regulates progenitor cell proliferation during adult zebrafish retina regeneration

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Abstract

Damage of the zebrafish retina triggers a spontaneous regeneration response that is initiated by Müller Glia (MG) dedifferentiation and asymmetric cell division to produce multipotent progenitor cells. Subsequent expansion of the progenitor pool by proliferation is critical for retina regeneration. Pax6b expression in the progenitor cells is necessary for their proliferation, but exact regulation of its expression is unclear. Here, we show that *miR-203* is downregulated during regeneration in proliferating progenitor cells. Elevated *miR-203* levels inhibit progenitor cell expansion without affecting MG dedifferentiation or progenitor cell generation. Using GFP-reporter assays and gain and loss of function experiments in the retina, we show that *miR-203* expression must be suppressed to allow *pax6b* expression and subsequent progenitor cell proliferation.

Keywords

miR-203; progenitor cell proliferation; *pax6b*; retina regeneration

Introduction

Retinal cell loss is the cause of many degenerative human diseases, including retinitis pigmentosa and age-related macular degeneration. In humans as well as other mammals, retinal cell loss is irreversible and ultimately leads to blindness. This is in stark contrast to the zebrafish retina, where damage by a variety of methods triggers a spontaneous regeneration response that restores not only lost retinal cell types, but also retina function (Fausett and Goldman, 2006; Vihtelic and Hyde, 2000; Yurco and Cameron, 2005). Intriguingly, Müller glia (MG), the cell type that initiates retina regeneration, are not specific to the zebrafish retina, but are common to all vertebrates (Lamba et al., 2008).

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Competing Interests
The authors declare no financial or non-financial competing interests.

However, mammalian MG normally respond to injury by undergoing reactive gliosis, which leads to scarring (Bringmann et al., 2009). Treatment of explants with transcription factors and select growth factors can induce mammalian MG to mount a regeneration response but the response is extremely limited (Karl et al., 2008; Ooto et al., 2004; Pollak et al., 2013). Understanding the mechanisms controlling retina regeneration in the zebrafish is a starting point to design strategies to trigger regeneration in the mammalian retina.

Recently, a number of genes and signaling pathways that control important steps in zebrafish retina regeneration have been identified. Ascl1a and Lin-28 activate MG dedifferentiation and cell cycle reentry (Ramachandran et al., 2010), while Pax6a and Pax6b are essential for proliferation of MG-derived progenitor cells (Thummel et al., 2010). Precise regulation of these genes is central to ensure efficient regeneration. miRNAs regulate gene expression by binding to recognition sites in the 3′ untranslated regions (UTRs) of target mRNAs triggering both mRNA decay and repression of translation (Kloosterman and Plasterk, 2006). Distinct subsets of miRNAs have been shown to regulate proliferation, maintenance of pluripotency, cell specification, and differentiation (Melton et al., 2010; Wang et al., 2008). miRNAs have also been implicated in regulating regeneration in a number of species (Sehm et al., 2009). In zebrafish, miRNAs regulate regeneration of a variety of tissues including the fin, heart, spinal cord and retina (Park et al., 2011; Ramachandran et al., 2010; Thatcher et al., 2008; Yin et al., 2012; Yin et al., 2008). In this study, RNA sequencing and analysis of specific cell types during zebrafish retina regeneration revealed that *miR-203* expression must be downregulated during retina regeneration to enable proliferation of MG-derived progenitor cells. Downregulation of *miR-203* allows upregulation of *pax6b*, which is required for increased progenitor cell proliferation and the formation of clusters of replicating progenitor cells associated with dedifferentiated MG. Our results demonstrate for the first time that miRNAs play a key role in the zebrafish retinal regeneration response after MG dedifferentiation and during the proliferation of MG-derived progenitor cells.

Materials and Methods

Fish maintenance

Zebrafish were maintained in 14h light and 10h dark cycles at 28.5°C. *Albino* fish were received from David Hyde (University of Notre Dame), *tg:1016tuba1a:gfp* were received from Daniel Goldman (University of Michigan) (Fausett and Goldman, 2006). Other fish lines used were *tg:gfap:gfp* (Bernardos and Raymond, 2006) and wild type AB. Embryos for microinjections were obtained from matings of AB fish. All experiments were performed with the approval of the Vanderbilt University Institutional Animal Care and Use Committee (Protocol # M/09/398).

Adult zebrafish light lesioning

Constant instense light lesioning was performed as previously described (Vihtelic and Hyde, 2000). Briefly, adult fish were dark adapted for 14 days, transferred to clear tanks placed between two fluorescent lights with light intensity at ~20,000 lux and the temperature maintained at 30–33°C. Zebrafish were subjected to light lesioning from 16h–3 days.

RNA isolation, RT-PCR, Taqman realtime PCR

Total RNA was isolated from control and light damaged zebrafish retinas using TRI Reagent®. For semi quantitative PCR, oligo-dT primers (Life Technologies) were used to synthesize cDNA using MMLV reverse transcriptase (Promega). PCR was performed using Phusion DNA polymerase (NEB) on a MyCycler thermal cycler (Biorad). GAPDH was used as a loading control. For quantitative real time PCR (qPCR), RNA was DNase treated (Rapid Out, Thermo Scientific), converted to cDNA using Maxima first strand cDNA synthesis kit (Thermo Scientific) and qPCR was performed using SYBR Green (Biorad). All qPCR primers spanned exon-exon junctions (IDT). miRNA realtime PCR was perfomed using Taqman probes as per the manufacturer's instructions (Life Technologies). Relative RNA expression during regeneration was determined using the ΔΔCt method and normalized to 18s rRNA levels and U6 snRNA levels for mRNAs and miRNAs respectively. Real time PCR was performed on a Biorad CFX 96 Real time system. Primer sequences are listed in Supplemental Table 1.

Morpholino and miRNA injection and electroporation

Lissamine tagged morpholinos (MOs) (Gene Tools) were injected and electroporated into adult zebrafish eyes prior to light lesioning as described (Thummel et al., 2008b). The following 3′-Lissamine-tagged MOs were used:

Gene Tools standard control MO:	5′-CCTCTTACCTCAGTTACAATTTATA-3′
pax6b MO:	5′-CTGAGCCCTTCCGAGCAAAACAGTG-3′
miR-203a MO:	5′-CAAGTGGTCCTAAACATTTCAC-3′

Duplex mature miRNAs (Thermo scientific) were injected and electroporated into eyes either prior to start of light lesioning (Figure 2, 3, 5), or 51h after light lesioning (Figure 4) using the same procedure as the MOs but with reversed electrode polarity. For initial experiments, we used RNAs that contained a Dy-547 fluorescent tag at the 3′ end. Double stranded mature miRNAs were synthesized with 3′-UU overhangs for the following target sequences:

miR-203: 5′-GUGAAAUGUUUAGGACCACUUG-3′

control: 5′-AAAAACAUGCAGAAAAUGCUG-3′

Electroporation was performed using the Gene Pulser Xcell™ Electroporation Systems (Biorad).

