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The role of microglia in the neurogenesis of zebrafish retina

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ABSTRACT

Microglia are cells from non-neuronal lineages that reside in the central nervous system. In zebrafish, early macrophages migrate from the yolk sac to the brain and retina at 26–30 hour post fertilization (hpf) and transform into microglia at 55–60 hpf. The migration of macrophages into the central nervous system requires signaling by macrophage colony stimulating factor-1 receptor (csf-1r), which is encoded by the gene *fms*. In this study, we show that the targeted knockdown of csf-1r with morpholino oligonucleotides delays migration of macrophages from the yolk sac to the retina, and this delay in macrophage migration results in microphthalmia, delay in cell cycle withdrawal among retinal progenitors and the absence of neuronal differentiation. When embryos were allowed to survive beyond the time when morpholino-dependent translation inhibition is lost, microglia re-occupy the retina and neuronal differentiation partially recovers. Our data demonstrate that microglia are required for normal retinal growth and neurogenesis. This study provides new insight into the neurogenic role of microglia during retinal development in zebrafish.

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1. Introduction

Microglia are cells from non-neuronal lineages that reside in the CNS. It is generally accepted that microglia are phagocytic cells involved in establishing host defense mechanisms against invading agents and the clearance of endogenous cell debris in the CNS [1,2]. Depending upon the nature of the injury, microglia may either be neuroprotective or neurotoxic or both [3]. The function of microglia in the retina is similar to that elsewhere in the CNS.

In zebrafish, early macrophages differentiate in the yolk sac; some then join the blood circulation, while others migrate to the head of the embryo. Macrophages that colonize the retina and forebrain migrate to the cephalic mesenchyme and then secondarily invade the nervous system. These cells first enter the retina between 26 and 30 hpf. The migration of macrophages from the yolk sac to the brain and retina requires signaling by macrophage colony stimulating factor-1 receptor (csf-1r). The *fms* gene, which codes for csf-1r, is expressed in all macrophage lineage cells and is crucial for their colonization of embryonic tissues [4,5].

Recently, a concept has emerged that in both the normal and injured brain, microglia function to regulate neurogenesis [6,7]. Array studies performed in our lab identified *progranulin-a* (*pgrn-a*, GenBank: NM_001001949) as a gene that was strongly upregulated during the proliferative phase of photoreceptor regeneration in zebrafish [8]. Furthermore, this gene was expressed exclusively by microglia in the CNS and in the macrophage lineage cells outside of the CNS. However, there is still a lack of knowledge about the function of microglia during retinal development.

In this study, embryonic zebrafish were used to experimentally investigate the function of microglia during retinal neurogenesis. Following the targeted knockdown of csf-1r, the migration of macrophage to the retina and brain is delayed. This results in microphthalmia and a delay in the differentiation of retinal neurons. Labeling mitotic cells with BrdU revealed that retinal progenitors have an extended cell cycle period and a reduced rate of cell cycle exit. Escape from the inhibition of csf-1r knockdown results in the partial recovery of neuronal differentiation. Collectively, these data indicate that microglia function as essential regulators of neurogenesis in the embryonic and larval retina of zebrafish.

2. Materials and methods

2.1. Experimental animals

AB strain zebrafish were used in this study. The animals were maintained in aquaria at 28.5 °C with a 10/14 h dark/light cycle. Embryos were collected after natural spawns, housed at 28.5 °C, and staged by hours post fertilization (hpf). Protocols for all procedures using animals were approved by the University Committee for Use and Care of Animals at the University of Michigan and

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the Institutional Animal Care Committee at Nankai University and conform to National Institutes of Health guidelines.

2.2. Morpholino oligonucleotides and microinjections

Morpholino oligonucleotides, either complementary to the translation start site of zebrafish *fms* mRNA sequence (GenBank: AF240639) or containing a 5 bp mismatch (control morpholinos), were synthesized by Gene Tools, LLC (Philomath, OR). The sequence of the morpholino oligonucleotides is 5′-AAGAGCGCGAA GAACATCTCAGAGC-3′ (antisense start codon were underlined), and the sequence of the mismatch oligos is 5′-AAcAcCGCcAAcAAC ATCTCAcAGC-3′ (mismatch nucleotides were underlined). Morpholinos were diluted in 1× Danieau buffer [9] at 1 ng/μl. Embryos were injected with 4 ng morpholino oligonucleotides at the 1–4-cell stage. To suppress off-target *p53*-mediated apoptosis [10], we co-injected a zebrafish *p53* oligo at 2 ng with the *fms* morpholino oligonucleotides. The antisense sequence for the *p53* oligos is 5′-GCGCCATTGCTTTGCAAGAATTG-3′ (www.gene-tools.com).

