# Nestin Regulates Müller Glia Proliferation After Retinal Injury

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**Purpose.** The proliferative and neurogenic potential of retinal Müller glia after injury varies widely across species. To identify the endogenous mechanisms regulating the proliferative response of mammalian Müller glia, we comparatively analyzed the expression and function of nestin, an intermediate filament protein established as a neural stem cell marker, in the mouse and rat retinas after injury.

**Methods.** Nestin expression in the retinas of C57BL/6 mice and Wistar rats after methyl methanesulfonate (MMS)-induced photoreceptor injury was examined by immunofluorescence and Western blotting. Adeno-associated virus (AAV)-delivered control and nestin short hairpin RNA (shRNA) were intravitreally injected to rats and Müller glia proliferation after MMS-induced injury was analyzed by BrdU incorporation and immunofluorescence. Photoreceptor removal and microglia/macrophage infiltration were also analyzed by immunofluorescence.

**Results.** Rat Müller glia re-entered the cell cycle and robustly upregulated nestin after injury whereas Müller glia proliferation and nestin upregulation were not observed in mice. In vivo knockdown of nestin in the rat retinas inhibited Müller glia proliferation while transiently stimulating microglia/macrophage infiltration and phagocytic removal of dead photoreceptors.

**Conclusions.** Our findings suggest a critical role for nestin in the regulation of Müller glia proliferation after retinal injury and highlight the importance of cross species analysis to identify the molecular mechanisms regulating the injury responses of the mammalian retina.

Keywords: nestin, Müller glia, proliferation, microglia, phagocytosis

In lower vertebrates, like zebrafish, retinal Müller glia have a robust regenerative capacity; in response to injury, they re-enter the cell cycle, de-differentiate into highly proliferative progenitors, and re-differentiate into all types of neurons.[1] In mammals, however, the regenerative capacity of Müller glia is extremely limited and they undergo reactive gliosis instead of neurogenesis after injury. Various attempts have been made to stimulate neurogenesis of mammalian Müller glia. Mitogenic stimulation,[2–5] inactivation of the Hippo pathway,[6,7] or overexpression of proneural transcription factors[8–11] have been reported to promote proliferation and neurogenesis of mammalian Müller glia. However, in vivo reprogramming of mammalian Müller glia remains extremely challenging due to the presence of strong endogenous mechanisms repressing their proliferative and neurogenic competence.[12,13] Defining the endogenous mechanisms regulating glial responses to injury would aid in promoting regenerative potential of the mammalian retina and designing effective strategies to treat degenerative retinal diseases in humans.

Most studies involving the neurogenic potential of mammalian Müller glia have been conducted in rodent species, such as mice and rats. Müller glia in mice, especially the commonly used B6 strain, have extremely limited proliferative potential after injury in vivo,[14,15] whereas rat Müller glia are capable of proliferation in response to various types of retinal injury.[16–18] Müller glia in both mice and rats upregulate cyclin D1 after injury; however, the increase in cyclin D1 levels is much more robust in rats compared to mice and upregulation of cyclin E and A, essential for G1/S transition, is observed only in rats.[17,19] Thus, although injury may trigger cell cycle re-entry of Müller glia in both species, mouse Müller glia may be arrested in early G1 phase due to lack of sufficient mitogenic signals required for G1 progression and G1/S transition.[14,15] Intriguingly, the phagocytic activity of Müller glia also varies between the two species and phagocytic signals, such as phosphatidylserine and Rac1, regulate various injury-induced glial responses including proliferation, phagocytosis, and gliosis.[20] However, the mechanism underlying the species difference in the proliferative ability of mammalian Müller glia remains largely unknown. Comparative analysis of the two rodent species with different proliferative potential of Müller glia may help delineate the endogenous mechanisms regulating glial responses to injury.

Nestin is an intermediate filament protein, established as a marker of neural stem/progenitor cells.[21–24] In the retina, nestin is highly expressed in retinal progenitor cells

(RPCs) and immature Müller glia whereas it is downregulated in differentiated neurons and Müller glia.[25,26] After retinal injury, nestin is re-upregulated in reactive Müller glia, which has been considered as a sign of dedifferentiation.[15,16,18,26,27] However, the precise role of nestin in Müller glia after injury remains unexplored. In the course of our study characterizing methyl methanesulfonate (MMS)-induced photoreceptor injury in both mice and rats, we found that nestin is highly upregulated in Müller glia in rats but not in mice. Only rat Müller glia were capable of proliferation after MMS-induced injury implicating functional links between nestin expression and Müller glia proliferation. We thus conducted RNAi-mediated knockdown of nestin in the rat retina in vivo and found that loss of nestin inhibited Müller glia proliferation after injury. We also found that nestin knockdown stimulated microglia/macrophage infiltration and phagocytic removal of dead photoreceptors. Our data reveal a critical role for nestin in the regulation of Müller glia proliferation after retinal injury and underscore the utility of cross species analysis to predict and functionally validate the molecular mechanisms regulating the injury responses of the mammalian retina.

## Materials and Methods

### Animals and Induction of Retinal Degeneration

Male Wistar rats (Charles River Laboratories, Yokohama, Japan) and C57BL/6J mice (Sankyo Labo Service Corporation, Tokyo, Japan) were used. Photoreceptor degeneration was induced by intraperitoneal administration of MMS (Nacalai Tesque, Kyoto, Japan) at a dose of 75mg/kg[28] or N-methyl-N-nitrosourea (MNU; Sigma-Aldrich Corp., St. Louis, MO, USA) at a dose of 70 mg/kg,[17] as described previously. The animals were enucleated after euthanasia with $CO_2$ gas and the retinas subjected to the analyses below. All animal experiments conformed to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional animal care committee of Tokyo Women's Medical University.

### Construction of Vectors

The sequence of rat nestin short hairpin RNA (shRNA) was TCTGAGGCTCCCTGTCCTCCA. Non-targeting control shRNA (MISSION shRNA: SHC002) was purchased from Sigma (St. Louis, MO, USA). The shRNA and the U6-promotor were ligated into adeno-associated virus (AAV) vectors (Takara, Kyoto, Japan). AAV shH10 (addgene, Watertown, MA, USA) and pHelper (Takara) were transformed into HEK293T (Takara) and AAV was purified using AAVpro Purification Kit Maxi/Midi (Takara). The AAV titration protocol[29] was used to determine the number of genome-containing particles of an AAV prep using real-time PCR. About $1 \times 10^{11}$ Vg/μL of AAV was titrated for injection in 4 μL volume in each eye.