Immunohistochemistry, BrdU labeling, TUNEL assay and *in situ* hybridization

Adult zebrafish eyes were collected and fixed in 4% paraformaldehyde for 2–5h at room temperature. Following fixation, eyes were cryoprotected overnight in 30% sucrose/1X PBS at 4°C, before embedding in Shandon cryomatrix (Thermo Scientific) for sectioning. Embedded samples were kept at −80°C until sectioning. 10–12 micron sections were obtained using a cryostat (Leica), collected on charged Histobond slides (VWR), dried and stored at −80°C until used. For immunohistochemistry (IHC), slides were thawed for 30 min on a slide warmer, rehydrated in 1X PBS and blocked (3% Donkey serum, 0.1% TritonX-100 in 1X PBS) for 1–2h at room temperature before incubating with primary

antibodies overnight at 4°C. Primary antibodies were mouse anti-PCNA monoclonal antibody (1:500, Sigma), mouse anti-glutamine synthetase monoclonal antibody-clone GS8 (1:200, Millipore), rat anti-BrdU monoclonal antibody (1:500, Abcam), rabbit anti-GFP polyclonal antiserum (1:1000, Torrey Pines Biolabs) and mouse anti-β-catenin antibody (1:500, BD Bioscience). After primary antibody incubation, sections were washed 3 times in 1X PBS/0.1% Tween-20 for 10 min each followed by 1–2h incubation in secondary antibody and nuclear stain TOPRO 3 (1:1000, Invitrogen) at room temperature. Secondary antibodies were donkey anti-mouse AF488 (1:200), donkey anti-mouse AF647 (1:200), donkey anti-rat Cy3 (1:100) and donkey anti-rabbit AF488 (1:200)(Jackson Immuno). Slides were washed in 1X PBS/0.1% Tween-20, 3 times for 10 mins, followed by a 5 min PBS wash before drying and coverslipping with Vectashield (Vector labs). Nuclear β-catenin was detected following citrate buffer antigen retrieval, as previously described (Ramachandran et al., 2011). For PCNA IHC, slides were boiled in 10mM sodium citrate buffer containing 0.05% Tween-20 (pH 6) for 20 mins, cooled at room temperature for 20 min and then quickly washed in 1X PBS prior to blocking. After IHC, slides were examined using an LSM 510 Meta inverted confocal microscope (Zeiss). For BrdU labeling, adult fish received a 25ul (20mM) IP injection of BrdU (Sigma) 3h prior to sacrifice. For BrdU IHC, sections were pretreated with 2N HCl for 20 min at 37°C and then quenched in 100mM sodium borate 2 times for 5 min each. For TUNEL assays, sections were processed as for IHC. TUNEL assays were performed using the TMR red *in situ* cell death detection kit (Roche). Digoxigenin labeled LNA probes (Exiqon) were used for *miR-203 in situ* hybridizations. A final probe concentration of 500nM and hybridization temperature of 42°C was used. Hybridization and wash conditions were previously described (Ramachandran et al., 2010). Slides were examined using a Leica DM6000B microscope.

Western blots

Protein lysates from 1dpf embryos were prepared as described previously (Flynt et al., 2007). Briefly, 1dpf embryos were dechorionated, deyolked and sonicated in lysis buffer supplemented with PMSF. For adult retinas, dorsal retinas were dissected from 7 fish per treatment group and sonicated in lysis buffer. Insoluble debris was removed by centrifugation and 20ug and 40ug of protein was separated on 12% polyacrylamide gels, for embryo lysates and adult retina lysates respectively. Proteins were transferred to PVDF membranes and probed with antibodies against GFP (1:1000, Torrey Pines), Pax6 (1:4000, Covance), Actin (1:100, Santa Cruz) and α-tubulin (1:1000, Abcam). For visualization, HRP-conjugated secondary antibodies (1:5000, GE Healthcare) were used followed by detection with ECL (Perkin Elmer). Imaging was performed on an LAS4000 Chemiluminescent CCD Imager (GE Healthcare) and blots were quantified using LAS4000 ImageQuant software (GE healthcare). GFP levels were normalized to α-tubulin levels and the GFP/tubulin ratio was determined for different injection conditions. Actin served as loading control for Pax6 Western blots.

Fluorescence activated cell sorting (FACS)

FACS was used to purify GFP^+ cells from the retinas of undamaged *tg:gfap:gfp* fish and *tg:1016tuba1a:gfp* fish that were exposed to 72h of light damage following a procedure adapted from Qin et al. (Qin and Raymond, 2012). Briefly, retinas were pooled from 15

undamaged *tg:gfap:gfp* fish and 20, 72h light treated *tg:1016tuba1a:gfp* fish. Dissected retinas were treated with activated papain/dispase (Worthington) and incubated at 28°C for 30 min on a nutator for dissociation. Dissociated cells were pelleted by centrifugation, resuspended in DNaseI solution (Sigma) and gently tapped to complete tissue dissociation. Cells were triturated briefly, filtered through 35-micron filters and then sorted using BD FACSAria III (BD Biosciences). Approximately 27,000 GFP^+ cells were obtained from 15 undamaged *tg:gfap:gfp* fish and 28,000 GFP^+ cells were obtained from 20 light-damaged *tg:1016tuba1a:gfp* fish. As quality control for FACS, qPCR was used to confirm MG specific glutamine synthetase and progenitor cell specific *ascl1a* expression (Supplemental Figure 1). FACS was performed in the VUMC Flow Cytometry Shared Resource.

Molecular cloning and embryo microinjections

The *pax6b* 3′UTR was amplified from cDNA by PCR with the following primers:

pax6b-3′utr-fp: 5′-ACTAGTAAGGAACAACAGCCATTGTG-3′

pax6b-3′utr-rp: 5′ CTGTCTTGCAGATATTTCAATTTAACCTCGAG-3′

The 3′ UTR was cloned into the $pCS2^+$ plasmid downstream of the GFP coding sequence. Capped RNA was transcribed from these reporter constructs using SP6 mMessage mMachine (Life Technologies). Zebrafish embryos at the single-cell stage were injected with 50pg of mRNA with or without 100pg of synthetic duplex *miR-203a* (Thermo Scientific). Titrations were performed to determine the lowest effective injection concentrations.

Cell counts and statistical analyses

For confocal microscopy, only retina sections that comprised optic nerves were used. All cell counts were done in the central-dorsal retina, at a linear distance of ~300 microns from the optic nerve. 3–8 retinas were used in every experiment. In Figures 4, 5 and 6, progenitor clusters were defined as groups of more than 3 closely adherent $PCNA^+$ cells. In Figures 2–5 and 6O–P data are represented as mean +/− standard error of the mean (s.e.m) and significance was calculated using the non-parametric Mann-Whitney U test. Student-t tests were used to calculate significance for qPCR data and Western blots.