2.3. Acridine orange labeling

The embryos were dechorionated at 24 hpf and placed in acridine orange solution (5 μg/ml in embryo medium) (Sigma) for 15 min followed by extensive washes in embryo medium. The embryos were anesthetized and viewed under a fluorescence dissecting microscope, and cells in the retina that were positively labeled with acridine orange were counted.

2.4. Western blot analysis

The specificity of the translation-blocking morpholinos was tested by Western blot analysis. Thirty to forty embryos were lysed in buffer with protease inhibitors (Complete Mini, Roche, Mannheim, Germany), and protein concentrations were quantified by using the BCA Protein Assay Kit (Complete Mini, Roche, Mannheim, Germany). The proteins were separated in a 12% SDS–PAGE gel and were transferred to a nitrocellulose membrane (Sigma–Aldrich, St. Louis, MO). The membrane was then blocked in 5% nonfat dry milk in PBS for 2 h and incubated with the following antibodies: anti-csf-1r (1:500; Anaspec, Fermont, CA) and anti-GAPDH (1:1000; Millipore, Billerica, MA). The blots were rinsed with PBS and incubated with peroxidase-conjugated goat anti-rabbit antibodies (1:10,000; Sigma–Aldrich, St. Louis, MO) for 1 h. The bound antibody was visualized using the enhanced chemiluminescence assay (Kodak Chemiluminescence BioMax Film, Rochester, NY).

2.5. Immunohistochemistry

Embryos were fixed overnight in 4% paraformaldehyde, cryoprotected in 20% sucrose in 0.1 M phosphate-buffered saline (pH 7.2), frozen in OCT (Sakura Finetek, Torrance, CA). Cryosections (10 μm) were mounted on glass slides for immunohistochemistry or *in situ* hybridization. Immunohistochemistry was performed using standard procedures. In brief, sections were rinsed in 0.1 M phosphate-buffered saline and 0.5% Triton X-100 (PBST), incubated with 20% normal sheep serum (NSS) in PBST, and incubated overnight at 4 °C in primary antibodies diluted in 2% NSS–PBST. The primary antibodies and concentrations used were as follows: mouse monoclonals Zn12 (1:200), Zpr1 (1:200), and Zpr3 (1:200) all from the Zebrafish International Resource Center (ZIRC, University of Oregon, Eugene, OR) for labeling ganglion cells, cones, and rods, respectively. After washing with PBST, the sections were incubated for 1.5 h at room temperature in PBST containing 2% NSS and fluorescently labeled secondary antibodies (Molecular Probes, Eugene, OR), diluted at 1:500. The sections were counterstained with a 1:1000 dilution of DAPI (4′,6-diamidino-2-phenylindole) (Sigma) to label the nuclei. Following this step, sections were washed extensively in PBST, and sealed with mounting media and glass coverslips. Ten animals were processed at each time point.

2.6. BrdU labeling

Bromodeoxyuridine (BrdU; Sigma) was used to label mitotically active cells. The embryos were systemically labeled with BrdU at 72 hpf by soaking them for 20 min in 5 mM BrdU and 15% DMSO in 4 °C E3 medium as described previously [11]. BrdU was detected with a monoclonal antibody (Becton Dickinson Immunocytochemistry Systems, San Jose, CA) diluted 1:100. The total cell population was labeled using DAPI (Sigma) diluted at 1:1000 to label nuclei. Twenty retina sections from ten embryos in each group were counted to determine the ratio of BrdU-positive cells to all nuclei.

2.7. In situ hybridization

All embryos were grown in 0.003% 1-phenyl-2-thiourea (PTU, Sigma) to block pigmentation and mediate visualization until 72 hpf. *In situ* hybridization was performed on whole mount embryos or cryosections using a standard protocol [12]. Briefly, sense and antisense riboprobes were synthesized from linearized plasmids, and digoxigenin (DIG)-labeled probes were generated by *in vitro* transcription using the DIG RNA labeling kit (Roche Diagnostics, Indianapolis, IN). Two probes were used in this study. Microglia were labeled using an mRNA probe for *pgrn-a*. The cDNA encoding *pgrn-a* was linearized with *Sma*I, and the riboprobes were synthesized with T3 polymerase. An mRNA probe for *athnal-homologue5* (*ath5*) was used as a marker to explore whether the neurogenesis initiated on time. The cDNA encoding *ath5* was linearized with *Apa*I, and the riboprobes were synthesized with SP6 polymerase. For whole mount *in situ* hybridization, probe with a concentration of 1 ng/μl was added to Eppendorf tubes and hybridized overnight at 55 °C. For the *in situ* hybridization of the sections following pre-hybridization, 100 ng *pgrn-a* probe in 100 μl of hybridization buffer was pipetted onto each slide, coverslipped, and hybridized overnight at 55 °C. Negative controls were riboprobes encoding the sense strand of the respective cDNAs, which failed to hybridize. The next day, the embryos or sections were washed, and digoxigenin was immunolabeled using an alkaline-phosphatase-conjugated antibody (Roche). NBT/BCIP (Roche) served as the enzymatic substrate on the third day.