### Intravitreal Injection

To make nestin knockdown retinas in vivo, AAV-delivered shRNAs were injected intravitreously into rats 2 weeks before photoreceptor degeneration by MMS administration. Rats were intraperitoneally anesthetized with 0.375 mg/kg of medetomidine, 2.0 mg/kg of midazolam, and 2.5 mg/kg of butorphanol as previously reported.[30] A small penetrating incision was made into the vitreous cavity using 30 gauge needle and 4 μL volume of 1 × PBS containing nestin shRNA or control shRNA was injected into the vitreous using a NanoFil syringe (World Precision Instruments, Sarasota, FL, USA).

### Immunofluorescence

Immunofluorescence staining was performed as described previously[31] with partial modifications. PBS containing 0.5% Triton X-100 was used to permealize the tissue and antibodies listed (Supplementary Table S1) were diluted by Blocking-one (Nacalai tesque). The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Immunofluorescence images were collected using a confocal laser scanning microscope (LSM710; Carl Zeiss, Jena, Germany) or a fluorescence microscope (Eclipse E600; Nikon Instruments, Tokyo, Japan).

### TUNEL Assay

To visualize cell death, TUNEL assay was conducted by using an in situ cell death detection kit, TMR red (Roche, Mannheim, Germany) in accordance with the manufacturer's instruction.

### BrdU Assays

To label S-phase cells in vivo, 100 mg/kg 5-bromo-2′-deoxyuridin (BrdU; Sigma) was injected intraperitoneally 2 hours before euthanasia. For a long term analysis, BrdU was injected 5 times with approximately 12 hour intervals from 48 hours to 94 hours after MMS treatment. For BrdU immunolabeling, the sections were treated with HISTO VT (Nacalai tesque) at 70°C for 20 minutes and 2M HCl at room temperature for 30 minutes before incubation with primary antibodies.

### Western Blotting

Western blotting was carried out as described previously.[32] In brief, tissues were lysed with RIPA buffer (composed of 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, and 50 mM Tris-HCl pH8.0) supplemented with a protease inhibitor. The lysates were resolved by SDS-PAGE. Antibodies listed in Supplementary Table S1 were diluted by Blocking-one (Nacalai tesque). Immunoblots were visualized with the enhanced chemiluminescence reagent Immunostar (WAKO) and detected with LumiVision PRO 400EX (AISIN, Aichi, Japan).

### Quantitative Reverse Transcription PCR

Total RNA of each retina was isolated by using the ISOGENII (Nippon Gene CO., LTD, Toyama, Japan). Reverse transcription was conducted by ReverTra Ace quantitative PCR (qPCR) RT Master Mix with qDNA Remover kit (Toyobo, Osaka, Japan). Quantitative RT-PCR was performed by using THUNDERBIRD SYBR qPCR Mix (Toyobo) and Step One Plus (Applied Biosystems, Tokyo, Japan). Primers for nestin (forward 5′-CCCTGAAGTCGAGGAGCTG-3′ and reverse 5′-CTGCTGCACCTCTAAGCGA-3′), glial fibrillary acidic protein (gfap; forward 5′-CGGAGACGTATCACCTCTG-3′ and reverse 5′-AGGGAGTGGAGGCGTCATTCG-3′) and TATA-binding protein (Tbp: forward 5′-TGGGATTGTACCACAGCTCCA-3′

and reverse 5′-CTCATGATGACTGCAGCAAACC-3′) were used. Expression levels were determined by the standard curve method.

### Cell Counting

Cell counting was conducted on the vertically sliced retinas. More than seven fields per animal were analyzed under a confocal laser scanning microscope (LSM710), excluding the periphery of the tissue. Each field was taken with a 20 times objective lens. To count cells, manual or the automatic counting tool of ComDet, plugin of ImageJ (National Institutes of Health, Bethesda, MD, USA) was used.

### Statistical Analysis

Statistical analysis was conducted by the paired *t*-test or 1-way analysis of variance (ANOVA) followed by Tukey's post hoc comparisons using the JMP software (SAS Institute, Cary, NC, USA). A value of $P < 0.05$ was considered statistically significant.

## Results

### MMS-Induced Retinal Injury in Mice and Rats

We first conducted TUNEL staining to characterize the time course of retinal degeneration induced by MMS treatment (75 mg/kg) in both mice and rats. In mice, most photoreceptors in the outer nuclear layer (ONL) became TUNEL-positive by day 2 and were gradually removed by day 7 (Fig. 1), consistent with the time course of MNU-induced photoreceptor injury.[17] However, we unexpectedly found numerous TUNEL-positive cells in the inner nuclear layer (INL), ganglion cell layer (GCL), and the RPE of the mouse retinas peaking at days 3 and 4, indicating degeneration of non-photoreceptor cells (Fig. 1). To clarify the identity of these cells, we performed immunolabeling for syntaxin (amacrine/ganglion cell marker), Sox9 (Müller glia marker), and Otx2 (bipolar cell marker) in combination with TUNEL (Supplementary Fig. S1A). Of all TUNEL-positive cells in the INL/GCL, approximately 40% were bipolar cells, approximately 30% were amacrine/ganglion cells, and only 5% were Müller glia (Supplementary Fig. S1A). TUNEL-positive RPE cells were identified by the location and the presence of pigments (Supplementary Fig. S1B). Despite the presence of TUNEL-positive RPE cells at day 4, the overall structure of the RPE was preserved at day 7 (Supplementary Fig. S1B). When mice were treated with a lower dose of MMS (60 mg/kg), TUNEL-positive cells were decreased in number and their localization confined to the ONL (Supplementary Fig. S1C). In rats, the effects of MMS were similar to those of MNU[17]; most photoreceptors became TUNEL-positive by day 2 and these degenerated cells were rapidly eliminated by day 3, which contrasted markedly with more gradual clearance of photoreceptor debris in mice (Fig. 1). In rats, MMS-induced cell death was observed only in the ONL (Fig. 1).

### Nestin is Upregulated in Müller Glia After Photoreceptor Injury in Rats But Not in Mice

Although nestin upregulation in Müller glia has been reported in a variety of retinal injury models,[16,18,33–35] none of the previous reports described species difference in nestin expression. We thus examined nestin expression in Müller glia after MMS-induced photoreceptor injury in rats and mice. Cell cycle re-entry of Müller glia was also analyzed by the BrdU incorporation assay (Fig. 2A). Nestin expression was not detected in the control retinas of both mice and rats (Fig. 2B). Although no apparent increase in the nestin levels was observed in the mouse retinas after injury, there was a dramatic increase in nestin expression in Müller glia processes in the rat retinas, with peak levels around day 3 after MMS treatment (Fig. 2B). Co-labeling of BrdU with the Müller glia marker Sox9 was observed at day 2 in the rat retinas, but no BrdU incorporation was detected in mouse Müller glia after injury (Figs. 2B, 2C). We also examined nestin expression in the mouse and rat retinas after MNU-induced photoreceptor injury and obtained equivalent results (Supplementary Fig. S2A). The expression of other glial intermediate filaments, vimentin and glial fibrillary acidic protein (GFAP), and cytoplasmic Müller glia marker glutamine synthetase (GS), was also analyzed after MMS injury and compared between mice and rats. The expression of GFAP and vimentin increased in Müller glia processes after injury in both mice and rats whereas the GS levels did not change significantly in both species (Supplementary Figs. S2B, S2C). Western blotting confirmed the immunohistochemical results (Fig. 2D). Together, nestin was strongly upregulated in rat Müller glia during their proliferative response after injury while its expression was low or undetectable in mouse Müller glia, which remained quiescent after injury.