Results

miR-203 is downregulated in proliferating progenitor cells during retina regeneration

To identify differentially expressed miRNAs during regeneration of the light-damaged zebrafish retina, we performed a high-throughput sequencing screen. We identified 35 miRNAs that showed altered expression during regeneration (Harding, R., Rajaram, K., Bailey, T.J., Hyde, D.R., and Patton, J.G., manuscript in preparation). Among these, the *miR-203* family was found to be downregulated (Figure 1A), a finding that intrigued us because we previously showed that *miR-203* downregulation is necessary for zebrafish caudal fin regeneration (Thatcher et al., 2008). Similarly, *miR-203* is downregulated in proliferating progenitor cells during mouse skin development and regeneration (Lena et al., 2008; Viticchie et al., 2012; Yi et al., 2008). To determine the role of *miR-203* in retina

regeneration, we examined its spatial expression pattern in undamaged and regenerating retinas using *in situ* hybridization with an LNA probe targeting mature *miR-203* (Figure 1B, C). Sections from undamaged retinas showed *miR-203* expression in the Ganglion Cell Layer (GCL), most of the Inner Nuclear Layer (INL) and the photoreceptor outer segments; with slightly lower expression in the Outer Nuclear Layer (ONL) (Figure 1B). In contrast, *miR-203* expression was markedly reduced in light-damaged retinas that were actively regenerating (Figure 1C). The reduction was most striking in the INL, whereas the relative *miR-203* levels in the GCL were overall similar or only slightly reduced between damaged and undamaged retinas.

Following light-induced injury, Müller glia respond by dedifferentiating and undergoing a single asymmetric division to produce progenitor cells that then continue to divide (Nagashima et al., 2013). As regeneration proceeds, clusters of proliferating progenitor cells occupy the INL along the processes of dedifferentiated MG (Fausett and Goldman, 2006; Nagashima et al., 2013; Thummel et al., 2008a). We sought to determine whether *miR-203* levels are reduced in proliferating progenitor cells. To test this, we used fluorescence activated cell sorting (FACS) to isolate specific retinal cell types from two different transgenic lines: one expressing GFP in post-mitotic MG from undamaged retinas (*tg:gfap:gfp)* (Bernardos and Raymond, 2006), and the other expressing GFP in dedifferentiated MG and proliferating progenitor cells from actively regenerating retinas (*tg: 1016tuba1a:gfp*) (Fausett and Goldman, 2006) (Supplemental Figure 1). Quantitative real-time PCR (qPCR) for *miR-203* in these FACS-purified cell types revealed a ~70% reduction in *miR-203* expression levels in proliferating progenitor cells relative to undamaged MG (Figure 1D). Together, these experiments indicate that *miR-203* expression is downregulated in proliferating progenitor cells in the regenerating retina.

miR-203 overexpression affects proliferation during regeneration

Next, we examined if downregulation of *miR-203* was necessary for retina regeneration. We performed gain-of-function studies (Supplemental Figure 2) by experimentally increasing the levels of *miR-203* or a control miRNA prior to the onset of light damage. In our hands, 51h of light exposure in *albino* fish corresponds to the peak of dedifferentiated MG cell cycle reentry and the generation of progenitor cells, while 72h of light exposure corresponds to progenitor cell proliferation in the regenerating retina (unpublished). Thus, we overexpressed *miR-203* or a control miRNA in dark-adapted *albino* fish retinas prior to the start of intense light damage (0h light exposure) and then assessed their effects on PCNA expression at 51h and 72h (Figure 2A). Compared to the control, *miR-203* overexpression resulted in a significant decrease ($p<0.001$; $n=8$) in the number of $PCNA^+$ proliferating progenitor cells at 72h, but not at 51h (Figure 2B,C). Additionally, ONL rod progenitor proliferation was unaffected by *miR-203* gain-of-function (Figure 2D). These data suggested that *miR-203* overexpression affects progenitor cell proliferation but not MG proliferation. To verify this, we injected zebrafish that were overexpressing either control miRNA or *miR-203* with BrdU to specifically label proliferating cells at 51h. We labeled MG processes using an antibody against glutamine synthetase (GS). Immunohistochemistry revealed GS^+ MG incorporating BrdU (Figure 3A, arrows). *miR-203* gain-of-function did not affect MG proliferation (Figure 3B) or rod progenitor proliferation (Figure 3A, arrowheads; Figure 3C).

Taken together, these data indicate *miR-203* overexpression does not delay MG cell dedifferentiation or proliferation during retina regeneration, but specifically affects progenitor cell proliferation.

To test if suppression of *miR-203* was sufficient to trigger retina regeneration, we injected and electroporated either control morpholinos (MOs) or MOs targeting mature *miR-203* into undamaged *1016tuba1a:gfp* transgenic fish eyes. This transgenic line specifically expresses GFP in dedifferentiated MG and progenitor cells. Compared to control MO treatment, loss of *miR-203* did not cause significant changes in MG dedifferentiation or overall retinal proliferation (data not shown). Based on these results, we hypothesize that *miR-203* downregulation is necessary for progenitor cell proliferation, but is not sufficient for initiation of MG dedifferentiation or for progenitor cell generation.

miR-203 inhibits progenitor cluster formation

To further test the hypothesis that progenitor cell proliferation and not progenitor cell generation is regulated by *miR-203*, we utilized the *1016tuba1a:gfp* transgenic line that specifically expresses GFP in dedifferentiated MG and progenitor cells. Constant intense light exposure elicited a regeneration response in these fish that followed a timeline consistent with that observed in *albino* zebrafish (unpublished). Overexpression of *miR-203* in these fish did not alter *tuba1a:GFP* transgene expression or BrdU incorporation at 51h (Figure 3D). We also detected β-catenin accumulation in $GFP^+/BrdU^+$ MG in both control and *miR-203* overexpressing retinas (Figure 3D, arrows). β-catenin stabilization is required for MG proliferation and progenitor cell generation (Ramachandran et al., 2011). Thus MG proliferation and progenitor cell generation is unperturbed by *miR-203* gain-of-function.

In contrast, *miR-203* overexpression resulted in a significant reduction in the total number of $PCNA^+$ progenitor cells at 72h ($p<0.03$; *n=4*) (Figure 4C, G, J). We also observed a striking reduction in the number of dedifferentiated MG associated with clusters of at least four $PCNA^+$ progenitor cells ($p< 0.03$; *n=4;* Figure 4K; arrows in Figure 4B–I; nuclei in higher magnification Figure 4C′, G′). With increased *miR-203*, we found that most dedifferentiated MG were associated with only 1–3 $PCNA^+$ progenitor cells (arrowheads in Figure 4F–I; nuclei in higher magnification Figure 4G″). These data are consistent with the hypothesis that *miR-203* inhibits progenitor cell proliferation and the formation of large progenitor clusters. TUNEL staining demonstrated that these effects were not simply due to toxicity associated with *miR-203* overexpression (Supplemental Figure 3).