2.8. Photography and image analysis

Images of the immunohistochemistry were photographed with a Leica TCS SP5 confocal microscope. Images of the *in situ* hybridization were photographed with a Nikon DMX1200 digital camera mounted on a Nikon Eclipse E800 epifluorescent compound microscope. The larvae for whole mount *in situ* hybridization of cryosections were observed with an Olympus SZX10 dissecting microscope. The resulting images were compiled in Adobe Photoshop CS2 (Adobe, San Jose, CA, USA) and resized and occasionally modified for contrast and brightness using the image-adjustments-contrast–brightness setting. All of the images within an experiment were manipulated in the same manner. Immunostaining and image analysis was conducted identically for each experimental group to minimize the possibility of introducing variability between samples. Image J 1.38X software (Wayne Rasband, NIH, http://rsb.info.nih.gov/ij/) was used to convert the fluorescent images of the BrdU to 8-bit grayscale prior to thresholding, positive staining and determining the positive area of each image. Excel was then used to calculate the percentile of the positive area on each

slide. The values were averaged across similarly treated retinas, and statistical analysis was performed by ANOVA.

3. Results and discussion

3.1. The csf-1r knockdown results in the delayed migration of macrophages into the retina and microphthalmia

In this study, we used an *fms*-targeted morpholino to inhibit the translation of the *fms* gene. Because changes in the cell cycle often result in the activation of apoptosis and increased cell death, we first examined whether cells undergo apoptosis in *fms* morphants at 24 hpf. Generally, the overall death rate in the developing zebrafish retina is very low (1–2%). By the co-injection of *fms* and *p53* morpholinos, we did not observe a significant increase in the levels of cell death in the *fms* morphant retina at 24 hpf by acridine orange labeling in either the retina or the whole body (figure not shown). This result suggested that cell death did not contribute to the retinal phenotype observed in *fms* morphants.

A western blot test showed injection of the *fms* MO resulted in specific suppression of csf-1r protein expression at 72 hpf in the *fms* morphants (Fig. 1A). The delayed migration of the macrophages from the yolk sac to the retina was verified by whole mount *in situ* hybridization with the microglia-specific mRNA probe *pgrn-a*. In un-injected and mismatch control animals *pgrn-a* was expressed in microglia in the brain and retina at 72 hpf (Fig. 1C, D). However, following knockdown of csf-1r, *pgrn-a*-expressing cells were present on the surface of the yolk sac, but there were no labeled cells in the brain or retina (Fig. 1E). These results indicate we were able to exclude microglia from the brain and retina until 72 hpf, thereby providing a model for evaluating the effects of microglia on the developing retina.

Compared to un-injected and mismatch control embryos, two differences were found in the gross appearance of *fms* morphants at 72 hpf (Fig. 1B). The first difference was less pigmentation in the bodies of *fms* morphants. The function of the zebrafish *fms* gene was first investigated in the *Danio rerio* pigment pattern mutant *panther* line [4]. *fms* is expressed and required by *D. rerio* xanthophore precursors for their ventralward migration and differentiation, and promotes the normal patterning of melanocyte death and migration during adult stripe formation [13]. Our result is consistent with this previous study in that when we knocked down the *fms* gene, the morphants had a lighter pigmentation. The second finding was that *fms* morphants had remarkably smaller eyes (Fig. 1B) than did un-injected and mismatch controls. However, the *panther* line did not show gross abnormalities in their eyes [4]. In *panther* larvae, a mean number of three macrophages penetrated the optic tectum and had differentiated into microglia at 72 hpf [5]. In our study, the *fms* morphants we identified had no microglia detected in the brain or retina by whole mount *in situ* hybridization with a *pgrn-a* mRNA probe specific to microglia. Our results suggested that even a few microglia (i.e., 1–3) may be sufficient to promote normal development of the retina and eye.

3.2. The onset of neurogenesis and neuronal differentiation are delayed following knockdown of csf-1r

Neurogenesis in the zebrafish retina is initiated in a small, discrete patch adjacent to the optic stalk in the ventronasal retina and then follows an order from the inner section to the outer [14,15]. Subsequent cellular differentiation then propagates away from this patch as a wave throughout the neuroepithelium [16]. The first retinal progenitors to withdraw from the mitotic cycle at 28 hpf lie in a small cluster in the ganglion cell layer [17]. From 48 hpf, the cells in the outer nuclear layer begin to withdraw from the mitotic cycle and lamination has spread across the retina [18]. Most major classes of retina cells, including photoreceptors, can be identified at 72 hpf [19].