### Nestin is Downregulated During Müller Glia Differentiation

Because the proliferative and neurogenic potential of Müller glia is known to decrease with age,[36,37] we next investigated expression of nestin and other Müller glial markers during postnatal development in rats. At P0, intense nestin immunoreactivity was detected in the cellular processes extending vertically across the retina (Supplementary Fig. S3A), consistent with nestin expression in RPC.[26] Nestin expression was diminished and became confined to the inner processes of Müller glia by P7, followed by further reduction by P21 (Supplementary Fig. S3A). The expression of GFAP and vimentin, localized in astrocytes and Müller glia, respectively, did not change significantly during development (Supplementary Figs. S3B, S3C). In contrast, GS expression was dramatically increased during the second postnatal week as Müller glial differentiated (Supplementary Fig. S3C). Western blotting showed nestin expression during the first postnatal week and its drastic reduction thereafter, confirming the immunohistochemical results (Supplementary Fig. S3D). Upregulation of GS and rather stable expression of vimentin during Müller differentiation were also confirmed by Western blotting (Supplementary Fig. S3D). We also analyzed nestin expression in the mouse retinas during postnatal development by immunofluorescence and Western blotting. Nestin immunoreactivity was intense at P0, but decreased toward maturity, showing a developmental pattern similar to the rat retinas (Supplementary Fig. S4). Together, nestin was abundantly expressed in RPC and immature Müller glia, but its levels decreased dramatically as Müller glia mature with age.

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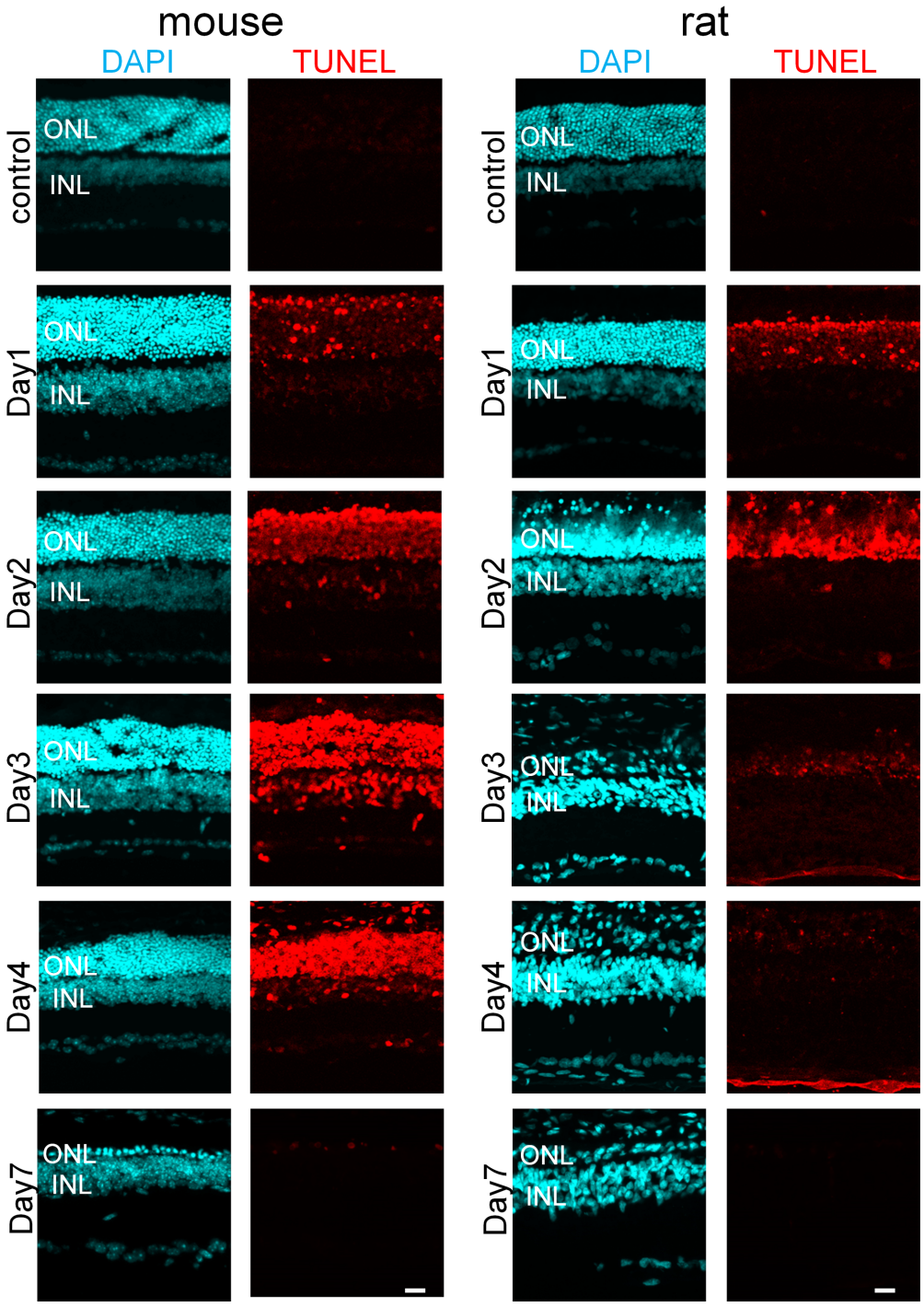


Figure 1. TUNEL staining in the mouse and rat retinas after MMS treatment (75 mg/kg). Most photoreceptors in the outer nuclear layer (ONL) become TUNEL-positive by day 2 in both species. Many TUNEL-positive cells are also found in the inner nuclear layer (INL) at day 3 in mice. Note that dead photoreceptors are rapidly removed by day 3 in rats while photoreceptor removal is more gradual in mice. Scale bars = 20 µm.