Since MG dedifferentiation precedes progenitor cell proliferation during regeneration, we altered the timing of *miR-203* overexpression to more precisely define its role. Thus, we overexpressed *miR-203* at 51h, a time point after most MG have dedifferentiated and asymmetrically divided to generate progenitor cells (Figure 5A). Under these conditions, *miR-203* overexpression resulted in a significant reduction ($p<0.03$; *n=3–5* retinas; Figure 5C) in the number of $PCNA^+$ progenitor cells compared to control miRNA injections (Figure 5B). Large clusters (>3) of $PCNA^+$ progenitor cells with elongated cell bodies were almost undetectable in *miR-203* overexpressing retinas ($p<0.03$; *n=3–5*; Figure 5D; Figure 5B). Taken together, these results support the hypothesis that *miR-203* inhibits progenitor

cell proliferation, and that downregulation of *miR-203* is necessary to generate large clusters of progenitor cells during retina regeneration.

miR-203 regulates retina regeneration through *pax6b*

To determine what mRNA targets are regulated by *miR-203* during progenitor cell proliferation, we used target prediction algorithms including Targetscan (Lewis et al., 2005). Several potential *miR-203* targets were identified and tested for direct targeting (see Discussion; Supplemental Table 2). Among these, *pax6b* emerged as a likely target (Supplemental Figure 4B). Previous studies showed that Pax6b is expressed in proliferating progenitor cells during retina regeneration and is required for the formation of large clusters of progenitor cells (Thummel et al., 2008a). To determine if *miR-203* can directly regulate *pax6b*, we performed GFP reporter assays in zebrafish embryos (Flynt et al., 2007) (Figure 6A–C, Supplemental Figure 4B). The 3′UTR of *pax6b* was cloned downstream of the GFP coding sequence and *in vitro* transcribed mRNAs from these reporter constructs were injected into single-cell stage zebrafish embryos in the presence or absence of exogenous *miR-203*. At 1dpf, co-injection with *miR-203* reduced GFP levels by ~40% relative to the control injections (Figure 6B,C) ($p<0.008$, n=3). Consistent with previous studies (Thummel et al., 2010; Thummel et al., 2008a) and consistent with our finding that *miR-203* levels decrease during regeneration, we detected upregulation of *pax6b* transcripts in RNA isolated from whole retinas during regeneration (Figure 6D and Supplemental Figure 5). More precisely, we determined the levels of *pax6b* in FACS-purified cells and observed significant enrichment of *pax6b* mRNA in proliferating progenitor cells compared to post-mitotic MG from undamaged retinas ($p < 0.008$; n=3) (Figure 6E). These data are consistent with the hypothesis that *miR-203* regulates *pax6b*.

If *miR-203* regulates *pax6b*, then overexpression of *miR-203* should mimic morpholino knockdown of *pax6b*. Previously, Thummel et al. (2010) showed that loss of *pax6b* reduced the number of $PCNA^+$ and $BrdU^+$ cells in *albino* fish subject to intense light damage (Thummel et al., 2010). To test whether overexpression of *miR-203* only affects progenitor cell proliferation by regulating *pax6b*, we injected and electroporated either standard control MOs or *pax6b* MOs into the eyes of *1016tuba1a:gfp* transgenic zebrafish prior to the start of light lesioning (Figure 6F). Under these conditions, MG dedifferentiation was unaffected (Figure 6G, K), and consistent with previous results, there were fewer $PCNA^+$ progenitor cells in the *pax6b* MO-treated retinas at 72h compared to controls ($p<0.002$; n=6–7; Figure 6H, L, O). Interestingly, there was a significant decrease in the number of progenitor clusters (arrows in Figure 6I, M) associated along the processes of dedifferentiated MG in *pax6b* MO-injected retinas ($p<0.002$; n=6–7; Figure 6P). Additionally, knockdown of *pax6b* yielded clusters with only 1–3 progenitor cells per dedifferentiated MG (arrowheads in Figure 6M) relative to the larger clusters observed in standard control morphants. This indicates that loss of Pax6b significantly reduces progenitor cell proliferation and phenocopies the *miR-203* gain-of-expression result.

The reporter experiments and the Pax6b loss-of-function experiments indicate the *miR-203* regulates *pax6b*. To test whether overexpression of *miR-203* would decrease Pax6b levels *in vivo*, we analyzed Pax6b protein levels in the dorsal retina after *miR-203* or control miRNA

gain-of-function. Consistent with the hypothesis that *miR-203* regulates Pax6b, we observed a ~30% reduction in Pax6b protein levels (Supplemental Figure 6). We attempted to directly rescue the *miR-203* gain-of-function phenotype by injection and electroporation of *pax6b* mRNA but unfortunately we could not achieve mRNA delivery.

Discussion

Here, we identify *miR-203* as a novel regulator of zebrafish retina regeneration. We demonstrate that *miR-203* represses *pax6b*, which is necessary for progenitor cell expansion. During retina regeneration, *miR-203* levels must be reduced to allow progenitor cell amplification and cluster formation via *pax6b* expression. Elevated *miR-203* or loss of *pax6b* expression during retina regeneration inhibits progenitor cell proliferation and significantly impairs progenitor cluster formation. This reveals a novel regulatory mechanism that controls progenitor cell proliferation, a key step in retina regeneration.

miR-203 inhibits proliferation

miR-203 functions as negative regulator of proliferation and stem cell properties in a number of cancers (Viticchie et al., 2011; Wang et al., 2013; Wang et al., 2012; Yu et al., 2013), as well as in mouse skin development and regeneration (Jackson et al., 2013; Lena et al., 2008; Viticchie et al., 2012; Yi et al., 2008). *miR-203* is also important for zebrafish regeneration. We previously showed that downregulation of *miR-203* is necessary to initiate blastema formation following caudal fin amputation (Thatcher et al., 2008). During retina regeneration, *in situ* localization showed that *miR-203* levels are significantly reduced in the INL upon light damage and qPCR revealed a nearly 70% reduction in *miR-203* levels in progenitor cells compared to undamaged post-mitotic MG (Figure 1). This is highly reminiscent of the role of *miR-203* in mouse skin development, where *miR-203* expression is restricted to the differentiated and basal layers of newly stratified skin epithelium, but conspicuously absent in the proliferating progenitor compartment (Yi et al., 2008). Overexpression of *miR-203* induced skin progenitors to exit the cell cycle, which severely reduced the pool of proliferating progenitors (Yi et al., 2008). During zebrafish retina regeneration, *miR-203* downregulation in progenitor cells is also necessary for their proliferation (Figures 2, 4, 5). We restricted our analysis of the effects of *miR-203* to the first 72h post injury. It will be interesting to determine the fate of the proliferating progenitor cells in retinas overexpressing *miR-203*, as well as the effects of long-term suppression of *miR-203* on regeneration.

miR-203 inhibits progenitor expansion by targeting *pax6b*

Intense light treatment causes dying photoreceptors to express TNFα, which induces MG dedifferentiation (Nelson et al., 2013). Dedifferentiated MG express *ascl1a*, activate Wnt signaling, and undergo asymmetric cell division to produce progenitor cells (Nagashima et al., 2013; Ramachandran et al., 2011). These progenitors undergo multiple rounds of cell division and form clusters, which associate along the processes of the dedifferentiated MG and span the length of the INL (Thummel et al., 2010). Ectopic expression of *miR-203* before starting intense light exposure in the transgenic *1016:tuba1a:gfp* line did not affect overall MG dedifferentiation or the first cell divisions needed to generate progenitor cells

(Figure 3 and Supplemental Figure 3). However, relative to control retinas, we detected a significant reduction in the number of dedifferentiated MG associated with progenitor clusters that had >3 associated progenitor cells (Figure 4). Instead, most of the dedifferentiated MG were associated with only 1–3 progenitor cells. This reduction was apparent when *miR-203* was overexpressed specifically during the progenitor expansion phase in the regenerating retina (Figure 5).