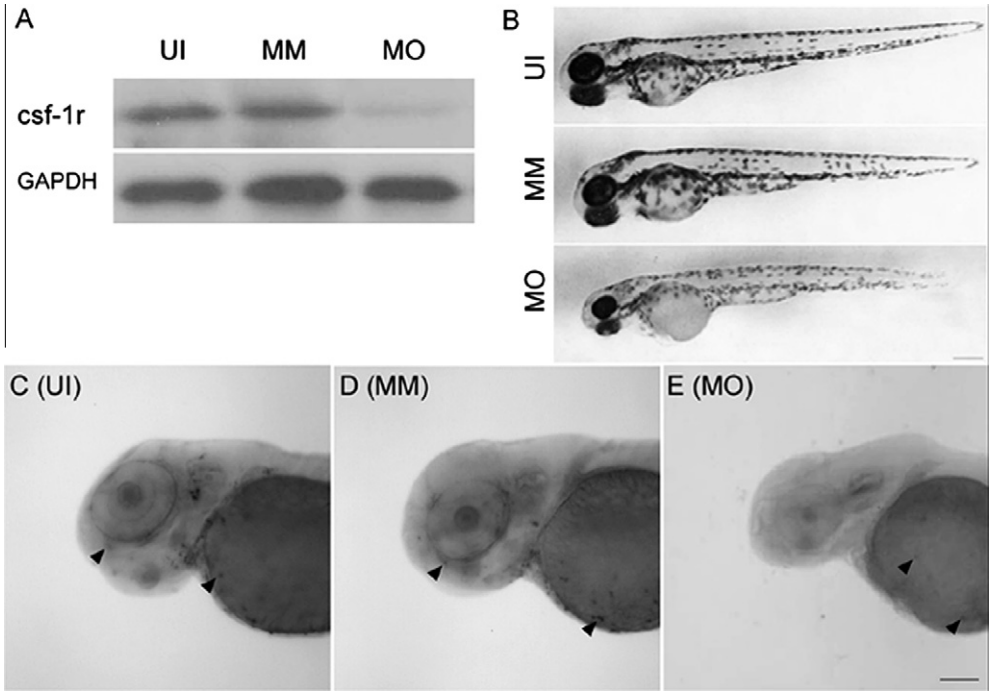

Fig. 1. *fms*-Targeted morpholino oligonucleotides inhibit the migration of microglia from the yolk sac to the retina and brain at 72 hpf. Panel A is the result of a western blot with the csf-1r antibody at 72 hpf. Panel B illustrates the gross development of un-injected (UI), mismatch control (MM) and *fms* morphant (MO) larvae. *fms* morphant larvae are slightly smaller than are un-injected and mismatch controls, and have less pigmentation of the body at 72 hpf. However, the *fms* morphants have dramatically smaller eyes than do the other two groups. Panels C–E show a whole mount *in situ* hybridization with the riboprobe *progranulin-a* (*pgrn-a*), which is specific to microglia. For un-injected and mismatch control larvae, the microglia (*arrowheads*) were detected in the yolk sac, retina and brain at 72 hpf. Note the absence of microglia in the brain and retina in *fms* morphants (E) and the presence of microglia in the yolk sac (*arrowheads*). Scale bar: B, 250 μm; C–E, 100 μm.

Athnal-homologue5 (*ath5*) is a bHLH transcription factor that is expressed in a wave-like pattern in cells immediately after their final mitosis, and is required for the specification of the first-born retinal neurons (retinal ganglion cells) [17,20]. In this study, we used *ath5* as a marker at 28 hpf to explore whether the differentiation of ganglion cells initiated on time. *In situ* hybridization revealed that in un-injected and mismatch control retinas at 28 hpf, the expression of *ath5* matches that described previously (see [14,15]; Fig. 2A and B). In contrast, following knockdown of csf-1r, at 28 hpf there was no *ath5* mRNA expression (Fig. 2C). These data suggested that neuroepithelial cells were defective in the process of neurogenesis at the early time point, and the differentiation of ganglion cells was disrupted in the *fms* morphant retina.