### Nestin Knockdown Impairs Injury-Induced Proliferation of Rat Müller Glia In Vivo

The above results indicate the possibility that nestin may regulate the proliferative potential of Müller glia. To investigate the role of nestin in the injury-induced responses of Müller glia, we conducted loss-of-function experiments using nestin shRNA in the rat retinas. AAV-delivered control and nestin shRNA were intravitreally injected to rats 2 weeks before MMS treatment (Fig. 3A). Although we observed the highest proportions of BrdU-positive Müller glia (approximately 30%) at day 2 when examined on a day-by-day basis (Fig. 2C), a more detailed examination indicated that the highest proportions (approximately 70%) of Müller glia incorporate BrdU at 53 to 56 hours after MMS treatment (Supplementary Fig. S5). Thus, the proliferative response of

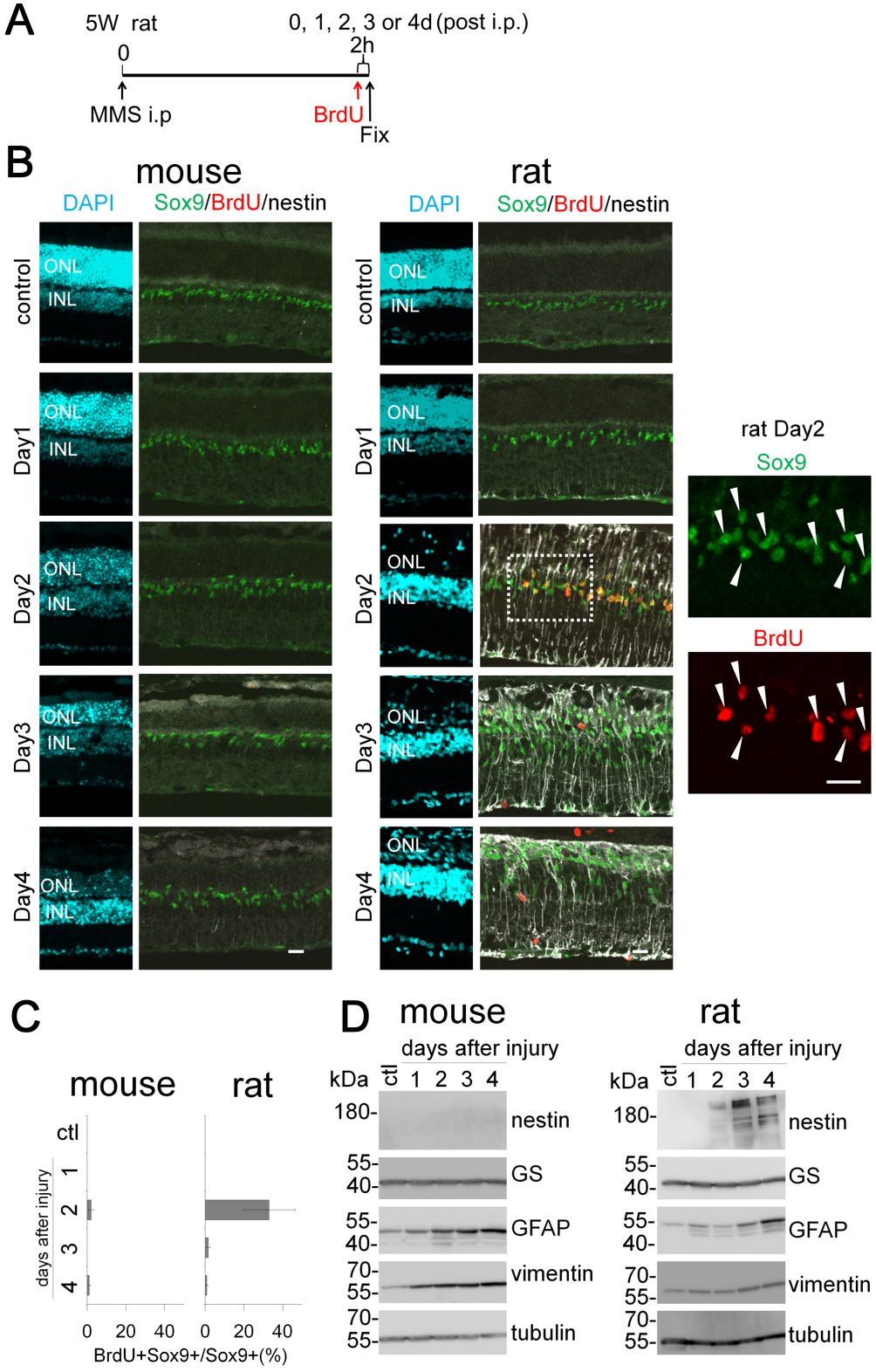


**Figure 2.** Cell cycle reentry and nestin expression in Müller glia after MMS-induced injury. (**A**) Diagram showing the timing of BrdU injection. (**B**) Triple immunofluorescence for Sox9 (Müller glia marker), BrdU (S phase marker), and nestin in the mouse and rat retinas after MMS treatment. Higher magnification of the rat retina at day 2 showing colocalization of Sox9 and BrdU (arrowheads). ONL, outer nuclear layer; INL, inner nuclear layer. Scale bars = 20 µm. (**C**) Quantitation of BrdU-positive Müller glia in mice and rats. Each bar represents the mean ± SD ($n = 3$). (**D**) Western blotting for glial-specific proteins including nestin, glutamine synthetase (GS), glial fibrillary acidic protein (GFAP), and vimentin in the MMS-treated mouse and rat retinas. Tubulin serves as an internal control.