Progenitor cell proliferation during retina regeneration is under the control of *pax6* genes. While *pax6b* controls the first cell division of the MG-derived progenitor cells to generate 4 cell clusters, *pax6a* is required for further amplification of these 4 cell clusters (Thummel et al., 2010). It was previously reported that MO-mediated loss of *pax6b* prevents cluster formation by inhibiting the first cell division of the MG-derived progenitor cells. This is similar to the inhibition of progenitor cell proliferation and cluster formation phenotype we observed with *miR-203* gain-of-function (Figures 4, 5), and consistent with *miR-203* targeting of *pax6b* using reporter and qPCR assays (Figure 6). Similar to *miR-203* gain-of-function, we detected a reduction in progenitor cluster number upon MO-mediated reduction of *pax6b* (Figure 6). Moreover, overexpression of *miR-203* during the progenitor cell proliferation phase resulted in a ~30% reduction in Pax6b protein levels in dorsal retinas (Supplemental Figure 6). A more elegant approach would be to rescue the *miR-203* overexpression phenotype by co-injection of *pax6b* mRNA. Unfortunately the ability to achieve delivery and expression of mRNAs by injection and electroporation into retinas has not been reported and proved technically challenging in our hands.

Regulation of Pax6b

Although the role of Pax6b in progenitor cell expansion is well established, the exact mechanism that regulates its expression is not clear. Knockdown of HB-EGF and *ascl1a* reduced the total amount of *pax6b* mRNA in the retina (Ramachandran et al., 2011; Wan et al., 2012) and stabilization of β-catenin upregulated *pax6b* levels (Ramachandran et al., 2011). Since *pax6b* is upregulated in proliferating progenitor cells, regulatory mechanisms must exist to precisely control its expression in these MG-derived progenitors. An intriguing hypothesis is that Wnt activation in asymmetrically derived progenitor cells suppresses *miR-203* leading to upregulation of *pax6b* and subsequent progenitor cell expansion.

Regulation of *miR-203*

While *miR-203* must be downregulated to allow progenitor cell proliferation, the mechanism of its downregulation remains unknown. In human keratinocytes, *miR-203* expression is activated by PKC and inhibited by EGF treatment (Sonkoly et al., 2010). We previously showed that during zebrafish development, *miR-203* expression is activated by suppressing hedgehog signaling (Thatcher et al., 2007). Studies in cancer also suggest epigenetic control of *miR-203* expression via promoter DNA methylation (Bueno et al., 2008; Diao et al., 2013; Taube et al., 2013). In zebrafish, both *miR-203* genes (*miR-203a* and *miR-203b;* Supplemental Figure 4A) are monocistronic, contain their own promoters and reside on separate chromosomes, suggesting possible transcriptional and epigenetic control of *miR-203* gene expression. A recent study detected the existence of dynamic DNA methylation in MG and progenitor cells during zebrafish retina regeneration (Powell et al.,

2013). Inhibition of DNA methylation during progenitor cell expansion inhibited proliferation, suggesting that genes that inhibit progenitor cell proliferation might be epigenetically suppressed. Moreover, HB-EGF induction in the dedifferentiated MG is necessary for progenitor cell production and retina regeneration (Wan et al., 2012). It is tempting to speculate that *miR-203* expression in progenitor cells could be negatively regulated via both HB-EGF and promoter methylation, thus allowing progenitor cell proliferation and cluster formation.

Targets of *miR-203*

Several genes that are critical for retina regeneration are predicted to be *miR-203* targets, including HB-EGF, Lin-28, and Insm1a (Supplemental Table 2). We found that *pcna* and *lef1*, which are both important for regeneration, are *bona fide miR-203* targets (unpublished data) (Thatcher et al., 2008). Interestingly, *miR-203* gain-of-function prior to retina damage does not inhibit PCNA expression early (51h of light), but does reduce PCNA expression at later stages (72h of light). This suggests that PCNA may not be a primary *miR-203* target during retina regeneration, but rather is a consequence of *miR-203* repressing DNA replication through its inhibition of the progenitor cell cycle. During zebrafish caudal fin regeneration, targeting of *lef1* by *miR-203* was central to both initiation and termination of regeneration (Thatcher et al., 2008). However, we have not found that targeting of *lef1* by *miR-203* affects retina regeneration (data not shown). In mouse skin development, the main target for *miR-203* is ΔNp63, a member of the p53 family and a regulator of proliferation in epithelial cells and various cancers (Dotto, 2012; Lee and Kimelman, 2002; Parsa et al., 1999; Rivetti di Val Cervo et al., 2012; Senoo et al., 2007; Truong et al., 2006; Yang et al., 1999). In zebrafish, *p63* expression is upregulated during optic nerve regeneration (Saul et al., 2010). However, we detected very low levels of *p63* expression in FACS-purified post mitotic MG and progenitor cells from undamaged and regenerating retinas, respectively, suggesting that *p63* is not an important target for *miR-203* in the retina.

Limitations and implications of miRNA gain-of-function experiments

A caveat of our *miR-203* overexpression studies is that all retinal cells, not just MG, take up the miRNA (Supplemental Figure 2). Hence, it is formally possible that inhibition of progenitor cell proliferation is a result of gain of *miR-203* in cell types other than the MG-derived progenitor cells, that the effects we observe may not be cell autonomous. Since *miR-203* can regulate many targets and Pax6 proteins are also expressed in amacrine and bipolar cells, it is possible that *miR-203* inhibition of Pax6b or additional targets in these retinal neurons feeds back and inhibits progenitor cell proliferation by additional secondary mechanisms. It is therefore possible that the loss of progenitor cell proliferation due to excess *miR-203* could be the result of the function of *miR-203* in progenitor cells, retinal neurons or both.

miRNAs and regeneration

Early zebrafish retina regeneration can be divided into distinct steps requiring precise gene expression changes as MG dedifferentiate, undergo asymmetric division, and generate progenitor cells (Fausett and Goldman, 2006; Nagashima et al., 2013; Thummel et al.,

2008a). We show for the first time that *miR-203* must be downregulated to derepress *pax6b* and allow progenitor cell proliferation. Coupled with previous work showing that *let-7* plays a key role regulating MG dedifferentiation (Ramachandran et al., 2010), it seems likely that additional miRNAs will be identified that regulate not only early steps during regeneration, but also migration and re-differentiation as lost cell types are replaced. *miR-203* is an especially interesting case as it has now been demonstrated to play a key regulatory role in progenitor proliferation during development (Jackson et al., 2013; Lena et al., 2008; Yi et al., 2008), regeneration (Thatcher et al., 2008), and possibly adult neurogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Qiang Guan for zebrafish maintenance at the Vanderbilt University Stevenson Center Fish Facility. This study was supported in part by grants from the National Eye Institute–National Institutes of Health to J.G.P (R21 EY 019759) and D.R.H. (R01-EY018417) and the Center for Zebrafish Research, University of Notre Dame. K.R. was supported in part by the Vanderbilt International Scholar Program and by the Gisela Mosig fund in the Department of Biological Sciences.