Cell type-specific antibodies were used to evaluate neuronal differentiation at 72 hpf. The Zpr1 antibody labels a cell surface epitope on red/green-sensitive double cone photoreceptors in the zebrafish [21,22]. The Zpr3 antibody labels an antigenic region on the rod opsin protein [23]. The Zn12 antibody labels a cell surface epitope on ganglion cells [15]. In un-injected and mismatch

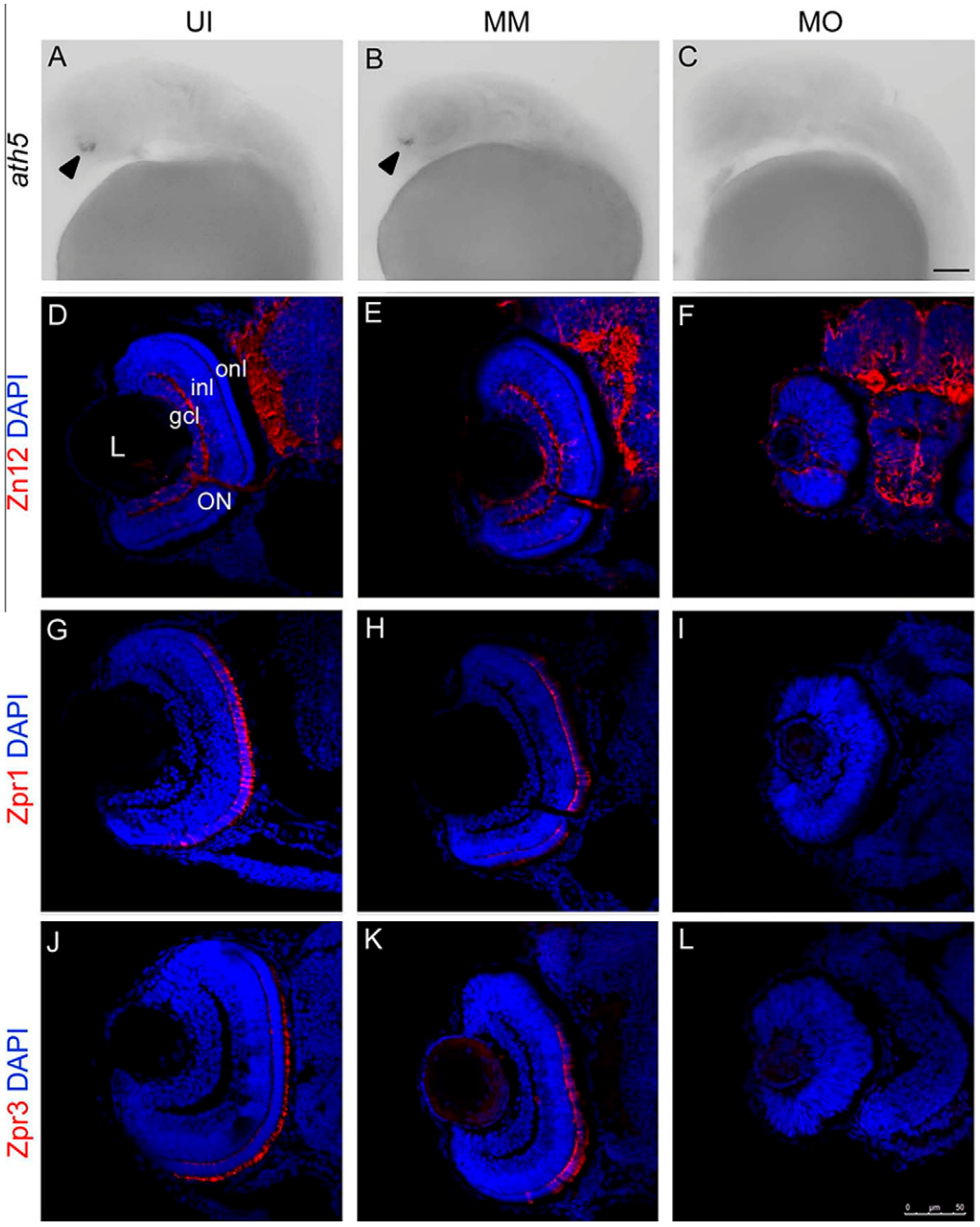

Fig. 2. *fms* Knockdown delays neurogenesis initiation at 28 hpf and affects neuronal differentiation at 72 hpf. Panels A–C show the *in situ* analysis of *ath5* expression during retina development in the retina of un-injected (UI), mismatch control (MM) and *fms* morphants (MO) at 28 hpf. Note that the expression of *ath5* was detected in the retina of un-injected and mismatch controls (*arrowheads*), but was absent in *fms* morphant retina. Panels D–L illustrate sections taken through the retinas at 72 hpf. Panels D–F illustrate Zn12 staining; panels G–I illustrate Zpr1 staining; panels J–K illustrate Zpr3 staining. Note that in the *fms* morphant retinas, only a handful of differentiated Zn12-positive cells were present at 72 hpf, possessed rudimentary optic nerves and had the appearance of an undifferentiated neuroepithelium. Neither Zpr1 nor Zpr3 was detected, while the retinas are well laminated and differentiated, showing strong expression of Zn12, Zpr1 and Zpr3 in the un-injected and mismatch control larvae at 72 hpf. L, lens; gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; ON, optic nerve. Scale bar: A–C, 50 μm; D–L, 50 μm.