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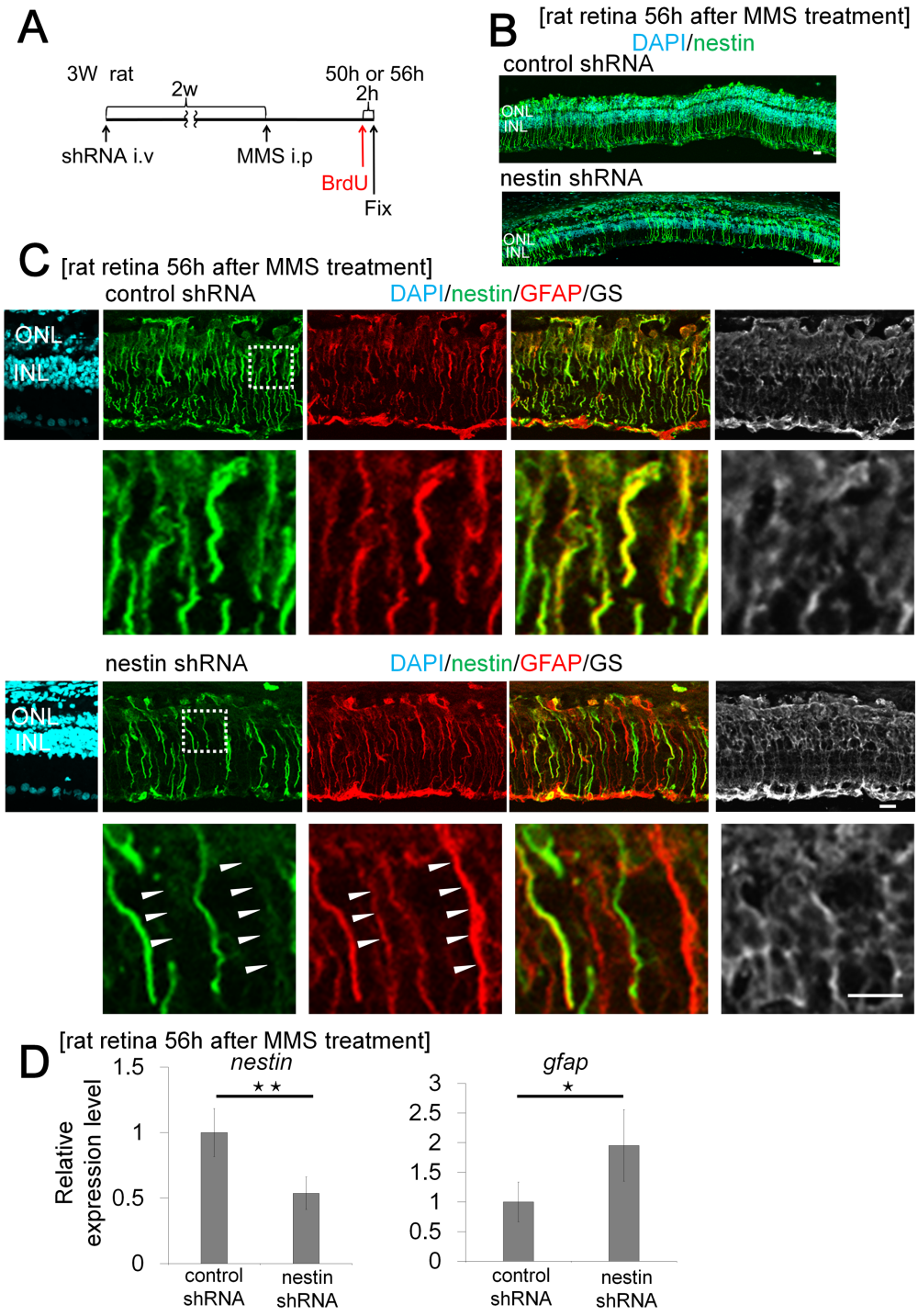


**Figure 3.** Inhibition of nestin expression by AAV-delivered nestin shRNA in the MMS-treated rat retinas in vivo. (**A**) Diagram showing experimental design. AAV-delivered control and nestin shRNA were intravitreally injected to rats two weeks before MMS treatment and the retinas were harvested 50 or 56 hours after MMS treatment. BrdU was injected 2 hours before euthanasia. (**B**) Nestin immunofluorescence showing mosaic patterns of nestin deletion in the knockdown retinas (56 hours after MMS treatment). (**C**) Triple immunofluorescence for nestin, GFAP, and GS in the rat retinas treated with control or nestin shRNA (56 hours after MMS treatment). Boxed regions are shown at higher magnification. *Arrowheads* denote Müller glia processes with nestin deletion. (**D**) Quantitative RT-PCR for nestin and gfap expression in the rat retinas treated with control or nestin shRNA (56 hours after MMS treatment). Each bar represents the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$. ONL, outer nuclear layer; INL, inner nuclear layer. Scale bars = 20 µm.

Müller glia was evaluated at two time points (50 and 56 hours), before and at the time of peak S phase (Fig. 3A). Nestin expression was successfully inhibited in a subpopulation of Müller glia after treatment with nestin shRNA but not control shRNA (Fig. 3B, Supplementary Fig. S6). In the retinas treated with control shRNA, nestin, GFAP, and GS were almost always colocalized in the Müller glia processes (Fig. 3C). However, in the retinas treated with