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Highlights

- *miR-203* expression is downregulated during retina regeneration
- Downregulation of *miR-203* is necessary for progenitor cell proliferation
- Excess *miR-203* impairs formation of progenitor clusters
- *miR-203* regulates proliferation by suppressing Pax6b expression

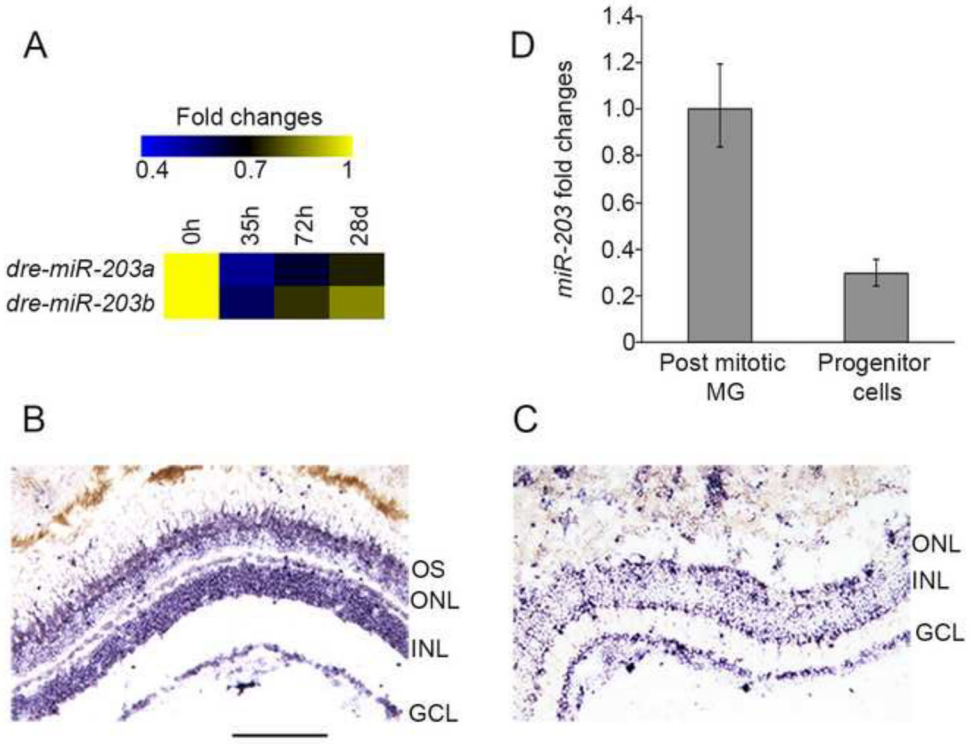

Figure 1. *miR-203* is downregulated in proliferating progenitor cells during retina regeneration

(A) Heat map showing fold changes in deep sequencing reads for *miR-203a* and *b* during retina regeneration. Fold changes relative to 0h read number are shown. (B–C) LNA *in situ* hybridization for *miR-203*. Sections from undamaged retinas show strong *miR-203* signals in the INL, GCL and photoreceptor outer segments (B). Staining is reduced in the INL of light damaged retinas (60h), but persists in the GCL (C). (D) qPCR for *miR-203* in FACS purified post mitotic Müller glia (MG) and dedifferentiated MG-derived progenitor cells from 72h light treated fish. Data represent mean +/− s.e.m from 15 undamaged fish and progenitor cells were purified from 20 light damaged fish. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 100um.

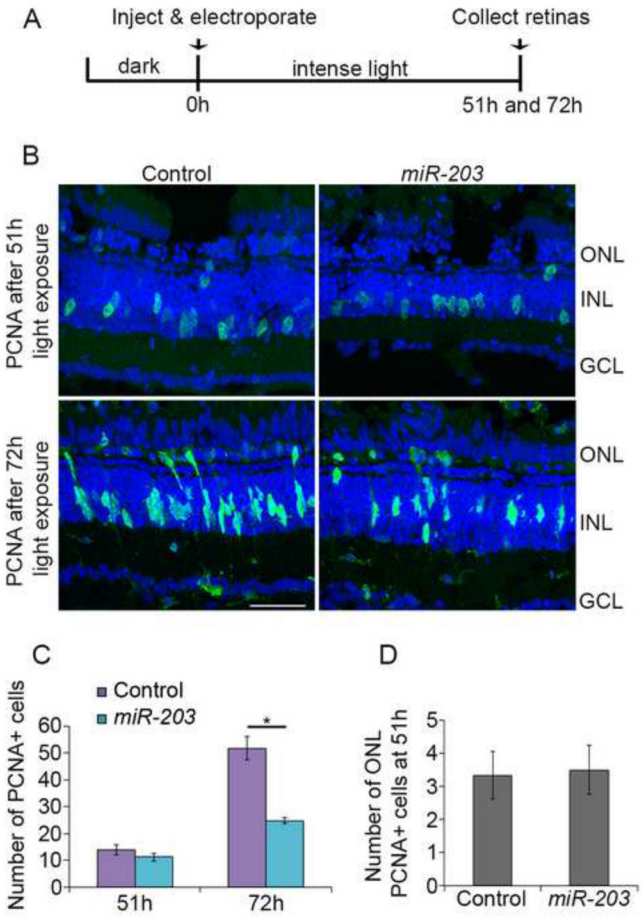

Figure 2. *miR-203* overexpression affects proliferation during regeneration

(A) Experimental scheme. (B) Control miRNA or *miR-203* was injected and electroporated into the left eyes of *albino* zebrafish before intense light exposure. Retinas were collected after 51h and 72h of light exposure, sectioned and immunostained using an antibody against PCNA (green). Nuclei were counterstained with TOPRO (blue). (C) Quantification of INL $PCNA^+$ cells. (D) Quantification of ONL $PCNA^+$ cells at 51h. Data represent mean +/− s.e.m (n= 8 fish); *, p< 0.001 by Mann-Whitney U test. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50um.