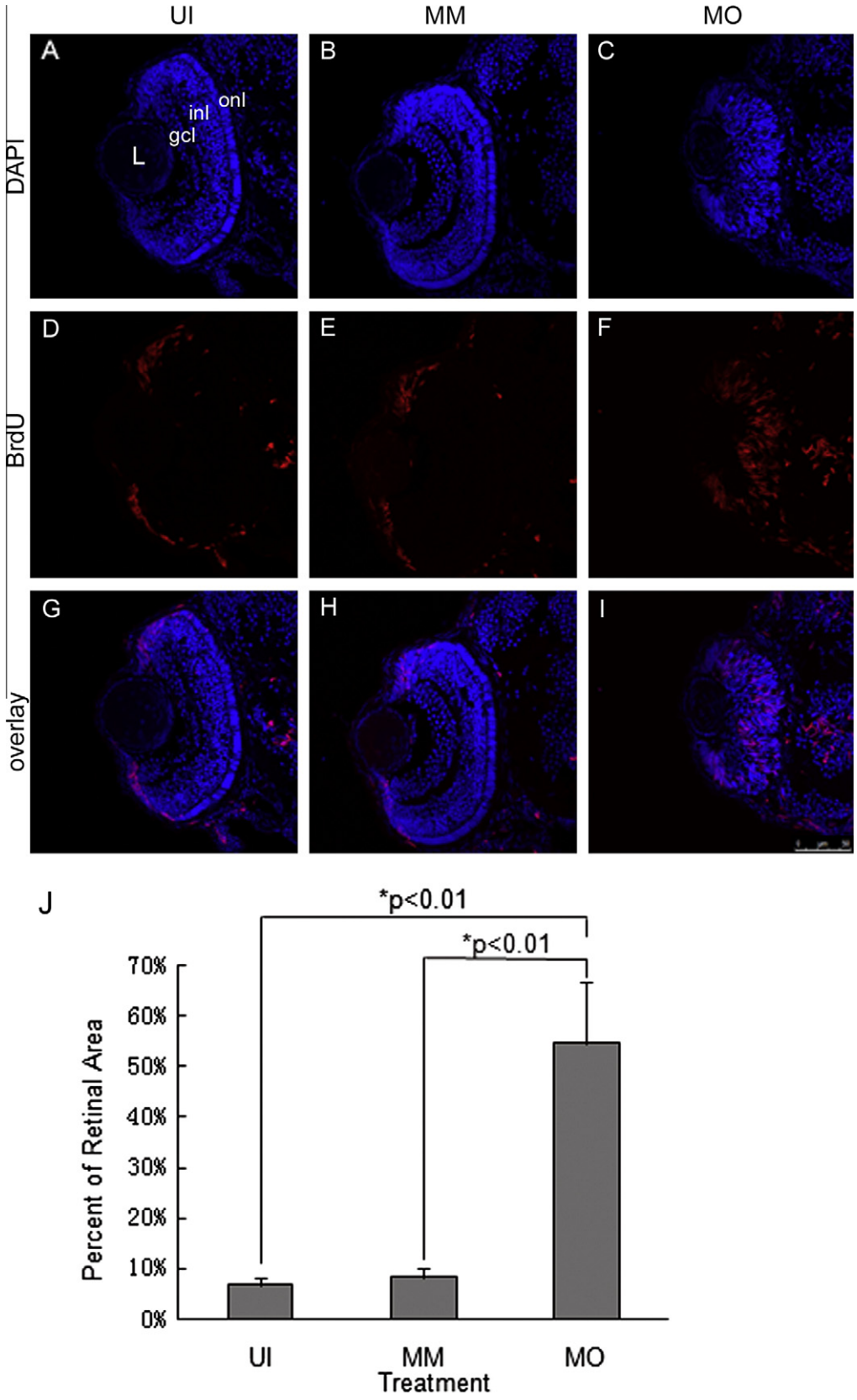

Fig. 3. The *fms* morphant retina remains as neuroepithelium at 72 hpf. Systemic BrdU was used to label mitotically active cells in the retinas of un-injected (UI), mismatch controls (MM) and *fms* morphants (MO) at 72 hpf, respectively. Note the expanded distribution of BrdU-positive cells in the *fms* morphant retina. L, lens; gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer. Scale bar: 50 μm. Panel J illustrates the quantitative comparison of the proportion of the retina occupied by BrdU-positive cells (n = 10 larvae/treatment group). There were statistically significant differences between the control and morphant retinas ($p < 0.01$; ANOVA).