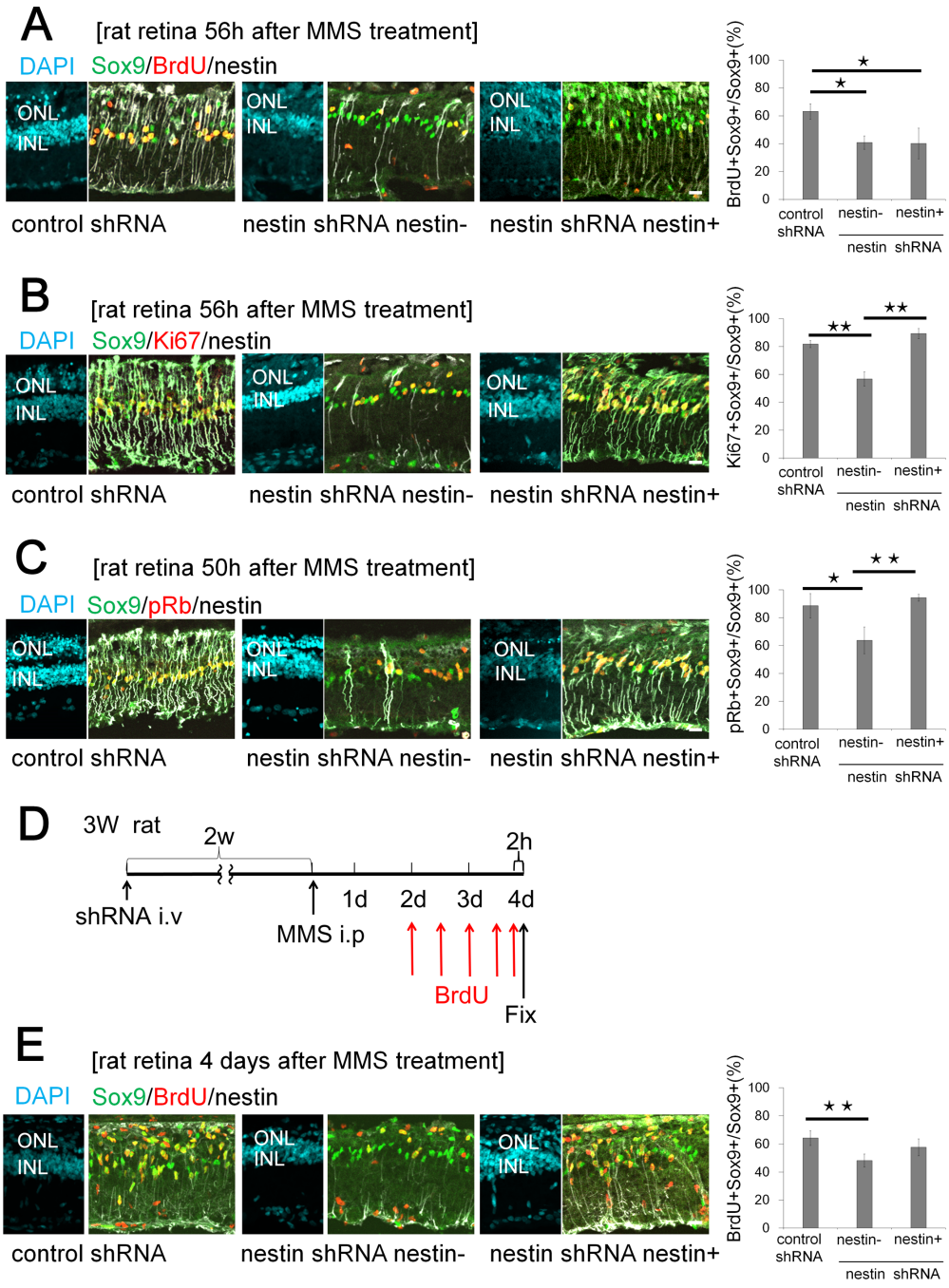


**Figure 4.** Inhibition of injury-induced proliferation of rat Müller glia by in vivo nestin knockdown. (**A**) Triple immunofluorescence for Sox9, BrdU, and nestin in the rat retinas treated with control or nestin shRNA (56 hours after MMS treatment). The proportions of BrdU-labeled Müller glia in the control retinas, the nestin-negative and nestin-positive regions of the knockdown retinas are shown in the graph. (**B**) Triple immunofluorescence for Sox9, Ki67, and nestin in the rat retinas treated with control and nestin shRNA (56 hours after MMS treatment). The proportions of Müller glia expressing Ki67 are shown in the graph. (**C**) Triple immunofluorescence for Sox9, phospho-pRb (pRb), and nestin in the rat retinas treated with control and nestin shRNA (50 hours after MMS treatment). The proportions of Müller glia expressing pRb are shown in the graph. (**D**) Diagram showing experimental design. Animals treated with shRNA and MMS received repeated BrdU injection and the retinas were harvested 4 days after MMS treatment. (**E**) Triple immunofluorescence for Sox9, BrdU, and nestin in the rat retinas treated with control and nestin shRNA (4 days after MMS treatment). The proportions of BrdU-labeled Müller glia are shown in the graph. Nestin- and nestin+ indicate nestin-negative and nestin-positive regions of the knockdown retinas, respectively. Each bar represents the mean ± SD ($n = 3$ or 4). *$P < 0.05$, **$P < 0.01$. ONL, outer nuclear layer; INL, inner nuclear layer. Scale bars = 20 µm.

nestin shRNA (knockdown retinas), the immunoreactivity for nestin, but not GFAP and GS, was lost in many Müller glia processes (Fig. 3C). We also quantitated the effects of nestin knockdown by quantitative RT-PCR. Although nestin knockdown was mosaic in nature (Fig. 3B, Supplementary Fig. S6), nestin expression in the whole retinas treated with nestin shRNA was reduced to approximately half of the control levels (Fig. 3D). Notably, gfap expres-

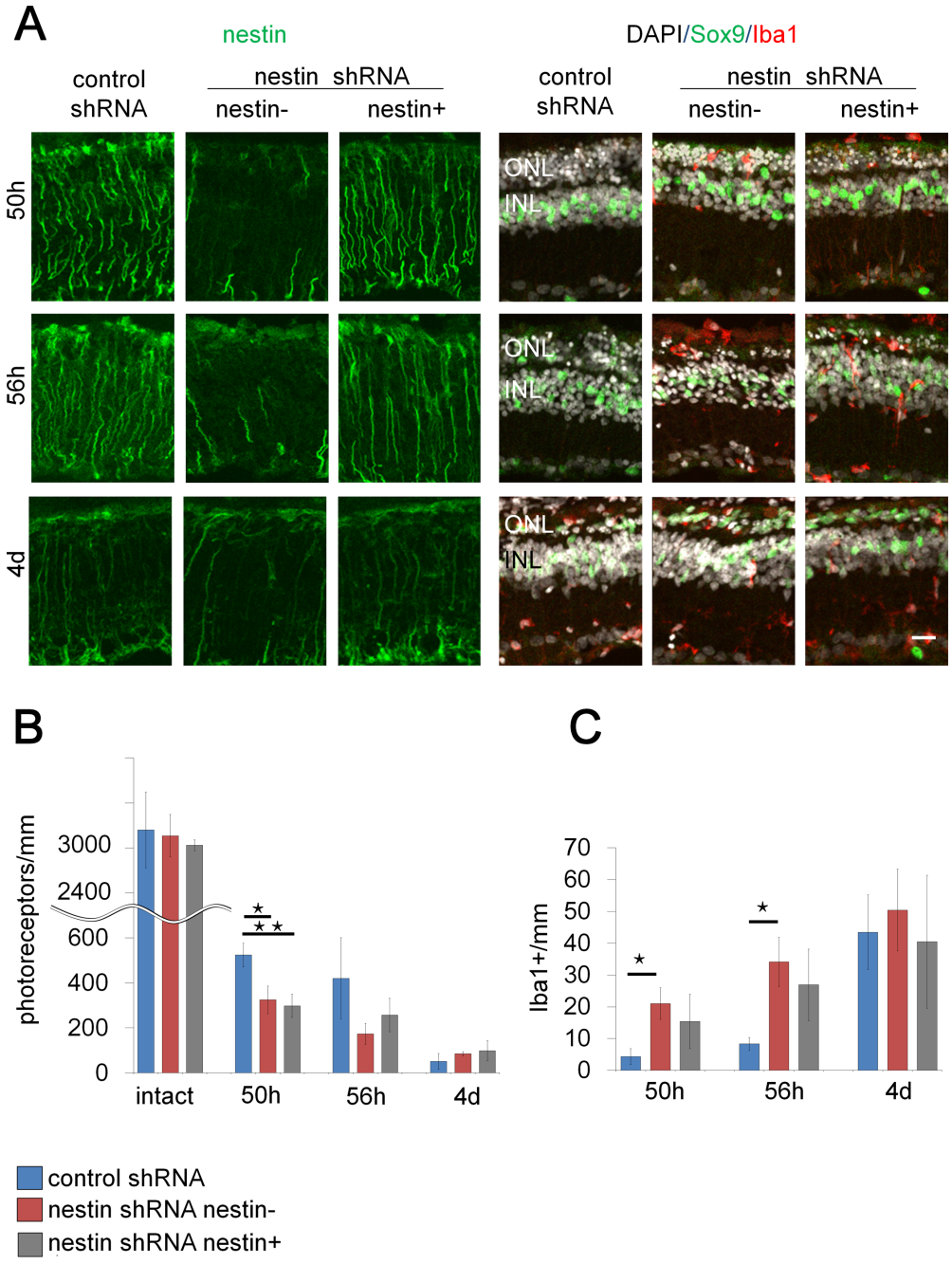


**Figure 5.** Nestin knockdown promotes photoreceptor removal and microglia/macrophage infiltration after MMS-induced injury in rats. (**A**) Triple immunofluorescence for nestin, Sox9, and Iba1 in the rat retinas treated with control and nestin shRNA at given time points after MMS treatment. (**B**) Density of DAPI-labeled photoreceptor nuclei in the ONL after MMS treatment. The numbers of Sox9-positive Müller glia and Iba1-positive microglia/macrophages were subtracted from the numbers of DAPI-labeled nuclei in the ONL. (**C**) Density of Iba1-positive microglia/macrophages after MMS treatment. Each bar represents the mean ± SD ($n = 3$ or 4). *$P < 0.05$. ONL, outer nuclear layer; INL, inner nuclear layer. Scale bars = 20 µm.