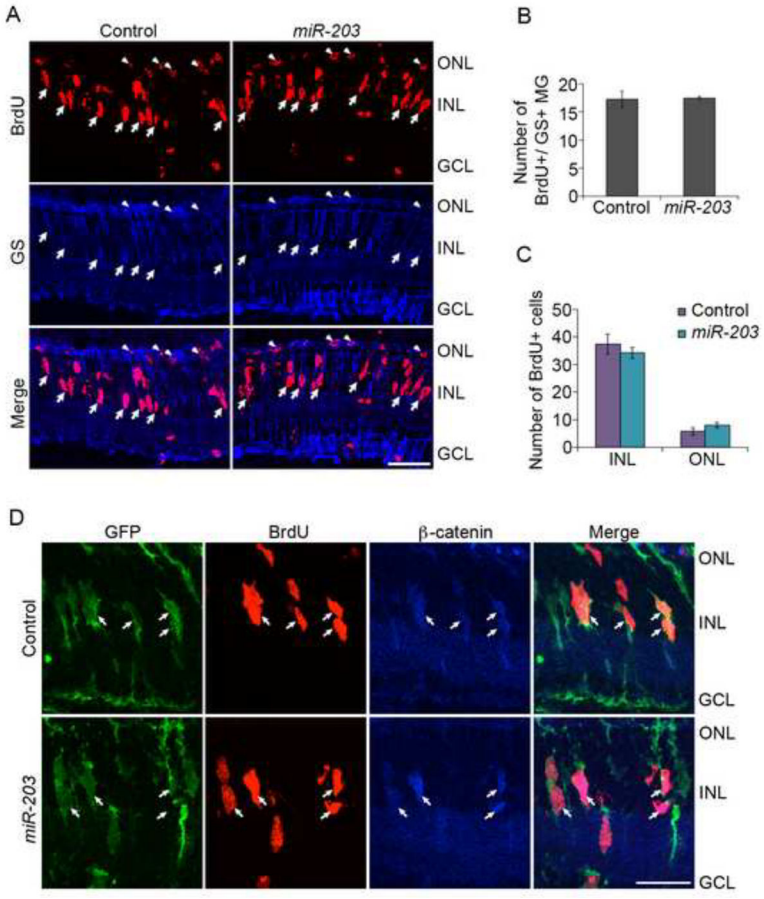

Figure 3. *miR-203* gain-of-function does not affect MG dedifferentiation or proliferation

Control miRNA or *miR-203* was injected and electroporated into the left eyes of *albino* (A) or *tg:1016tuba1a:gfp* (D) zebrafish before intense light exposure. BrdU was injected intraperitoneally after 48h of light exposure and retinas were collected at 51h of light exposure, sectioned and immunostained with antibodies against BrdU and GS in (A) and BrdU, GFP and β-catenin in (D). In (A), $BrdU^+$ nuclei (red) colabel with MG specific anti-glutamine synthetase (GS; blue) antibody (arrows). ONL rod precursors also incorporate BrdU (arrowheads). (B) Quantification of $BrdU^+/GS^+$ MG. (C) Quantification of total $BrdU^+$ cells in INL and ONL. Data represent mean +/− s.e.m (n= 4 fish). No statistical significance was detected using Mann-Whitney U test. In (D), $BrdU^+$ nuclei (red; arrows) colabel with β-catenin (blue) and GFP (green). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50um.

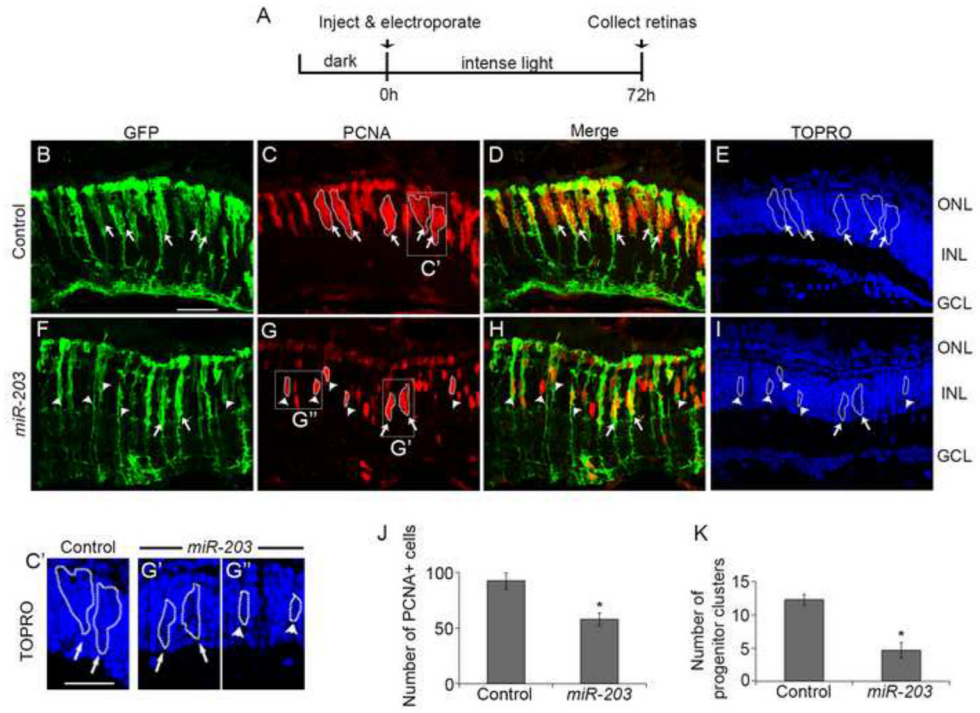

Figure 4. *miR-203* gain-of-function reduces the number of progenitor clusters

(A) Experimental scheme. (B–I) Control miRNA or *miR-203* was injected and electroporated into the left eyes of *tg:1016tuba1a:gfp* fish prior to intense light exposure. Following 72h of light exposure, retinas were sectioned and stained with antibodies against GFP (green; panels B, F) and PCNA (red; panels C, G); nuclei are counterstained with TOPRO (blue; panels E, I). *1016tuba1a:gfp* transgene expression was unchanged after *miR-203* overexpression (B, F). In the control miRNA treated retinas, large clusters of $PCNA^+$ cells associated along the processes of dedifferentiated MG (arrows in B–E). *miR-203* overexpression reduced the number of dedifferentiated MG associated with large progenitor clusters (arrows in F–I) but increased the number of dedifferentiated MG associated with 1–3 $PCNA^+$ cells (arrowheads in F–I). (C′ and G′) Higher magnification view of the progenitor clusters boxed in C and G respectively. (G″) Higher magnification view of boxed progenitors in G. (J) Quantification of $PCNA^+$ cells. (K) Quantification of number of dedifferentiated MG associated with progenitor clusters. Data represent mean +/− s.e.m (n= 4 fish); *, p< 0.03 by Mann-Whitney U test. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar (panels B–I) 50um; (panels C′, G′ and G″) 30um.