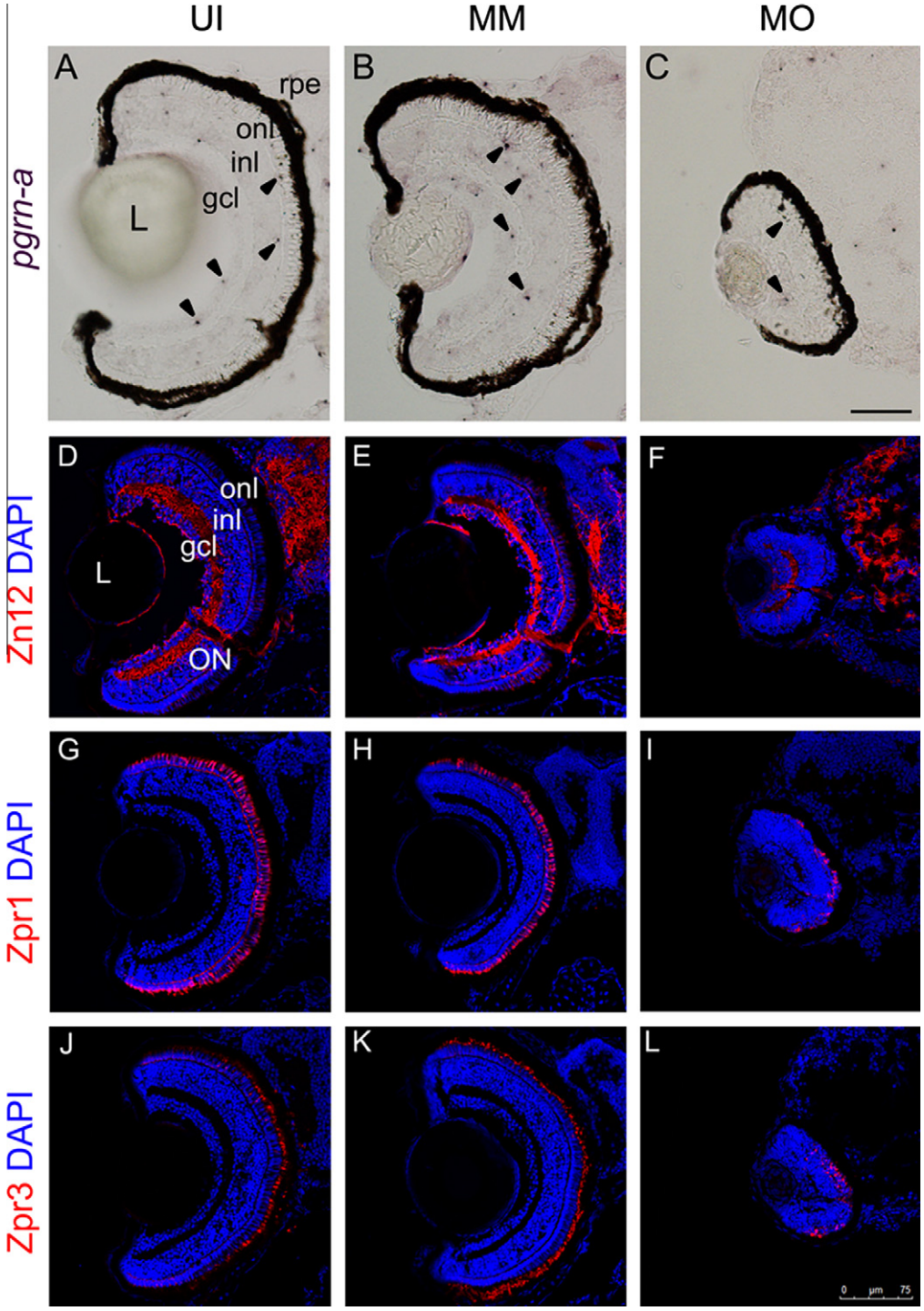

Fig. 4. Recovery of microglia migration into the retina and brain results in delayed cellular differentiation at 7 dpf. Panels A–C illustrate expression patterns of *progranulin-a* (*pgrn-a*) by *in situ* hybridization in un-injected (UI), mismatch controls (MM) and *fms* morphants (MO) at 7 dpf. Note that the microglia (*arrowheads*) have already migrated and colonized into the brain and retina in *fms* morphants at 7 dpf, although the morphants continue to have smaller eyes. Larvae are allowed to survive to 7 dpf and are stained with cell type-specific antibodies: Zn12 for ganglion cells (D–F), Zpr1 for cones (G–I) and Zpr3 for rods (J–L). The retinas of the *fms* morphants expressed the appositional cell type-specific markers (F, I, L). L, lens; gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigment epithelium; ON, optic nerve. Scale bar: A–C, 50 μm; D–L, 75 μm.