sion was increased approximately twofold by nestin shRNA (Fig. 3D).

We first assessed Müller glia proliferation at the peak S phase (56 hours) using the S phase marker BrdU and pan-cell cycle marker Ki67. The proportions of Müller glia immunolabeled for BrdU or Ki67 were quantitated and compared between the control and knockdown retinas. In the knockdown retinas, nestin-negative and nestin-positive regions were separately analyzed. BrdU-labeling in the nestin-negative regions of the knockdown retinas was significantly decreased compared to the control retinas (Fig. 4A). There were no significant differences between nestin-negative and nestin-positive regions within the knockdown retinas (Fig. 4A). The proportions of Ki67-positive Müller glia were significantly lower in the nestin-negative regions of the knockdown retinas compared to the

control retinas or the nestin-positive regions of the knockdown retinas (Fig. 4B). We further examined pRb phosphorylation at 50 hours after MMS treatment. Similar to Ki67, the percentages of phospho-pRb-positive Müller glia were significantly decreased in the nestin-negative regions of the knockdown retinas compared to controls or the nestin-positive regions of the knockdown retinas (Fig. 4C). To test the possibility that depletion of nestin only delayed cell cycle progression rather than reduced the number of Müller glia re-entering the cell cycle, we labeled proliferating Müller glia by repeated injection of BrdU and analyzed BrdU incorporation at 4 days after MMS treatment (Fig. 4D), when most Müller glia had exited the cell cycle (Fig. 2C). Again, the proportions of BrdU-positive Müller glia in the nestin-negative regions were significantly lower compared to controls and also exhibited a trend to be lower than those in the nestin-positive regions ($P = 0.064$), indicating that depletion of nestin impaired, rather than delayed, cell cycle re-entry of Müller glia (Fig. 4E).

As nestin has been shown to promote cell survival,[38,39] the observed reduction of Müller glia proliferation by nestin knockdown may have been caused by increased cell death of Müller glia. To test this possibility, we examined whether nestin knockdown affects cell death of Müller glia by double labeling for Sox9 and TUNEL. The proportions of TUNEL-positive Müller glia did not differ significantly between the controls and the nestin-negative and nestin-positive regions of the knockdown retinas at both 50 and 56 hours after MMS treatment (Supplementary Fig. S7A), indicating that nestin knockdown did not affect the survival of Müller glia at least at these time points. As previous reports suggested that nestin regulates cell motility and migration,[40–42] we also tested whether nestin knockdown affects migration of Müller glia after injury. Because the nuclei of many Müller glia migrate toward the ONL by day 4 after photoreceptor injury (Fig. 2B), the number of displaced Müller glia in the ONL and outer plexiform layer (OPL) was counted and compared between controls and nestin knockdown retinas. No significant difference in the proportion of displaced Müller glia was observed between controls, nestin-negative regions and nestin-positive regions of the knockdown retinas (Supplementary Fig. S7B).

### Nestin Knockdown Transiently Accelerates Microglia/Macrophage Infiltration and Photoreceptor Removal After Injury

We previously reported that degenerated photoreceptors are rapidly phagocytosed by Müller glia prior to the infiltration of microglia/macrophages in the MNU-treated rat retinas.[20] The present results also revealed that dead photoreceptors were rapidly eliminated in the rat retinas after MMS treatment (Fig. 1). As proliferation and phagocytic activity of Müller glia have been shown to be regulated by common molecular mechanisms,[20] we next analyzed the effects of nestin knockdown on the time course of photoreceptor removal based on the hypothesis that nestin may regulate the phagocytic activity of Müller glia. To this end, we quantitated the number of DAPI-labeled photoreceptor nuclei remaining in the ONL at various stages after MMS treatment. Because Müller glia and microglia/macrophages migrate into the ONL after photoreceptor injury, we conducted triple immunofluorescence for nestin, Sox9 (Müller glia), and Iba1 (microglia/macrophages; Fig. 5A), and the numbers of Müller glia and microglia/macrophages in the ONL were subtracted from DAPI-labeled cell counts. Before MMS treatment, there were no significant differences in the number of photoreceptor nuclei between controls, and nestin-negative regions and nestin-positive regions of the knockdown retinas (Fig. 5B). The numbers of photoreceptor nuclei substantially decreased in both control and knockdown retinas by 50 hours after MMS treatment; however, photoreceptor numbers in the knockdown retinas (both negative and positive regions) were significantly less than those in the control retinas (Fig. 5B). No significant differences were observed between the control and knockdown retinas at 56 hours and 4 days after MMS treatment. These results imply that nestin knockdown transiently promoted photoreceptor phagocytosis; however, lack of difference in the extent of photoreceptor removal between the nestin-negative and positive regions of the knockdown retinas argues against the possibility that loss of nestin cell-autonomously affected the phagocytic activity of Müller glia. We thus tested an alternative possibility that nestin knockdown may affect the activity of microglia/macrophages. Quantitation revealed that the numbers of Iba1-positive microglia/macrophages were significantly higher in the knockdown retinas (nestin-negative regions) compared to controls when examined at 50 and 56 hours after MMS treatment (Fig. 5C). Microglia/macrophages in the control retinas drastically increased in number by day 4, and thus no differences were observed between the control and knockdown retinas at this stage (Fig. 5C). There were no significant differences in microglia/macrophage number between the nestin-negative and positive regions of the knockdown retinas at any stages examined (Fig. 5C). Taken together, nestin knockdown transiently accelerated photoreceptor removal likely due to the activation of microglia/macrophage infiltration.