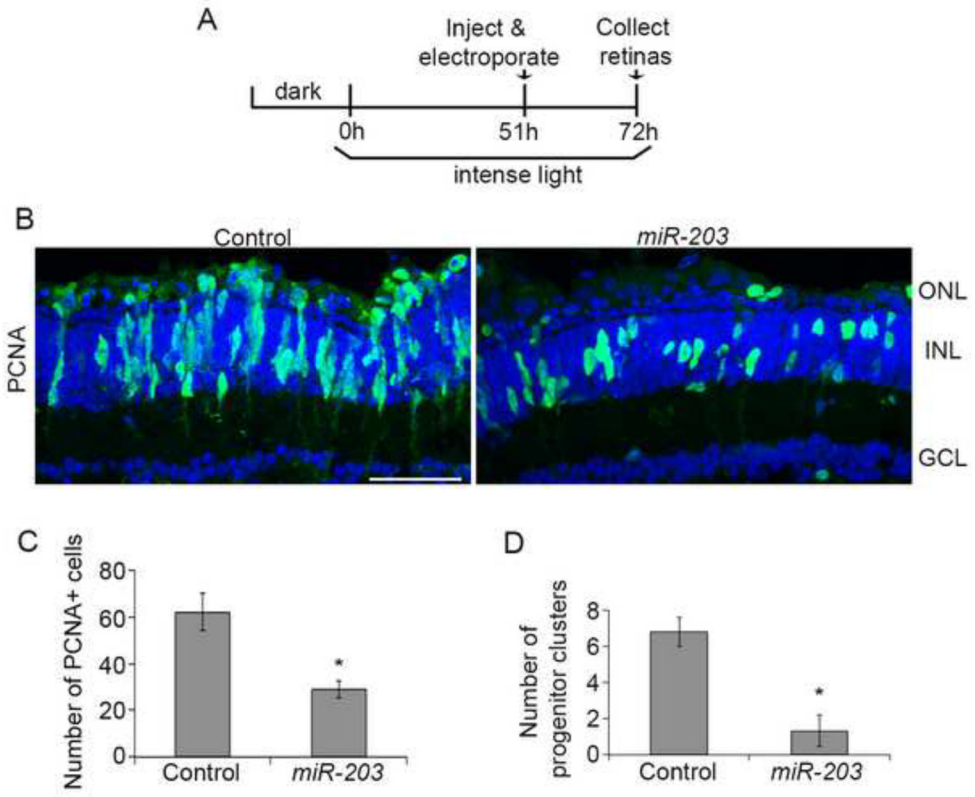

Figure 5. Excess *miR-203* during progenitor cell proliferation phase impairs progenitor cluster formation

(A) Experimental scheme. (B) Control miRNA or *miR-203* was injected and electroporated into the left eyes of *albino* zebrafish after MG dedifferentiation and progenitor cell generation (51h of light). After 72h, retinas were collected, sectioned and immunostained with antibodies against PCNA (green). Nuclei were counterstained with TOPRO (blue). (C) Quantification of $PCNA^{+}$ cells. (D) Quantification of progenitor clusters. Data represent mean +/− s.e.m (n= 3–5 fish); *, p< 0.03 by Mann-Whitney U test. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50um.

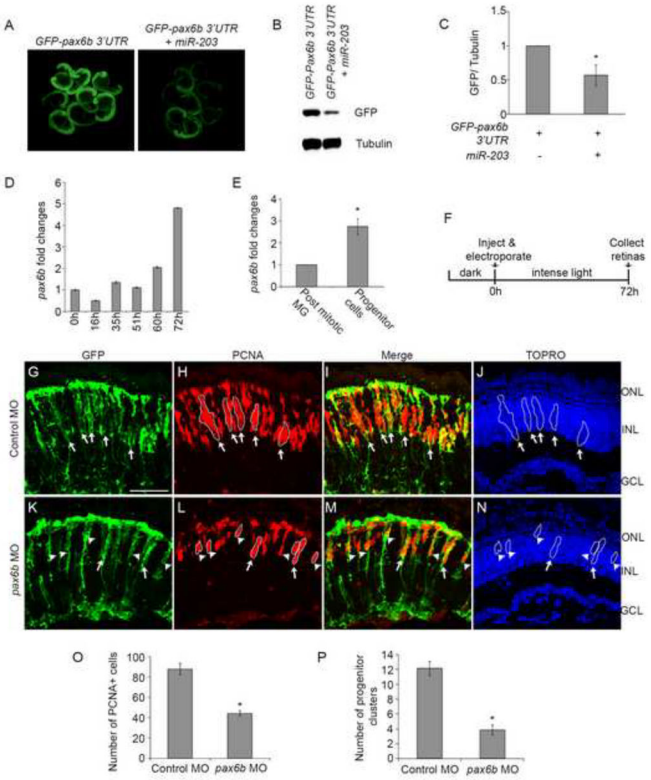

Figure 6. *miR-203* regulates retina regeneration through *pax6b*

(A–C) *miR-203* targets the 3′ UTR of *pax6b*. The *pax6b* 3′UTR was fused to the GFP coding sequence. Single-cell zebrafish embryos were injected with *in vitro* transcribed mRNA from the GFP fusion construct in the presence or absence of *miR-203*. (A) Representative fluorescent images of injected embryos at 1dpf. (B) Western blot for GFP and α-tubulin control was performed on lysates prepared from the injected embryos in (A). (C) Quantification of GFP/tubulin ratios from multiple western blots as in (B). Data represent mean +/− s.d. (n=3 separate injection experiments); *, p< 0.008 by Student t-test. (D–E) *pax6b* mRNA is upregulated during retina regeneration. (D) qPCR was performed to quantitate *pax6b* levels from whole retina RNA. (E) qPCR for *pax6b* in FACS purified post mitotic MG and progenitor cells from 72h light treated fish. Data represent relative *pax6b* mRNA levels and show mean +/− s.e.m from 3 separate FACS experiments (n=3); *, p< 0.008 by Student t-test. For each FACS experiment post mitotic MG were purified from 15 undamaged fish and progenitor cells were purified from 20 light damaged fish. Samples were assayed in triplicate. 18s rRNA served as endogenous RNA control. (F) Experimental scheme for morpholino (MO) knockdown experiments in panels G–P. (G–N) Control MOs or *pax6b* MOs were injected and electroporated into the left eyes of *tg:1016tuba1a:gfp* fish prior to intense light exposure. Following 72h of light exposure, retinas were sectioned and stained with antibodies against GFP (green; panels G, K) and PCNA (red; panels H, L); nuclei were counterstained with TOPRO (blue; panels J, N). In the control MO treated retinas, large clusters of $PCNA^{+}$ cells associated along the processes of dedifferentiated MG (arrows in G–J). *pax6b* MO treatment reduced the number of dedifferentiated MG associated with progenitor clusters (arrows in K–N) but increased the number of dedifferentiated MG

associated with 1–3 PCNA$^+$ cells (arrow heads in K–N). (O) Quantification of PCNA$^+$ cells. (P) Quantification of number of dedifferentiated MG associated with progenitor clusters. Data represent mean +/− s.e.m (n= 6–7 fish); *, p< 0.002 by Mann-Whitney U test. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar 50um.