control retinas, the three nuclear layers were well-formed that included differentiated RGCs and photoreceptors (Fig. 2D, E, G, H, J and K). In contrast, following knockdown of csf-1r there was little evidence of cellular laminae, there were only few differentiated RGCs and a rudimentary optic nerve (Fig. 2F) and no presence of differentiated photoreceptors (Fig. 2I, L). These data show that the differentiation of all of the major cell types in the retina largely absent following knockdown of csf-1r. In the above results, we have demonstrated that the absence of microglia in the brain and retina resulted in a severe delay in the differentiation of all major cell types (ganglion cells, cones and rods) in the developing retina until 72 hpf.

3.3. The delayed migration of microglia causes defects in cell proliferation

To understand the basis of the defect in retinal development in *fms* morphant embryos, we examined the cell cycle progression of retinal precursor cells in *fms* morphants by BrdU systemic labeling. This method labels all cells that are in the S-phase, and therefore, will mark proliferative progenitor cells [24]. In the un-injected and mismatch control embryos, retinal neuroepithelial cells are restricted to the ciliary marginal zone (CMZ) by 72 hpf, an area that remains proliferative for the life of the zebrafish. Labeling with BrdU showed that most of the cells of the central retina in un-injected and mismatch control larvae did not incorporate BrdU because they had become postmitotic, whereas cells located within the CMZ remained strongly labeled (Fig. 3D and E). In contrast, the majority of the cells in *fms* morphant retinas continued to incorporate BrdU at 72 hpf, including a large number of cells in the central retina (Fig. 3F). To quantitate these findings, we scored the percent of the total retinal cells that were BrdU-positive and revealed that there were no statistically significant differences between the un-injected and mismatch controls, but there was a significantly greater proportion of the retina that contained proliferating cells in *fms* morphants (Fig. 3J). This result suggested that the absence of microglia in the brain and retina results in defects in the proliferation of retinal progenitor cells, whereby the cell cycle in the progenitor cells is prolonged or/and cell cycle exit is compromised. Microglia regulate the cell cycle and influence cell-type fate and differentiation pathways in the zebrafish retina. There are at least two possible roles for the microglia pathway in regulating the cell cycle of retina progenitor cells: it could be required for normal S-phase progression and/or it should be required for S-phase exit and G_2/M progression [25]. Further experiments can be performed in *fms* morphant retinas to address this question, including checking the key regulators and genes for S/M transition or cell cycle analysis by flow cytometry.

3.4. Retinal neurogenesis partially recovers in fms morphants

The effect of a morpholino knockdown is temporary, and the effect disappears after 5 days. Morphants from a single clutch were randomly selected and sacrificed at 7 dpf. By performing *in situ* hybridization on sections using the microglia-specific mRNA probe *pgrn-a*, we observed that the microglia in both the brain and the retina in *fms* morphants at 7 dpf (Fig. 4C). These data suggest that the effect of this csf-1r morpholino was to temporarily delay the migration of the macrophages. We then checked the expression of Zn12, Zpr1 and Zpr3 in the un-injected, mismatch controls and *fms* morphants to evaluate the differentiation and development of the ganglion cells and photoreceptors at 7 dpf. Within the cohort examined at 72 hpf, there was little neuronal differentiation in the retina; in contrast, by 7 dpf, the retinas of the *fms* morphants were laminated, and the retinal neurons expressed the appropriate cell type-specific markers (Fig. 4F, I and L), although the eyes of the morphants continued to be smaller than those of the un-injected and mismatch controls.

Following the knockdown of *fms*, the retina progenitors fail to exit the cell cycle, and the retina persists as a proliferative neuroepithelium. However, escape from the inhibition of *fms* translation results in an incomplete recovery of the neuronal differentiation. The spatial patterns of gene expression in morphant retinas suggested that the neurogenesis was not stopped by the *fms* morpholino but was only delayed. The absence of microglia did not alter positional information, cell identities or cell fate decisions in the retina; rather, microglia were required for retinal progenitors to initiate the switch from proliferation to differentiation.

Overall, our data indicate that the cell cycle of retinal progenitor cells can be disrupted by microglia, although the competency of retinal progenitor cells is intrinsically determined. The further identification and testing of the molecular determinants of neuronal differentiation in the zebrafish retina should yield results that will have implications for understanding the regulation of microglia in the developing zebrafish retina.

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