## Discussion

Nestin has been established as a marker of neuronal stem/progenitor cells[21–24]; however, the role of nestin in the central nervous system (CNS) remains largely unknown. Studies involving genetic deletion of nestin reported that it is dispensable for the proliferation of embryonic neuronal progenitors[39,43–45] or adult neuronal stem cells.[46] On the other hand, transgenic mice overexpressing nestin showed an increase in brain size due to enhanced proliferation of neuronal progenitors.[47] Moreover, in vitro knockdown studies revealed that nestin is essential for the proliferation of neuronal progenitors[48,49] or tumor cells.[40,42] Thus, there seems to be a discrepancy between the results of the knockout studies and those using gain-of-function or RNAi-based knockdown approaches. The present study using the RNAi-based in vivo knockdown technique demonstrated that nestin is required for the proliferation of rat Müller glia after injury. The mechanism how nestin regulates Müller glia proliferation remains to be determined. It has been reported that nestin promotes G1/S transition of neuronal progenitor proliferation via activation of the PI3K pathway.[47,48] The present findings that nestin knockdown inhibited BrdU incorporation of Müller glia are consistent with the possibility that nestin regulates G1/S transition of Müller glia. However, our data suggest the involvement of more complex mechanisms underlying the nestin-mediated regulation of Müller glia proliferation. Müller glia proliferation evaluated

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by pan-cell cycle markers such as Ki67 and phospho-pRb was significantly decreased in the nestin-negative regions but not in the nestin-positive regions of the knockdown retinas, indicating the possibility that the effects of nestin knockdown may be cell-autonomous. In contrast, S phase entry of Müller glia assessed by BrdU was significantly decreased in both nestin-negative and positive regions in the knockout retinas, suggesting the involvement of non-cell-autonomous mechanisms. We speculate that nestin may cell-autonomously affect cell cycle re-entry of Müller glia but may regulate their S phase entry via non-cell-autonomous mechanisms, possibly via the effects on the secretion of mitogenic or antimitogenic factors. However, we cannot exclude the possibility that nestin levels in the nestin-positive regions of the knockdown retinas may be lower than control, which may affect S phase entry of Müller glia in these regions. Further studies are required to elucidate this issue. Although relevance of nestin in the survival[38,39,43] or migration[41] of neuronal progenitors has been suggested, such roles of nestin in Müller glia could not be confirmed in the present analyses.

The present study also revealed a possibility that nestin may regulate retinal immune responses after injury. Earlier studies suggested that microglia or blood-borne macrophages play a key role in the phagocytic removal of dead cells in the retina.[50–52] However, we and others have recently shown that Müller glia, rather than microglia/macrophages, are primarily responsible for the phagocytic cell removal after photoreceptor injury.[20,53] In the rat model of MNU-induced injury, the majority of dead photoreceptors are rapidly phagocytosed by Müller glia prior to robust infiltration of microglia/macrophages into the ONL.[20] The time course of photoreceptor injury and removal was very similar between the MNU and MMS models, suggesting that Müller glia play a predominant phagocytic role also in the MMS model. We first assumed that nestin knockdown might inhibit not only Müller glia proliferation but also their phagocytic activity, leading to a delay in photoreceptor removal. On the contrary, nestin knockdown further accelerated photoreceptor removal after MMS injury. It is likely that loss of nestin stimulated the phagocytic activity of Müller glia; however, despite the mosaicism of nestin knockdown, the ONL thickness decreased rather uniformly across the retina and the number of photoreceptor nuclei remaining in the ONL did not vary significantly between the nestin-positive and negative regions, arguing against the possibility that nestin knockdown cell-autonomously affected the phagocytic activity of Müller glia. We then demonstrated that microglia/macrophage infiltration was accelerated in parallel with enhanced photoreceptor removal. Although direct evidence is lacking, our data are consistent with the possibility that nestin knockdown stimulated microglia/macrophage infiltration accelerating phagocytic removal of dead photoreceptors. Microglia/macrophages in the brain and retina have been shown to express nestin.[54–56] However, it is unlikely that the present knockdown technique affected nestin expression in microglia/macrophages considering that ShH10 AAV vectors allow Müller glia-specific gene delivery.[57] Alternatively, we favor the possibility that loss of nestin in Müller glia compromised their roles in retinal immune responses leading to aberrant microglia/macrophage activation. Further investigations are required to determine the role of nestin in the regulation of immune responses after retinal injury.

The present study using the MMS-induced retinal injury model adds to previous evidence that rat Müller glia are more proliferative than mouse Müller glia in vivo.[17,19] Unexpectedly, we found that MMS treatment caused degeneration of some non-photoreceptor neurons (mostly bipolar and amacrine cells) as well as photoreceptors in mice while the same dose of MMS induced photoreceptor-specific degeneration in rats, indicating a species difference in the sensitivity of retinal neurons to MMS toxicity. Despite the apparently more robust neuronal cell death induced by MMS in mice, mouse Müller glia remained quiescent whereas rat Müller glia showed proliferative activity accompanied by prominent nestin upregulation. As proliferation and nestin expression are both characteristics of RPC, rat Müller glia may be considered more phenotypically similar to RPC compared to mouse Müller glia. Does this imply that rat Müller glia have a higher regenerative potential than mouse Müller glia? In a highly regenerative species like zebrafish, proliferation of Müller glia is essential for generation of neurogenic progenitors,[58,59] and strong links between proliferation and the ability to be reprogrammed have been suggested in general.[60,61] However, it remains unclear whether glial proliferation and nestin expression play any role in the reprogramming and neurogenic potential of mammalian Müller glia. Injury-induced proliferation of rat Müller glia does not generate neuronal progenitors, but instead results in Müller glial cell death possibly due to the DNA damage response.[17] Adult mouse Müller glia forced to re-enter the cell cycle in vitro upregulate gliogenic transcription factor Nfia without induction of reprogramming factors such as Pax6 and Vsx2.[19] Moreover, nestin in hippocampal astrocytes has been shown to inhibit adult neurogenesis via activation of the Notch pathway.[46] Thus, glial ability to proliferate and express nestin may not contribute to neurogenic reprogramming, but may serve to maintain the glial identity. Nevertheless, the present data that nestin knockdown upregulated GFAP expression also suggest the possibility that nestin may suppress gliotic responses of Müller glia. Because inhibition of Müller glia proliferation induces reactive gliosis in zebrafish,[59] nestin knockdown may indirectly activate GFAP expression via inhibition of Müller glia proliferation. This possibility is also supported by a previous report that proliferating astrocytes in culture express nestin but not GFAP and upregulate GFAP as they exit the cell cycle.[62] As recent attempts to stimulate the regenerative potential of mammalian Müller glia have been conducted mostly in mice, it would be interesting to examine whether rat Müller glia have a higher neurogenic potential than mouse Müller glia when stimulated by pro-neurogenic factors. Furthermore, comparative analyses of Müller glia functions between the two rodent species may help to elucidate the mechanisms regulating the complex responses of mammalian Müller glia to injury.

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